AGE RELATED PATTERNS IN THE TOLERANCE OF ZEBRAFISH (BRACHYDANIO RERIO) EXPOSED TO LETHAL LEVELS OF EITHER CADMIUM, ZINC OR THEIR MIXTURES

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ABSTRACT

AGE RELATED PATTERNS IN THE TOLERANCE OF THE ZEBRAFISH (BRACHYDANIO RERIO) EXPOSED TO LETHAL LEVELS OF EITHER CADMIUM, ZINC OR THEIR MIXTURES

BARBARA GALLIMORE

The discrete and multiple toxicities of cadmium and zinc at lethal levels, to the egg, post larval, juvenile and adult life cycle stages of the zebrafish (Brachydanio rerio) were investigated. The sensitivity of the test organisms to the toxicants, either discretely or in combination, decreased with age and increasing body size, with the exception of the egg stage. The newly fertilized eggs were equally if not more resistant to the toxicants than the adults in all cases, and the post larval stage was consistently the most sensitive.

Excluding the egg stage, the influence of body size of the test organism on susceptibility to the heavy metal contaminants was quantitatively expressed by the following allometric equation:

$Y = a + b \log (C/W^h)$, where Y represents quantal response in probits; C, the ambient toxicant concentration; W, the wet weight of the test organism; and h, an empirically determined weight related factor of 0.10 for zinc, 0.31 for cadmium and 0.32 for the mixture. The response of all test organisms to the mixture was consistent with that predicted by the model of concentration addition, i.e. where

the toxicity of a mixture is based on the potency of its constituents and their relative proportions in that mixture. The consistent expression of this mode of joint action throughout the life cycle stages tested may suggest the existence of a critical toxicant target, which is common to both heavy metal contaminants and which is non-specifically exhibited by all of the test organisms.

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INTRODUCTION

As water borne contaminants, cadmium and zinc most often occur simultaneously (Spehar et al., 1978; Spehar,1976). This concurrence reflects their coexistance in geological formations from which they are mobilized into surface waters by natural geological forces. In the past twenty years, man has tended to increase the rate of environmental mobilization by mining cadmium and zinc ores (E.P.A., 1976; Leland et al., 1978). At the same time, the introduction of industrial effluents, containing cadmium and zinc, into receiving waters has also increased (E.P.A., 1976).

Presently, government regulatory agencies establish water quality criteria on the basis of single aquatic contaminants. Although such standards may protect aquatic populations from the discrete toxicities of cadmium and zinc, their frequent concurrence in water systems may present a potential toxicological hazard.

Anderson & d'Apollonia, 1978, have described infra additive, additive and supra additive interactions of mixtures containing heavy metals. These models indicate that certain contaminants, at levels which are individually harmless, may interact in receptor organisms to cause toxic effects. Thus, in protecting aquatic populations from the potential dangers of co-existing multiple pollutants, a primary aim of aquatic toxicologists is to empirically assess the multiple toxicity risk of pollutant mixtures.

In the last decade, the results of life cycle toxicity tests have provided the most reliable basis for the establishment of water quality criteria for single aquatic contaminants (McKim, 1977). The embryo-larval and early juvenile test life cycle stages of a

variety of species, have consistently shown a greater susceptibility to toxicants than other life cycle stages. In reviewing fifty-six life cycle bioassays, forty-six of the M.A.T.C.'s (maximum acceptable toxicant concentration, i.e. the highest toxicant concentration that does not reduce survival potential) estimated by the embryo-larval and early juvenile exposures were virtually identical to the M.A.T.C.'s established by complete life cycle tests (McKim, 1977). Thus McKim has suggested focusing further research efforts on these more sensitive life cycle stages.

Life cycle toxicity tests investigating multiple toxicity patterns at representative life cycle stanzas may also supply the most reliable basis for the establishment of safe water quality standards for mixtures of aquatic contaminants. However, until a firm precedent which describes the consistency of multiple toxicant interactions throughout the life cycle has been established, research efforts should not only be focused at the least tolerant life cycle stanza.

Government regulatory agencies establish safe water quality criteria which are primarily based on toxicant bioassays involving the adult of the test species. These standards may not apply to water systems in which various life cycle stages of a species are represented. With a better understanding of multiple toxicity mechanisms, possibly rendered by the description of tolerance patterns throughout the life cycle of the test organism, regulatory agencies may establish more meaningful water quality criteria. Thus the risks inherent to the multiple contamination of natural water systems may be reduced.

The rainbow trout, Salmo gairdneri, has been widely used as a standard test species in aquatic bioassays representing the cold water salmonoid family. The rainbow trout, however, is not well suited for life cycle testing as the time required for one cycle (i.e. from egg to reproductive maturity) is impractically long (Sprague, 1973). Species such as the flagfish, Jordanella floridae, and the zebrafish, Brachydanio rerio, are more appropriate candidates for life cycle bioassays as they are easily reared under laboratory conditions and have a relatively short life cycle. Although these species are not native to the colder waters of the United States and Canada, they may act as models representative of the responses of a wide spectrum of fish. Sprague & Fogels, 1977, investigated the toxicity of certain aquatic toxicants to the zebrafish and rainbow trout and found that the tolerance of the two species was similar. Zebrafish were approximately 2.6 times as tolerant as rainbow trout. This difference in tolerance is negligible if considering the variation in susceptibility, to a given toxicant, between two experimental lots of rainbow trout (Sprague & Fogels, 1977). As such, the zebrafish, Brachydanio rerio, has been recommended as a standard test species for aquatic bioassays and is particularly well suited for life cycle testing.

In this study, four primary research objectives were met. A protocol for the successful breeding and raising of the zebrafish, under laboratory conditions, was established. A profile of the tolerance of selected life cycle stanzas exposed to cadmium, zinc and their mixtures was determined. The selected life cycle stages were representative of critical morphological or physiological

periods in the life cycle. The response patterns of each life cycle stage exposed to cadmium and zinc mixtures were compared to theoretical models of multiple toxicity interactions and the consistency of the empirical multiple toxicity response patterns throughout the life cycle was evaluated. Finally, a quantitative relationship between susceptibility to these heavy metals and certain factors which were " non-specifically" expressed by all life cycle stages tested was established. These factors are termed non-specific since they are common to all life cycle stages in spite of the distinct morphological and physiological characteristics exhibited by each stage.

MATERIALS AND METHODS

The zebrafish, Brachydanio rerio, is a small tropical fish native to the Ganges River. It is a Cypriniform representative of the family Cyprinidae (Eaton & Farley,1974a; Laale, 1977). The International Standards Organization has recommended this species as a standard test organism for aquatic toxicity testing (Sprague & Fogels, 1977). The zebrafish is inexpensive to buy, readily available and easily maintained in captivity.

The zebrafish is particularly well suited for reproductive and life cycle bioassays. With appropriate laboratory conditions large numbers of non adherent, transparent eggs may be produced. The developmental period from fertilization to hatch is of a ninty-six hour duration at 26° C. (Laale, 1977) . Sexual maturity is achieved in seventy-five days of culture at 25±1°C. providing food is not limiting (Eaton & Farley, 1974b). Thus, one complete life cycle may be completed in less than three months.

Breeding Procedures

The original parental line of zebrafish was obtained from Tropicarium, a tropical fish supplier in St. Bruno, Quebec. All free swimming life cycle stages of the zebrafish, i.e. those that were more than eight days old, were held in 50-litre glass aquaria, supplied with a continuous flow of dechlorinated, degassed tap water at a temperature of 24 ± 1°C.

Certain physico-chemical characteristics of incoming laboratory water and those of the source water as determined by the City of Montreal Filtration Plant are listed in Table I.

Table I. Water quality data

Analysis of laboratory water used in experiments

dissolved oxygen	mg/L (p.p.m.)	7.85 ± 0.48
temperature	°C	24 ± 1
pH		7.73 ± 0.17
total hardness	mg/L as $CaCo_3$	126.6±0.82

Analysis performed at the City of Montreal filtration plant

silica	mg/L SiO_2	37.4
calcium	mg/L Ca^{++}	37.4
magnesium	mg/L Mg^{++}	8.1
sulfates	mg/L SO_4^{--}	26
chlorides	mg/L Cl^-	27
sodium	mg/L Na^+	12.3
potassium	mg/L K^+	1.4
fluorides	mg/L F^-	0.15
iron	mg/L Fe^{+++}	0.012
carbon dioxide	mg/L CO_2	0.3

Spawning

After a quarantine period of four weeks, the original stock of breeding adults was separated into male and female groups. Several criteria were used to determine the sex of the fish. The mean wet weight and standard length of the adult female B. rerio is greater than that of the adult male and the body contour of the male is slimmer than that of the female. The silver stripes of the adult male are often augmented with colors of yellow and pink, while those of the adult female are not (Eaton & Farley, 1974a). The males have a larger anal fin than do the females and the latter exhibit a distinct genital papilla (Laale, 1977).

Breeding lots were composed by combining five male and ten female zebrafish. The ratio of one male to two females is optimum for spawning (Eaton & Farley, 1974a). Each of the five breeding lots was distributed respectively into a breeding trap suspended within 50-litre glass aquaria.

Zebrafish adults are known to cannibalize newly fertilized eggs at spawning. Breeding traps ensured the immediate separation of the adults from their clutches. The traps were rectangular boxes, the four walls of which consisted of plexiglass. The bottom of the trap was covered with nylon mesh having a pore size of 2mm. The top of the trap remained open allowing easy access. The entire device was suspended by glass rods in the breeding aquarium (Figure 1). Zebrafish eggs which have a mean diameter of 0.97mm (Laale, 1977) sank through the 2mm mesh following spawning, to settle safely on the floor of the aquarium.

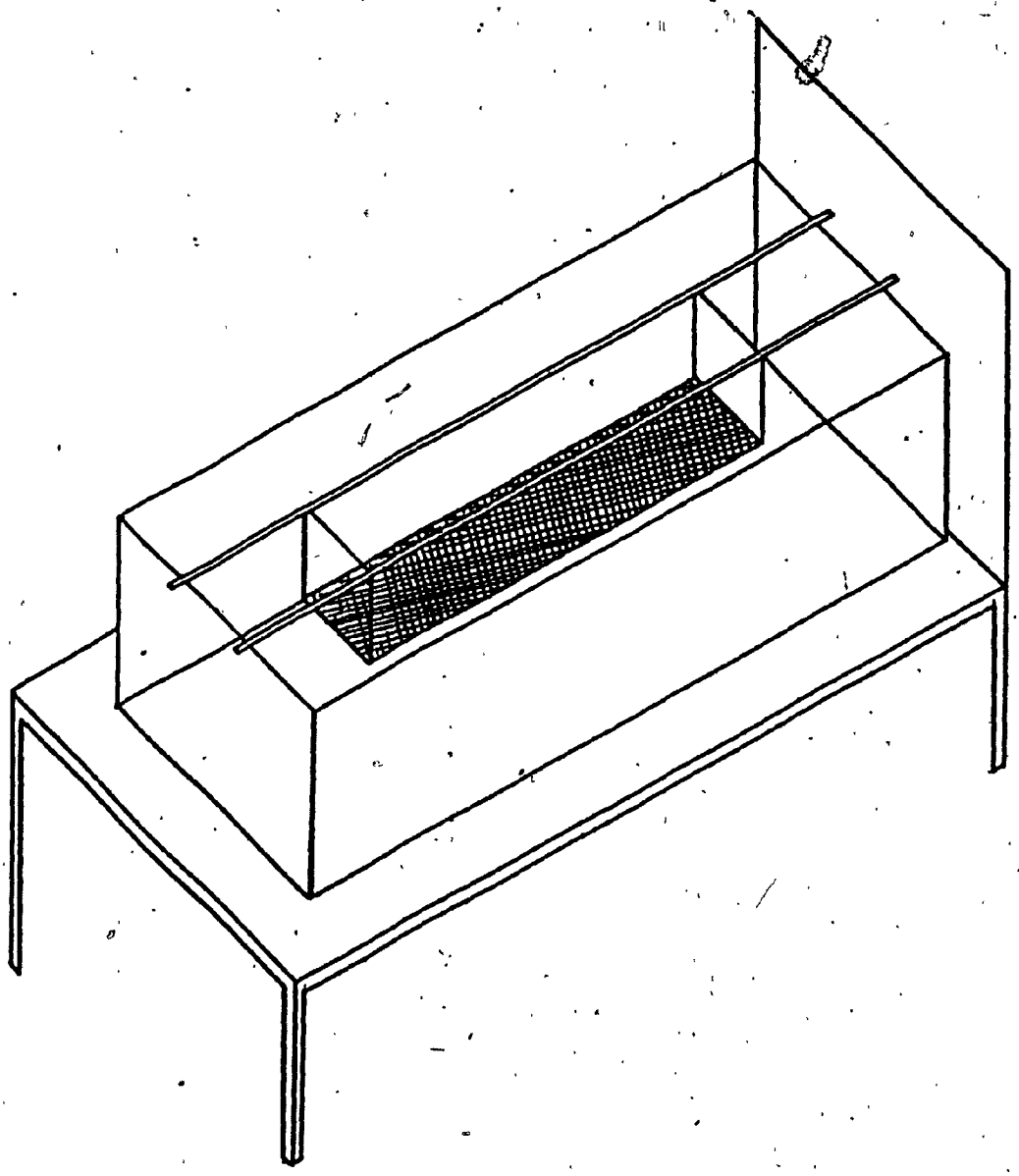


Figure 1. Diagram of the breeding trap suspended in an aquarium.

Temperature, photoperiod, food availability and food type are critical factors governing the activity of spawning (Laale, 1977). Brood stock were fed three times daily . In the morning fish were fed a mixture of beef liver and calf heart, in a ratio of 2:1, that had been minced, dried and pulverized. This mixture has been shown to promote the production of large numbers of viable eggs (Weinstein, 1978). Fish were fed in the afternoon and evening Tetramin staple tropical fish food and freeze dried Tubifex worms. Food wastes and feces, which had fallen to the bottom of the breeding tank during the day were siphoned out in the evening. This procedure limited the amount of debris that might have contaminated the surface of the eggs when spawned the following morning.

The photoperiod of the fish culture room was set at 14 hours light/10 hours dark (6:00 hr. / 20:00hr.). The zebrafish usually spawns with thirty minutes of the onset of light (Eaton & Farley, 1974a). The water temperature of the breeding tanks was 25°C. The optimum temperature for zebrafish spawning activity is between 22°C. and 25°C. (Laale, 1977).

Under these conditions, when adult males and females are together continuously they spawn at frequent and irregular intervals (Eaton & Farley, 1974a). Egg production was monitored over a six day period. The average production of eggs per female per day was 48.68 ± 14.2. During this period, random samples of approximately 100 eggs were examined daily to determine the rate of fertilization. Of the eggs examined 0.225% ± 0.45% were unfertilized.

Egg Collection and Incubation

Each morning, two hours after the onset of light, eggs from the breeding aquaria were collected. This period of time was deemed sufficient for spawning on any given day. Eggs were siphoned from the bottom of the breeding tanks and collected in the following manner. The outflow of the engaged siphon was positioned above a collection vessel(Figure 2). The collecting device consisted of a large reservoir on the top of which set two screens (Endecotts test sieves). The pore size of the upper screen was large enough to allow the eggs to pass through to the lower second screen but prevented the passage of large bits of debris. The pore size of the lower screen was just less than the diameter of the eggs but allowed the passage of small particles of debris into the reservoir. Repeated washing of the second screen tended to remove contaminants from the surface of the egg membrane and any other particulate matter. The eggs were finally flushed from the second screen into a pyrex petri dish. The eggs were distributed, in lots of approximately 100 into a series of plastic petri dishes containing tank water. Any remaining debris or dead embryos were removed with a disposible Pasteur pipette, the tip of which had been flame smoothed to prevent damage to the egg membranes. The culture dishes were covered with their respective lids, labelled with the date and placed into a Fisher Isotemp incubator at 25 $\pm$1^{o}C.

After twenty-four hours of incubation, cultures were re-examined and any dead embryos were removed. Incubation of the living embryos continued for a total period of eight days. Hatching occurred between seventy-two and ninety-six hours post fertilization

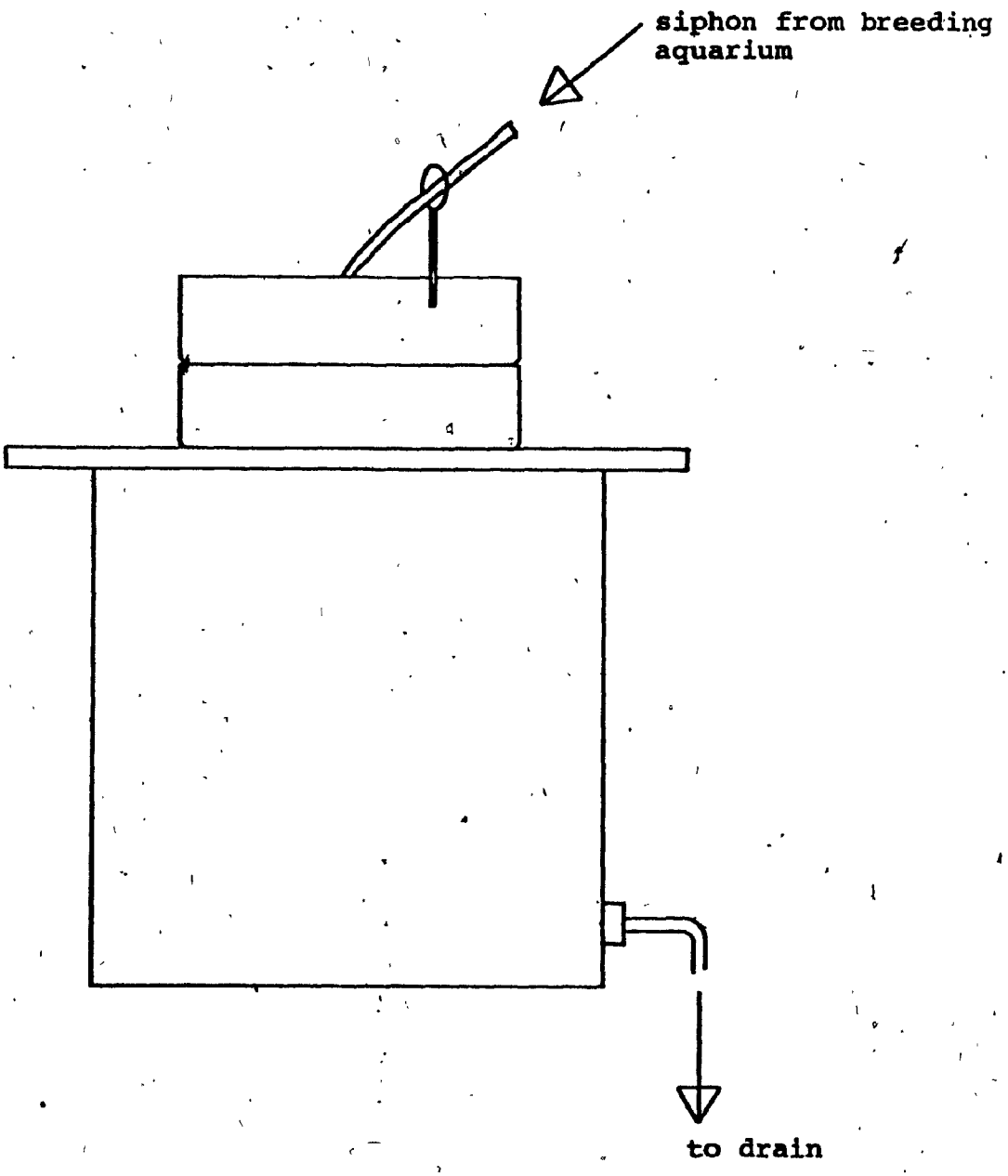


Figure 2. Diagram of the egg collecting device.

and yolk sac absorption was generally completed by the eighth day of incubation. Post hatch larvae were not fed during their incubation period.

Of 2,880 developing zebrafish that were cultured and monitored throughout their incubation, the average mortality rate during this eight day period was 24.73 ±8.65%. Virtually 95% (95.11 ±7.94%) of the deaths occurred within the first twenty-four hours of incubation.

Rearing Procedures

Subsequent to the absorption of the yolk sac, post larval fish were held in four, 50-litre, glass aquaria which were supplied with a continuous flow of water at a temperature of 24±1°C. A perforated plexiglass collar covered with 100 micron nylon mesh surrounded the drain pipe of each culture tank thereby preventing loss of post larval zebrafish.

The post larval culture tanks were seeded with algae and paramecia cultures (Eaton & Farley, 1974b). Food and fecal wastes that had been collected from the bottom of the breeding aquaria were also added to the culture tanks. After two weeks, a substantial community of various micro organisms was established. It was presumed that this infusoria provided a natural food source that supplemented their diet, for unless the infusoria existed in concurrence with post larval zebrafish, the survival rate of the latter was severely reduced. This requirement for the successful culturing of post larval zebrafish has also been observed by Eaton & Farley, 1974b.

Post larval fish were added to each of the culture tanks daily for a period of fourteen days. Their natural diet was supplemented daily by four to six feedings which consisted of Liquifry, Tetramin baby fish food for egg layers and Tetramin staple food for tropical fish, which had been finely ground with a mortar and pestle. With this natural and supplemented food regime, the mortality during this period was minimized. The survival success of this culturing program supplied an abundance of laboratory reared bioassay candidates.

Fish were held under these conditions in these culture tanks for four to six weeks. Then they were transferred to 50-litre glass holding aquaria that were also supplied by a continuous flow of water at a temperature of 24±1°C. Twice daily fish were fed Tetramin baby fish food and Tetramin staple tropical fish food. Excess food and fecal waste were siphoned from the bottom of the tanks daily.

Sexual maturity was reached at approximately ten weeks of culture (Laale, 1977). Some of the sexually mature adults were separated into lots of five males and ten females. These lots replaced the original store bought breeding culture. Only the offspring from the laboratory reared fish were used in the bioassays.

TEST ORGANISMS

The adult zebrafish used in the bioassays were between ten and sixteen weeks old and thus represented the sexually mature phase of the life cycle (Laale, 1977). Five to seven days before their use in toxicity tests, the adults were wet weighed and divided into size classes. The mean wet weight of the individual size classes ranged from 0.1 to 1.0g. and the range in wet weight of individuals within any one weight class never varied more than ±0.2g. from the mean.

Each lot of fish was held separately in an aquarium and allowed to acclimate until their use in the bioassays. Adult test fish were not fed during the bioassays and the twenty-four hour period prior to the bioassays. Each lot of test adult fish consisted of ten to twelve individuals.

Juvenile test subjects were selected from four to seven week old stock fish. This period of the life cycle represented a phase of rapid, exponential growth. The juveniles were reproductively immature (Laale, 1977). Twenty-four hours prior to the bioassay, juveniles were sorted into size classes. Since the weighing procedure, as per adults, was found to harm juveniles, the size classes were defined visually by body width and snout to tail length. Each lot of size defined fish was held separately in an aquarium and allowed to acclimate until the onset of the bioassay.

Test juveniles were placed in specially designed exposure vessels (Figure 3). Two sides and the bottom of these box-like containers consisted of plexiglass. The remaining and opposing two sides were covered with a nylon mesh which had a pore size of 100 microns. This design permitted the steady flux of water or toxicant

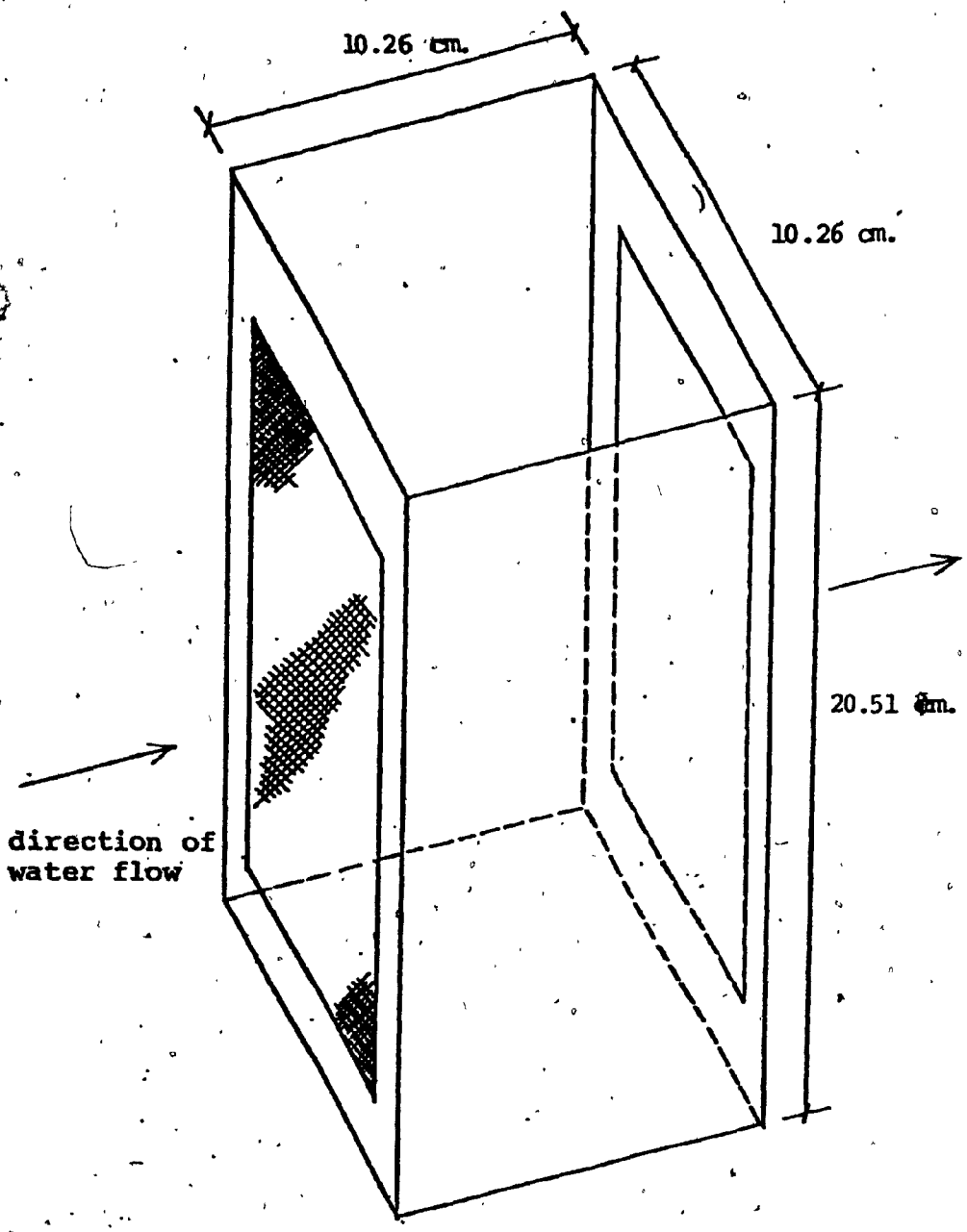


Figure 3. Diagram of the juvenile zebrafish exposure container.

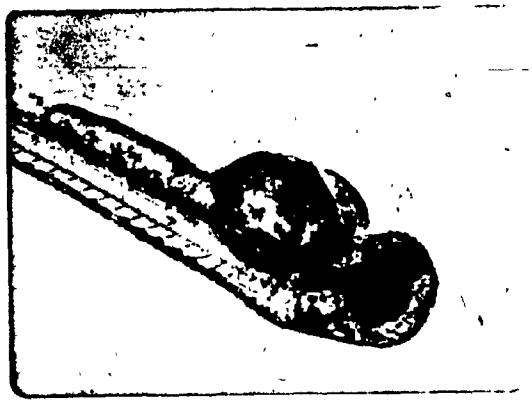
solution between the interior and exterior of the vessel. The top of the container was open allowing introduction of, access to and periodic inspection of test organisms.

These special containers sat on the floor of the larger 50-litre test tanks during a bioassay. The sides of the containers projected above the water line of the larger tank. Each lot of ten to twelve juveniles was introduced in separate exposure vessels at the onset of the bioassay. Juveniles were not fed for twenty-four hours before and during the bioassay.

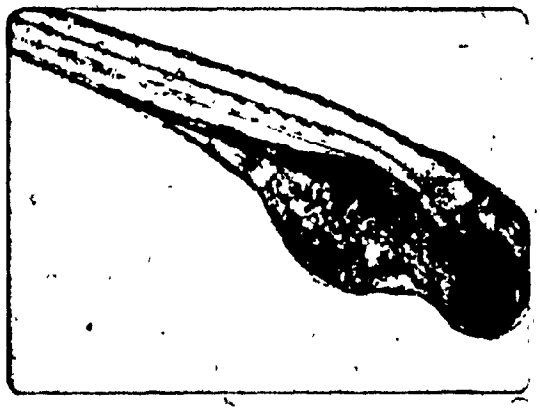
Juveniles in each experimental lot were wet weighed at death or at termination of the toxicity test. Before weighing, individuals were blotted dry with a Kimwipe tissue to remove any excess water on their surface. Living specimens were first anaesthetized with M.S. 222. The range in wet weight of individuals within any one size lot never varied more than ±10.6mg. from the mean. Expressed as percent variation, the weight of an individual within any one size lot never varied more than 55% from the mean (5.63 ± 3.1mg.). The mean wet weight of the juvenile test lots ranged from 2.8 to 62.5mg.

The age of all post larval zebrafish at the start of bioassays was eight days post fertilization. This age in the life cycle represents a transitional phase in nutrition from endogenous to exogenous food sources. Prior to eight days old and following hatching the yolk sac is observed to progressively diminish in size. At eight days the larval yolk sac is no longer visually apparent (Figure 4). According to Skidmore, 1967, individuals more than eight days old, either successfully forage for food within the next five days or begin to die of starvation.

four days



six days



eight days

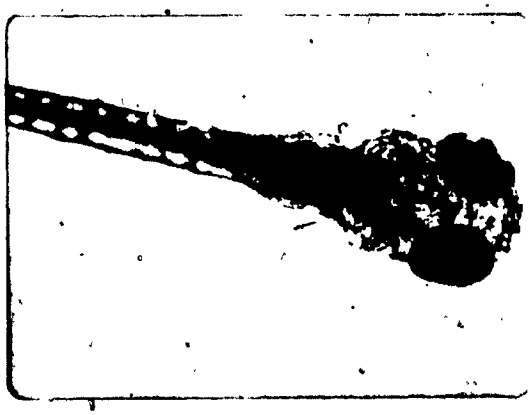


Figure 4. Photographs of larval zebrafish at four, six and eight days post fertilization.

During a bioassay, post larvae were held in specially designed containers which in turn were submerged in the larger 50-litre tanks. The containers consisted of a small piece of clear plexiglass tubing (Figure 5). The transparent walls of the exposure vessel allowed visual inspection of the individuals during the bioassay without necessitating the handling of the post larvae. The open ends of the tube were covered with a nylon mesh which had a pore size of 100 microns. This design allowed the unimpeded flux of water or toxicant solution between chambers as verified by dispersion of methylene blue solution from within the vessel. Access to the containers was gained through a hole in the center of the plexiglass wall. During the experiment, this hole was plugged with a cork. Post larval fish were introduced and removed via this port.

Ten to twenty post larval zebrafish were transferred from the incubator into each exposure vessel twenty-four hours prior to an experiment. The containers were then placed in a 50-litre acclimating aquarium and held under control conditions until the onset of the bioassay. Post larval fish were not fed prior to or during toxicity tests. Visually the size of eight day old fish did not appear to vary greatly. The wet weight of this group was represented by determinations based on ten individuals that were anaethetized with M.S. 222, blotted dry with Kimwipe tissues and weighed individually using a Metler M5 microgram-atic balance.

The egg stage in these experiments represented individuals that were tested initially during blastulation or early gastrulation (three to six hours post fertilization) and exposed through to hatching (Laale, 1977; Hisaoka & Battle, 1958). At 25 ± 1°C., hatching of control eggs occurs within the assigned period of the

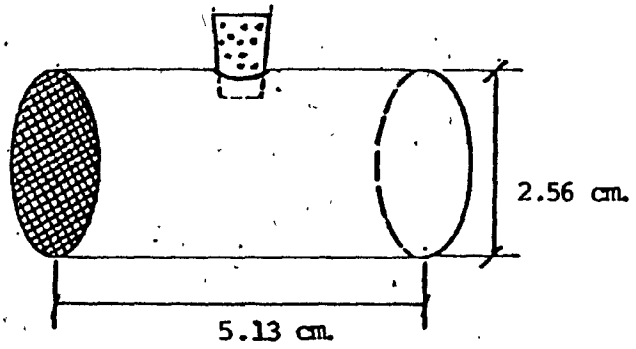


Figure 5. Diagram of the post larval zebrafish exposure container.

bioassay (Laale, 1977).

In the morning prior to a bioassay, eggs were collected from breeder tanks, carefully separated from any particulate matter on their membrane surface and examined for viability. Only fertilized eggs were selected for the various test lots. Each lot consisted of twenty to twenty-five viable eggs. During the bioassay the eggs were contained in exposure vessels identical to those housing the post larvae. This containment was necessary to prevent predation from copepods, which were normally present in the laboratory water supply. The egg containers were placed on the floor of the larger 50-litre test tanks.

DILUTER APPARATUS

Concentrated stock solutions of cadmium and zinc were prepared by dissolving reagent grade $CdCl$ and $ZnCl$ in glass distilled water. The concentrations of the toxicant stock solutions were between 10,000 and 50,000 p.p.m. depending on the required concentration range of each bioassay. The stocks were acidified to a pH of 2 by adding concentrated hydrochloric acid. The low pH of the toxicant stocks prevented precipitation of the stock solutions. Each toxicant's stock solution was held in a separate 18-litre Mariotte bottle (Grenier, 1960). The solutions dripped at a controlled rate from these containers into a funnel. A tube from the funnel transferred the solution to the diluter apparatus.

Bioassays of pure solutions of cadmium and zinc were carried out using a two phase serial diluter (Figure 6a). The hydraulic heads of the source water or toxicant solution in the first and middle chamber respectively were constantly maintained by standpipe drains. Toxicant, dripping at a constant rate, from the Mariotte bottle entered a funnel into which a required flow rate of water from the first chamber was delivered by a glass faucet. This funnel led into the second chamber or toxicant chamber of the diluting apparatus. Ten glass faucets of the first chamber delivered water at the required flow rates to the ten mixing chambers, which constituted the lower chambers. Ten glass faucets of the toxicant chamber delivered toxicant at specified flow rates to a corresponding chamber of a series of ten, in the immediately lower stage. The relative proportions of water and toxicant entering the lower stage determined the concentrations of toxicant that would be delivered from the

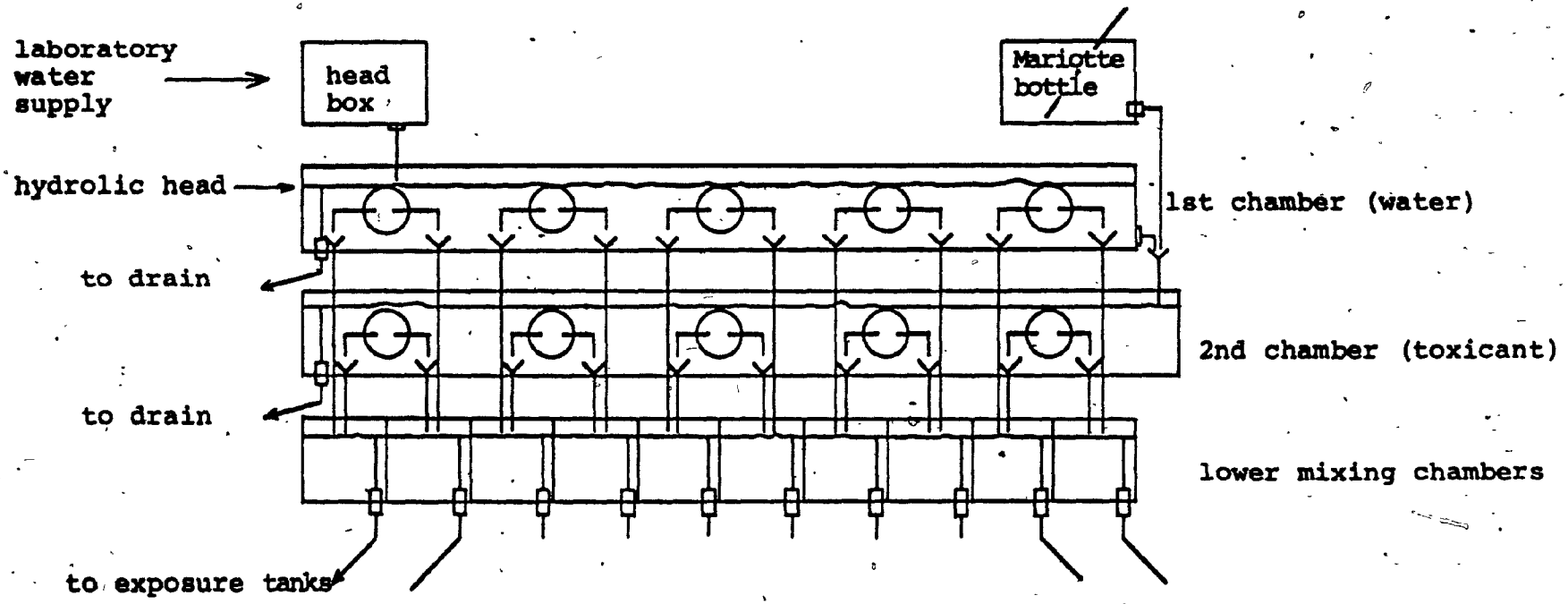


Figure 6a. Schematic diagram of the two phase serial diluter.

lower stage to the ten exposure tanks.

Cadmium and zinc multiple bioassays were conducted utilizing a three phase serial diluter (Figure 6b). The design of this diluter was essentially the same as that of the two phase diluter used for the discrete bioassays. However, instead of having only one toxicant chamber, the three phase diluter had two, each receiving either cadmium or zinc solutions from their respective Mariotte bottles.

Toxicant solution, flowing at a constant rate, from the cadmium Mariotte bottle, entered a funnel in which a required flow rate of water from the first chamber was also collected. The contents of this funnel flowed into the second chamber or cadmium chamber. Toxicant solution, flowing at a constant rate from the zinc Mariotte, entered a funnel in which a required flow rate of water from the third chamber was also collected. The contents of this funnel flowed into the fourth chamber or zinc chamber. Twelve glass faucets of the second chamber delivered the cadmium solution at specified flow rates into funnels that led to a corresponding funnel of a series of twelve at the immediately lower third chamber. Twelve glass faucets of the third chamber delivered water at specified flow rates to the corresponding funnels of the third chamber. The contents of the funnels of the third chamber flowed into a corresponding funnel of a series of twelve at the immediately lower fourth chamber. Twelve glass faucets of the fourth chamber delivered the zinc solution at specified flow rates to the corresponding funnels of the fourth chamber. The funnels of the fourth chamber led to a corresponding chamber of a series of

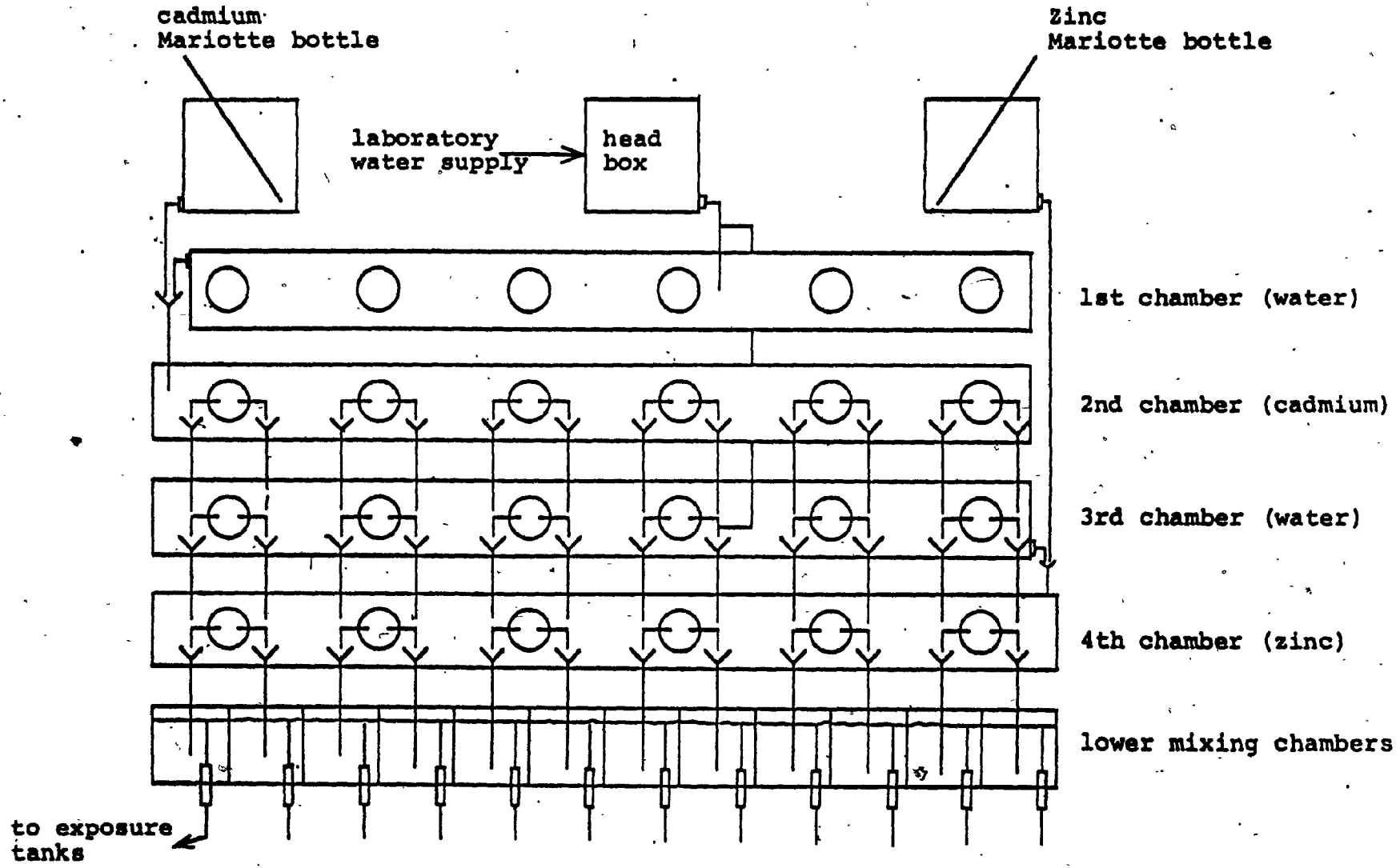


Figure 6b. Schematic diagram of the three phase serial diluter.

twelve in the immediately lower stage. The relative proportions of water, cadmium and zinc entering the chambers of the lower stage determined the concentrations of toxicants that would be delivered from the lower stage to the twelve exposure tanks.

Sample calculations determining the relative proportions of water and toxicant solutions necessary to establish given toxicant concentrations in the exposure tanks are as follows:

1) The flow rate of the cadmium solution from the cadmium Mariotte = V_1(mls/min).

2) The concentration of cadmium in the cadmium Mariotte is 10,000 p.p.m. = C_1.

3) The outflow of the cadmium solution from the second chamber is 800 mls/min. = V_2.

4) The required cadmium concentration of the second chamber is 50 p.p.m. = C_2.

$$V_1C_1 = V_2C_2$$

$$X(10{,}000) = 800(50)$$

$$X = 4 \text{ mls/min.}$$

1) The flow rate of the 50 p.p.m. cadmium solution from the second chamber into tank #1 = V_3 mls/min.

2) The concentration of cadmium in the second chamber is 50 p.p.m. = C_3.

3) The total outflow to tank #1 is 300 mls/min. = V_4. This flow rate ensures a 90% replacement of toxicant solution within six hours (Sprague, 1973).

4) The concentration of cadmium required in tank #1 is 4.5 p.p.m. = C_4.

$$V_3C_3 = V_4C_4$$
$$V_3(50) = 300(4.5)$$
$$V_3 = 27 \text{ mls/min.}$$

The cadmium concentration of exposure tank #1 is 4.5 p.p.m. This concentration results from the mixing of a 50 p.p.m. cadmium solution flowing at the rate of 27 mls/min. and water flowing at a rate of 273 mls/min.

The flow rates of water or toxicants entering the exposure tanks were regulated by adjusting the glass faucets at each stage of the serial diluter. The required flow rates were achieved by collecting the outflow of each faucet in a graduated cylinder for one minute. Each glass faucet was adjusted until the desired flow

rate was established.

The diluter apparatus was operational twenty-four hours before a bioassay was commenced. This period of time was sufficient to allow the required toxicant concentration to be established in each of the exposure tanks. The flow rates of water and toxicant solutions from the glass faucets of the diluter apparatus were checked and readjusted if necessary each day of the four day bioassay.

DESIGN OF LETHAL BIOASSAYS

All lethal bioassays were conducted for a period of ninety-six hours. Dissolved oxygen, pH and temperature of the exposure tanks were monitored daily. Dissolved oxygen was determined by the azide modification of the iodometric method (APHA et al., 1965). Water hardness and toxicant concentrations of the test solutions were determined using a Perkim Elmer 503 flame atomic absorption spectrophotometer. The physico-chemical characteristics of the solutions in the exposure tanks are listed in Table I.

Samples of toxicant solutions from the exposure tanks and water samples from the control tank were collected daily in 40 ml. glass test tubes. Samples were acidified by adding 100 ul. of concentrated hydrochloric acid per 40 ml. sample volume. This procedure minimized the surface absorption of the heavy metal solutions by the walls of the glass sampling tubes (Weinstein, 1978). Periodically, duplicate samples were collected from each tank. One of each sample was microfiltered with a 0.45 micron filter before the sample was acidified with concentrated hydrochloric acid. Toxicant concentrations of each sample were then measured. In no case was a significant difference observed between the toxicant concentrations in each of the duplicate samples. Thus, one may assume that the measured toxicant concentrations of both samples represented the soluble or dissolved form, excluding species of a particulate size (E.P.A., 1976).

During the bioassays, the mortality and time to death of the test organisms were monitored. The criteria for death of the adult zebrafish was cessation of opercular activity. Absence of the heartbeat was the criteria for mortality of the juvenile, post larval

and embryonic stages in which heartbeat had been initiated. The mortality of the embryonic stages prior to the initiation of heartbeat was determined by an opaque membrane. This opacity of the egg membrane occurred shortly after the death of all embryos (Skidmore, 1964).

The transparency of the plexiglass containers permitted direct microscopic examination of embryonic and post larval test organisms. During the bioassay the exposure vessels were transferred to pyrex petri plates which were filled with the aqueous medium of the respective exposure tank. The container was then transferred to the stage of a dissecting microscope with which the eggs and post larvae were examined. The number of deaths were recorded and dead individuals were removed. Exposure vessels were then promptly returned to their respective test tank.

Juveniles who were apparently moribund when examined within their exposure vessel, were removed and placed into pyrex petri dishes filled with medium from their respective exposure tanks. These individuals were examined more closely and under higher magnification with a dissecting microscope. In certain individuals the heart was observed to be still beating although weakly and slowly. In these cases the organism was promptly returned to the original exposure container. Deaths were recorded and dead individuals were removed.

DATA ANALYSIS

Lethal response data representing the cumulative percent mortality through ninety-six hours of exposure were fitted to linear regressions according to Finney, 1971. For this purpose the cumulative percent response of individuals in each test lot was converted to a probit unit (Appendix I). These quantities were then plotted against the mean ambient concentrations of cadmium and zinc. With the aid of a computer program (Weinstein, 1978), a linear regression was fitted to the determined coordinates. Ninety-five percent fiducial limits and correlation coefficients were computed for each regression. Each linear regression was described by the following equation:

$$y = a + b \log x \qquad (1)$$

where y = probit response

a = y intercept

b = slope

x = ambient concentration (mg/L) (Finney, 1971)

Rationale for an Empirical Approach to the Study of Multiple Toxicity

Theoretical models describing the quantal response of test organisms to mixtures of toxicants have been proposed by Bliss, 1939; and Plackett & Hewlett, 1952. 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.

Arbitrarily in these experiments, the concentration of the mixture of toxicants was expressed in equipotent units of the most potent constituent in the mixture. Thus the concentration of constituent "B" in the mixture may be expressed by an equipotent concentration of constituent "A"; the latter being the reference toxicant. Units of equipotency are determined for any two toxicants from their respective concentration response curves as follows:

$$\log_{10} B_A = \log_{10} B - (I - Y)\left(\frac{1}{b_A} - \frac{1}{b_B}\right) \qquad (2)$$

where,

B_A = Concentration of B in A equivalent units.

B = Concentration of B in the mixture.

I = The ordinate value at which the concentration response regression equations for A and B intersect.

Y = Probit response elicited by the magnitude of B as determined by the linear equation describing the concentration response relationship for B.

b_A = Slope of the concentration response regression equation for A.

b_B = Slope of the concentration response regression equation for B.

(Anderson et al., 1979)

The Model of Similar Joint Action or Concentration Addition

Concentration addition (Anderson & d'Apollonia, 1978) of constituents in a mixture occurs when the discrete toxicants have the same receptor site of critical target within the affected organism. One would expect the response of test organisms exposed to a mixture, whose constituents are all known hepatotoxins, to comply with the model of concentration addition. In such a mixture one constituent may be replaced by an equipotent amount of the other constituent without altering the potency of the mixture.

When exposed to a mixture of similarly acting toxicants, the distribution of susceptibilities of individuals, within randomly selected test lots, should be the same. As such, those organisms which are most susceptible to constituent "A" will also be most susceptible to constituent "B". The slope of the quantal response curve is representative of this distribution in susceptibility. Thus this characteristic of the discrete quantal response curves should not be significantly dissimilar for similarly acting constituents.

The potency of a mixture whose respective constituents conform to the model of concentration addition can be predicted from information describing the toxicity of the discrete constituents.

$$Y_c = a_A + b_A \log_{10} (A + B) \qquad (3)$$

where Y_c = Predicted response to the mixture in probits.

a_A = Y intercept of the regression equation describing the response of the organism to the reference toxic constituent "A", in the mixture.

b_A = Slope of concentration response regression line for constituent "A".

A = Concentration of constituent "A" in the mixture.

B = Concentration of constituent "B" in the mixture in "A" equivalent units.

(Bliss, 1939)

The Model of Independent Joint Action or Response Addition

When present as constituents in a mixture, toxicants having different receptor sites or critical targets within the test organism, act independently (Bliss, 1939). The toxic action of each of the components involves a unique and characteristic series of reactions that ultimately lead to the failure of a distinct vital system in each case (Bliss, 1939). The variance in susceptibility of the test organism to constituent "A" may or may not be correlated with the variance in susceptibility to constituent "B". Thus, similarity of the slopes of the discrete quantal response regressions is not a criteria for the response addition (Anderson & d'Apollonia, 1978) of constituents in a mixture.

The potency of a mixture containing independently acting toxicants may be predicted from the discrete quantal response regressions of the constituents. 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:

$$Pc = Pa + Pb - PaPb \qquad (4)$$

where,

Pc = Proportion responding to the mixture.

Pa = Proportion responding to "A" alone.

Pb = Proportion responding to "B" alone.

(Bliss, 1939)

When those animals most susceptible to "A" are also most susceptible to "B", then there is exact parallelism in susceptibility and r = 1.

$$Pc = Pa \text{ if } Pa > Pb \qquad (5)$$

$$Pc = Pb \text{ if } Pb > Pa$$

(Bliss, 1939)

If the correlation in susceptibility is completely negative, r = -1, then those individuals most sensitive to "A" will be most tolerant to "B" or vice versa and:

$$Pc = Pa + Pb \text{ if } Pa + Pb \leq 1 \qquad (6)$$

(Bliss, 1939)

Each independently acting constituent of a mixture evokes a response in the test organisms only when its ambient concentration exceeds that which just fails to elicit a response. Thus, if the concentration of one toxicant in the mixture is below threshold,

it should not contribute to the potency of that mixture. Similarly, if the concentration of one constituent exceeds that which elicits a 100% response, then the contribution of this toxicant to the total concentration of the mixture will be that which yields a probit response corresponding to 99.9%. These characteristics of response addition are considered in calculating the total concentration of the independently acting mixture by the following equation:

$$\log (CM) = \log M - \frac{(\log \frac{M}{A})(\log \frac{M}{B})}{\log \frac{M}{T}} \qquad (7)$$

where,

CM = The effective concentration of the mixture in "A" equivalent units.

M = Concentration of "A" which in pure solution evokes 99.9% response.

A = Concentration, if any, of "A".

B = Concentration, if any, of "B" in "A" equivalent.

T = Threshold concentration of "A" in pure solution, i.e. that below which no response will be elicited.

If "A" or "B" < T then "A" or "B" = 0

If "A" or "B" > M then "A" or "B" = M

(Anderson et al., 1979)

In accordance with the model of response addition, the predicted response of the test organism to the mixtures is a discontinuous concentration response relationship. For this reason, the statistical treatment of data with reference to this type of joint action is difficult. A Pearson chi square test may be implemented to compare the observed and predicted responses, however, this test ignores the errors of estimation of the linear regressions of the discrete constituents and, therefore, may exaggerate the significance of discrepancies from the curve (Finney, 1971). Furthermore, the application of a Pearson chi square test to the results of bioassays having a recommended sample size of ten to twelve individuals per exposure concentration necessitates the pooling of small observed response frequencies. This manipulation results in a significant decrease in the available degrees of freedom and may lead to erroneous conclusions.

The theoretical models of concentration and response addition are mutually exclusive. Additive constituents in a binary mixture of toxicants may act either at the same receptor or at different receptor sites within the test organism. In the absence of empirical data that conforms to the model of concentration addition the observed response may be better represented by the model of response addition. However, a sample size greater than that now recommended for toxicity tests, would be necessary to statistically support the representation of response addition.

Supra-additive Synergism, Infra-additive Antagonism

When the potency of a mixture of toxicants is not additive and does not conform to the theoretical models of concentration or response addition, it is not possible to predict the response of the test organism to the mixture from a knowledge of the quantal response relationships of each discrete constituent. If the empirical multiple toxicity data exceeds the predictions of additivity, than the joint action of the constituents in the mixture is supra-additive synergism (Bliss, 1939). If the empirical multiple toxicity data is less than the predicted additive potency then the joint action of the constituents in the mixture is infra-additive antagonism (Bliss, 1939).

Body Weight; A Modifying Factor

The effective dose of an administered drug may be modified by such processes as its absorption, distribution, metabolism and excretion, all of which are uniquely size dependent (Adolph, 1949). The effective dose may, therefore, be a complex function of the administered dose. In measuring the effectiveness of a drug, it has been advocated that the dose administered be normalized to the body weight of the recipient thereby, minimizing the variation in the effective dose between animals grouped according to body weight (Bliss, 1936; Rall & North, 1953).

The response of several species of fish to a number of aquatic contaminants has been expressed in relation to the body weight of test fish (Anderson & Weber, 1975; Spear & Anderson, 1975). This weight related tolerance pattern is mathematically expressed by the

following allometric equation:

$$y = a + b \log x/w^h \qquad (8)$$

where y = response (probits)

a = Y intercept

b = slope

x = ambient concentration (mg/L)

w = weight

h = an empirically derived weight related factor

(Anderson & Weber, 1975).

Thus when h = 0, the response of the organism is independent of body weight. When h = 1, there exists a direct proportionality between measured parameters. With every ten fold increase in the body weight of the test organism there is a corresponding decrease in the effective concentration to which the organism is exposed. When $0 > h < 1$ the exponent expresses a disproportionality between body weight and response.

Utilizing a computer program designed for the search of a weight related factor between ± 2.00, the concentration response data of all bioassays were analyzed (Weinstein, 1978). The weight related factor selected was that which generated the best correlation between response and effective concentration ($\log x/w^h$).

RESULTS

Discrete Toxicant Bioassay

The cumulative ninety-six hour response of the zebrafish egg, post larval, juvenile and adult test stages exposed to fixed concentrations of cadmium and zinc are listed in Tables II, III and Figures 7, 7a-7d, 8 & 8a-8d. These data were analyzed with the aid of a computer program designed for Finney's method of probit analysis (see Data Analysis, Materials & Methods). Regression equations delineating the response of the organism as a function of ambient concentration were generated for each of the four life cycle stages exposed to discrete solutions of cadmium and zinc (Equation 1). The derived regression equations and "t" values for the respective correlation coefficients are compiled in Table IV.

In the cadmium, zinc and mixtures bioassays, natural rates of embryo mortality of 20.8, 20.8 and 4% respectively were observed. Abbott's formula (Finney, 1971) might have been implemented to compensate for natural mortality rates, however, this correction may not have been applicable to these bioassay results. As seen in Tables II and III, the mortality rate of developing embryos exposed to low levels of cadmium and zinc is less than that of the controls. Pickering and Gast, 1972 and Eaton, 1973 have also reported a reduction in the mortality rate of developing fathead minnow, Pimephales promelas Rafinesque, embryos exposed to low levels of cadmium and mixtures of cadmium, zinc and copper. These low levels of the heavy metals may be prophylactic against fungal and bacterial contamination of the control embryos was not, therefore,

Table II. Lethal response data of the four life cycle stages of the zebrafish exposed to discrete solutions of cadmium

Life cycle stage of test organism	Mean assayed cadmium concentration (mg/L ± S.D.)	Mean wet weight of test organism (mg.± S.D.)	Number of organisms exposed	Observed % mortality at 96 hrs.
Egg	0	-	24	20.8
	16.03 ± 2.36	-	25	8.0
	16.03 ± 2.36	-	25	16.0
	22.47 ± 3.25	-	25	44.0
	22.47 ± 3.25	-	24	29.2
	22.90 ± 2.89	-	25	48.0
	22.90 ± 2.89	-	26	19.2
	29.60 ± 3.73	-	24	62.5
	29.60 ± 3.73	-	25	44.0
Post larvae	0	0.21 ± 0.13	20	5.0
	0.54 ± 0.10	0.21 ± 0.13	20	25.0
	0.54 ± 0.10	0.21 ± 0.13	20	15.0
	0.68 ± 0.09	0.21 ± 0.13	20	10.0
	0.68 ± 0.09	0.21 ± 0.13	20	30.0
	0.93 ± 0.31	0.21 ± 0.13	18	33.3
	1.43 ± 0.47	0.21 ± 0.13	18	44.4
	1.43 ± 0.47	0.21 ± 0.13	19	57.9
	2.20 ± 0.44	0.21 ± 0.13	20	75.0
	2.20 ± 0.44	0.21 ± 0.13	19	100.0
	2.60 ± 0.40	0.21 ± 0.13	20	95.0
	2.60 ± 0.40	0.21 ± 0.13	18	100.0
	3.30 ± 0.51	0.21 ± 0.13	18	94.4
Juveniles	0	-	10	0
	1.73 ± 0.25	7.70 ± 0.56	10	10.0
	2.58 ± 0.58	2.77 ± 1.15	10	10.0
	2.93 ± 0.34	5.72 ± 1.70	10	40.0
	3.95 ± 0.37	9.40 ± 0.85	10	20.0
	5.08 ± 0.53	9.72 ± 4.11	10	50.0
	6.38 ± 1.39	11.34± 4.32	10	70.0
	6.88 ± 1.07	10.35± 2.32	11	72.7
	9.33 ± 1.28	10.81± 3.85	10	90.0
	12.30 ± 1.34	16.66± 5.79	10	100.0
	16.65 ± 2.90	10.83± 3.67	10	100.0
Adult	0	500.0	12	0
	1.31 ± 0.19	500.0	12	0
	2.09 ± 0.31	500.0	12	0
	2.91 ± 0.33	500.0	12	0
	4.79 ± 0.49	500.0	12	8.33
	6.00 ± 0.73	500.0	12	8.33
	7.20 ± 0.98	124.5	10	20.00
	7.86 ± 1.0	500.0	12	33.33
	9.33 ± 1.28	124.5	10	70.00
	9.53 ± 1.48	124.5	10	40.00
	9.85 ± 0.87	500.0	12	66.66
	12.42 ± 1.51	124.5	10	100.0
	16.65 ± 2.90	174.5	9	100.0

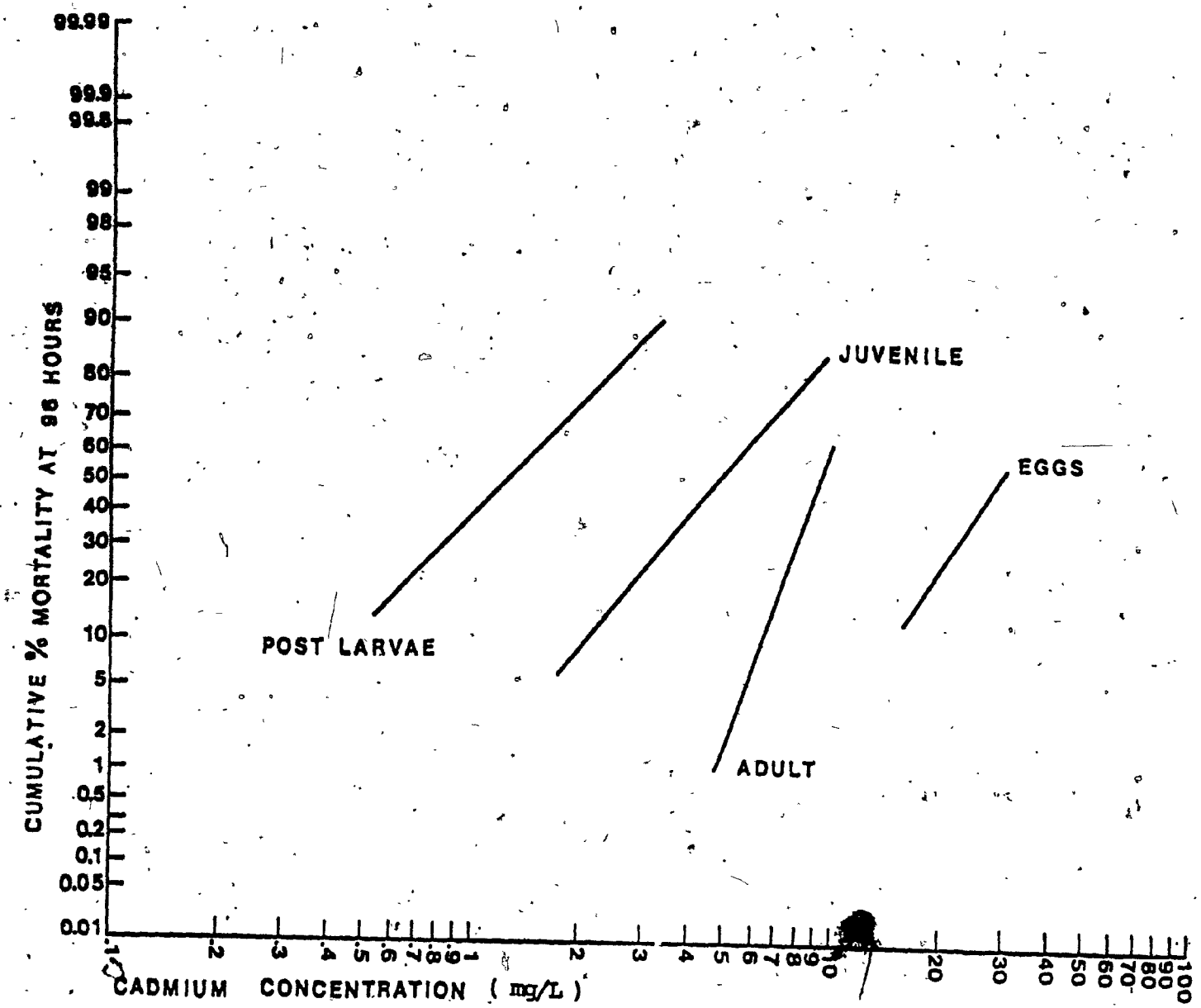


Figure 7. The response of the four test life cycle stages of the zebrafish exposed to discrete solutions of cadmium.

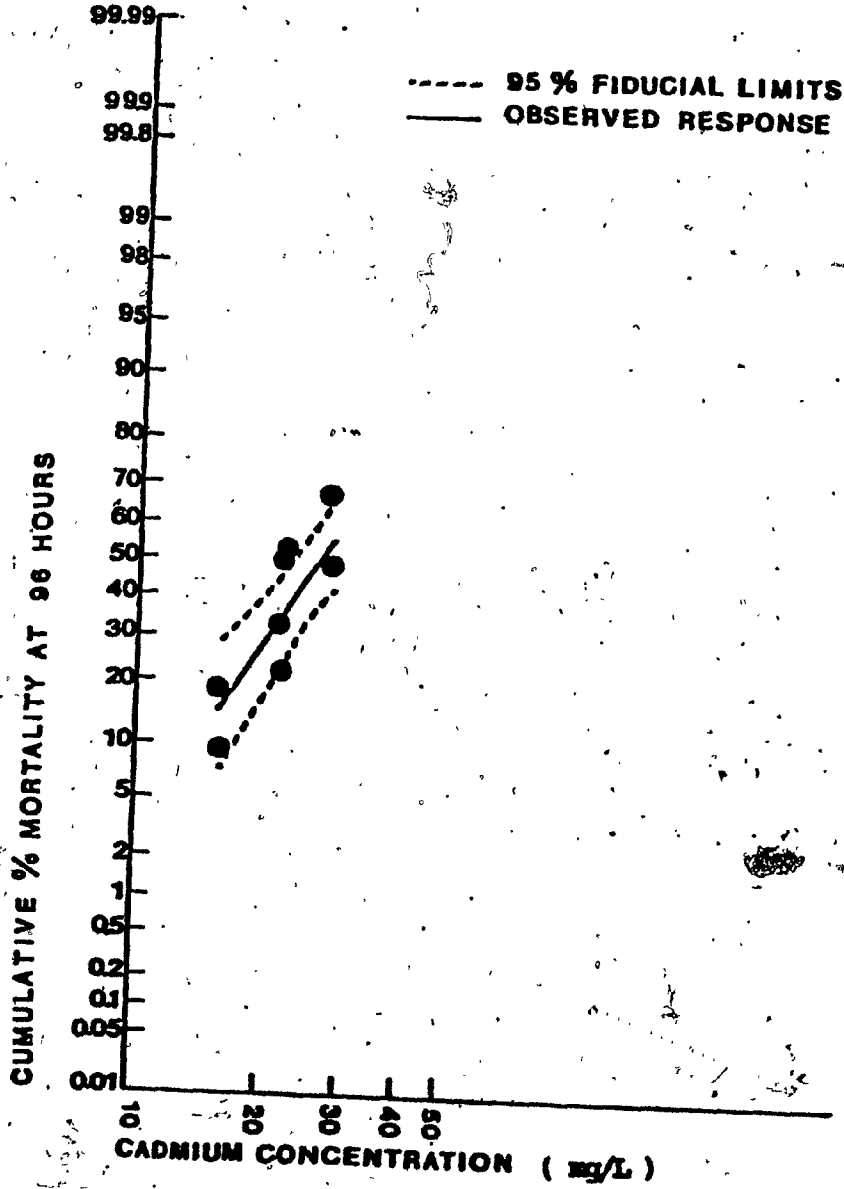


Figure 7a. The response of the zebrafish egg exposed to discrete solutions of cadmium.

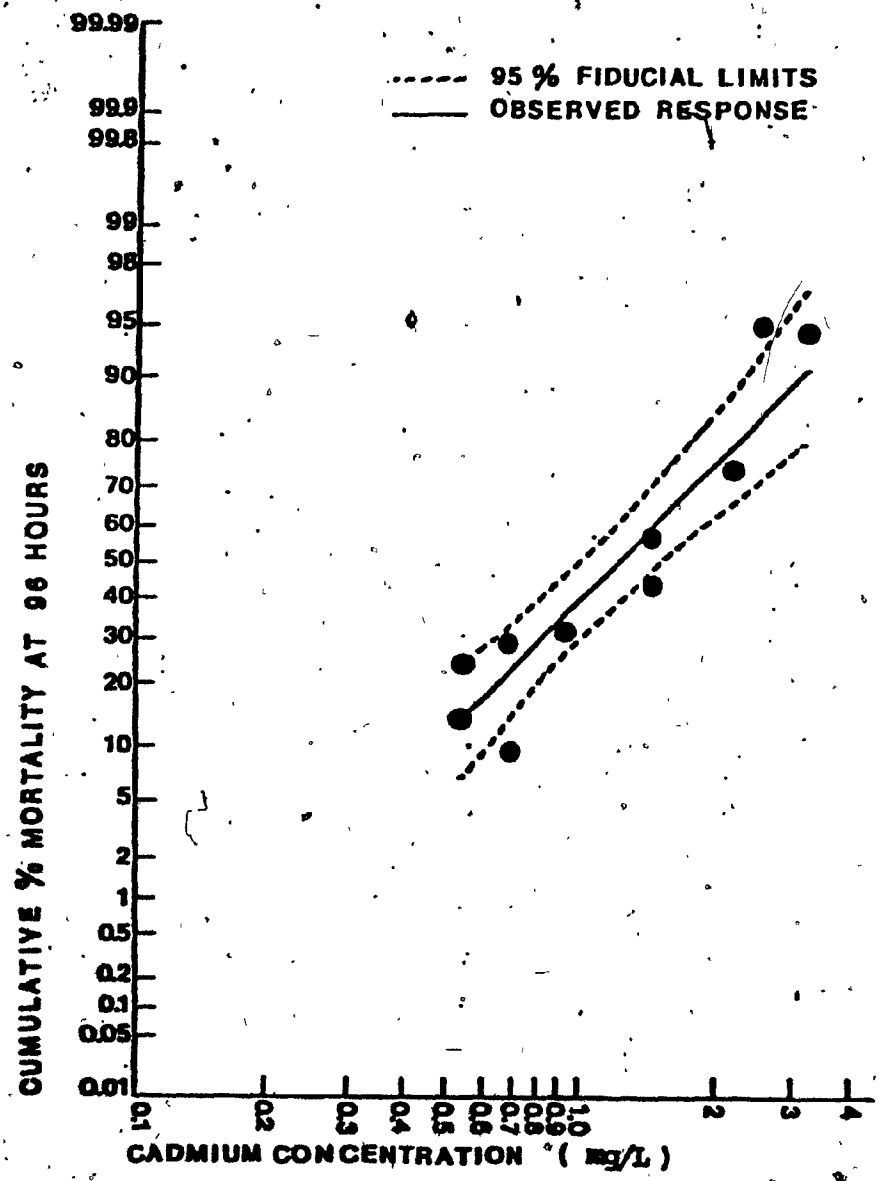


Figure 7b. The response of the post larval zebrafish exposed to discrete solutions of cadmium.

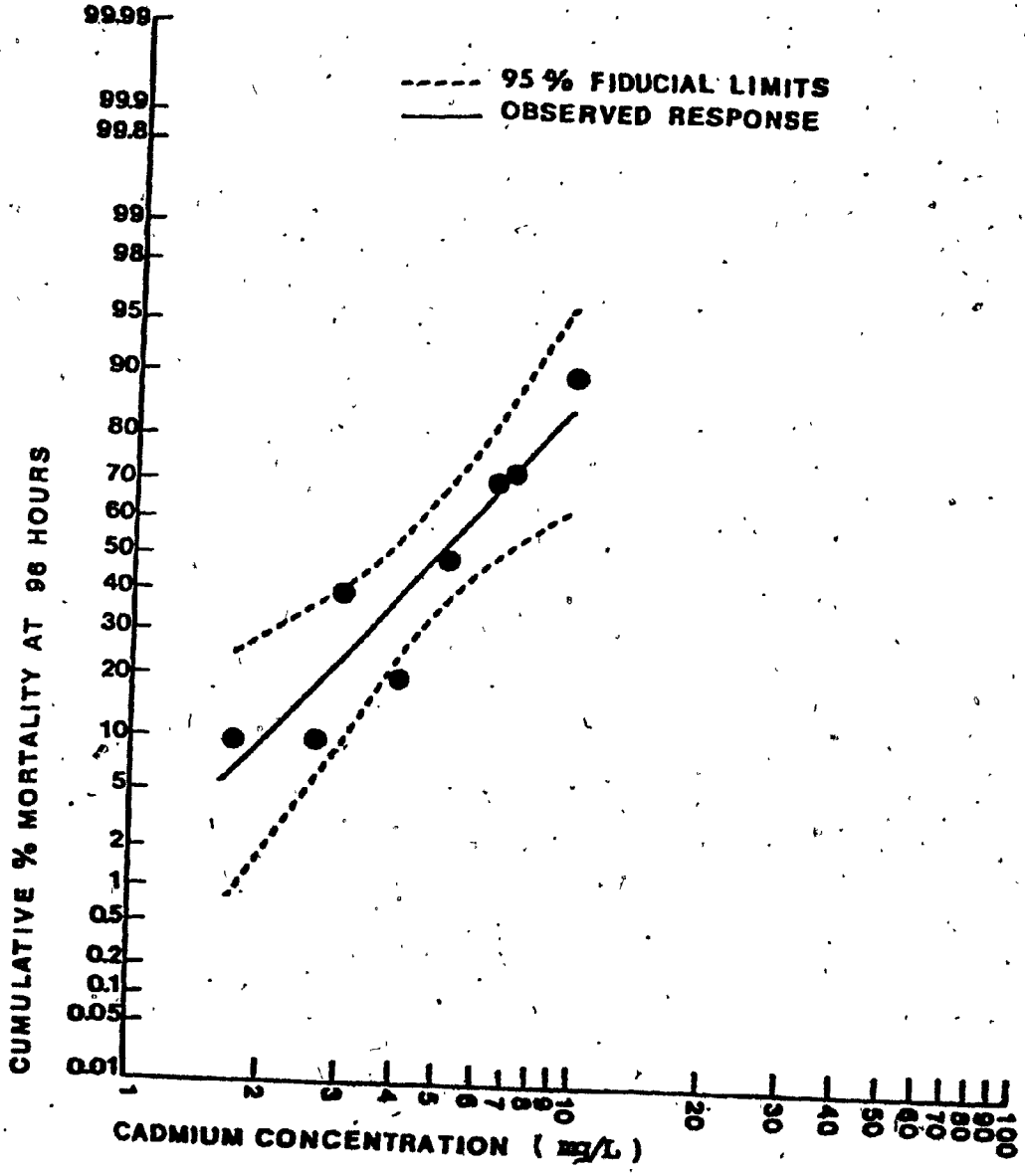


Figure 7c. The response of the juvenile zebrafish exposed to discrete solutions of cadmium.

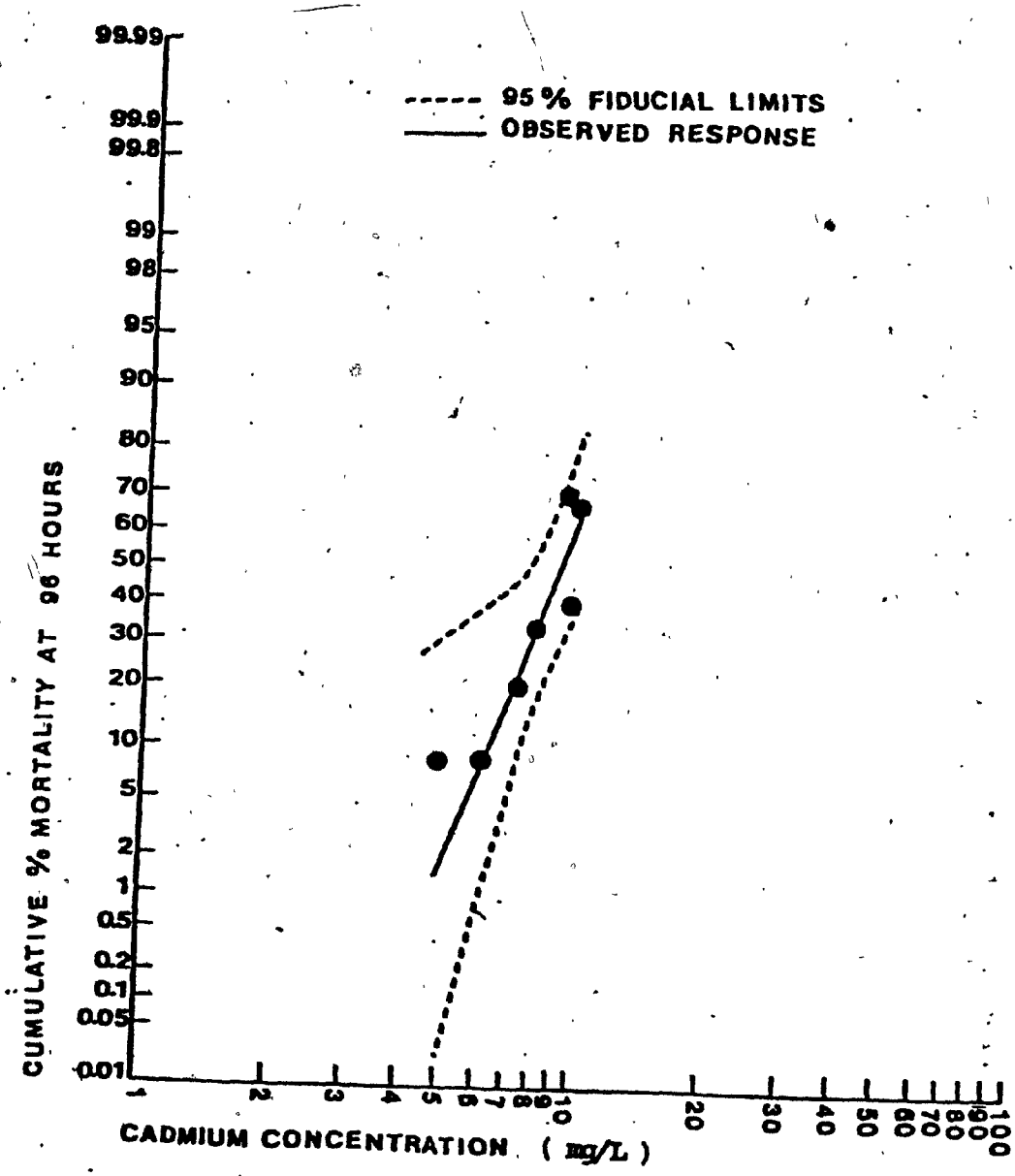


Figure 7d. The response of the adult zebrafish exposed to discrete solutions of cadmium.

Table III. Lethal response data of the four test life cycle stages of the zebrafish exposed to discrete solutions of zinc

Life cycle stage of test organism	Mean assayed zinc concentration (mg/L ± S.D.)	Mean wet weight of test organism (mg.± S.D.)	No. of organisms exposed	Observed % mortality at 96 hrs.
Egg	0	0	24	20.8
	6.83 ± 0.49	-	25	12.0
	9.10 ± 0.74	-	25	16.0
	9.10 ± 0.74	-	26	15.4
	11.60± 0.76	-	25	36.0
	13.60± 2.24	-	25	68.0
	14.40± 1.04	-	25	52.0
	14.40± 1.04	-	24	58.3
	15.98± 0.40	-	25	60.0
	21.63± 0.85	-	25	76.0
	23.85± 3.68	-	25	88.0
	28.15± 3.74	-	25	96.0
Post larvae	0	0.21 ± 0.13	20	0
	5.10 ± 0.70	0.21 ± 0.13	20	30.0
	5.10 ± 0.70	0.21 ± 0.13	19	21.1
	5.50 ± 0.63	0.21 ± 0.13	21	23.8
	5.50 ± 0.63	0.21 ± 0.13	19	15.8
	6.30 ± 1.10	0.21 ± 0.13	20	35.0
	6.30 ± 1.10	0.21 ± 0.13	20	40.0
	7.38 ± 0.22	0.21 ± 0.13	10	60.0
	7.90 ± 0.48	0.21 ± 0.13	20	35.0
	7.90 ± 0.48	0.21 ± 0.13	19	31.6
	9.04 ± 0.39	0.21 ± 0.13	10	50.0
	9.60 ± 1.23	0.21 ± 0.13	20	75.0
	9.60 ± 1.23	0.21 ± 0.13	21	66.6
	11.15± 0.49	0.21 ± 0.13	10	100.0
	15.60± 0.57	0.21 ± 0.13	10	100.0
Juvenile	0	-	10	0.0
	4.70 ± 0.58	19.80± 6.70	10	10.0
	6.69 ± 2.11	16.70± 4.78	10	30.0
	7.06 ± 0.63	10.67± 2.78	10	10.0
	8.05 ± 1.14	8.10 ± 2.26	10	20.0
	8.84 ± 0.71	8.17 ± 1.98	10	10.0
	9.20 ± 1.35	-	10	0.0
	12.95± 1.70	4.10 ± 1.85	10	50.0
	14.40± 0.80	6.40 ± 0.67	10	80.0
Adults	0	-	10	0
	9.00 ± 0.08	449.50	10	0
	12.00± 0.66	149.50	10	0
	12.03± 0.43	249.50	9	11.1
	15.00± 1.44	175.00	10	80.0
	15.20± 1.42	175.00	10	40.0
	15.45± 0.29	149.50	10	30.0
	15.48± 0.35	349.50	10	30.0
	18.83± 1.10	449.50	10	90.0
	19.75± 0.59	149.50	10	60.0
	21.23± 1.51	175.00	10	90.0

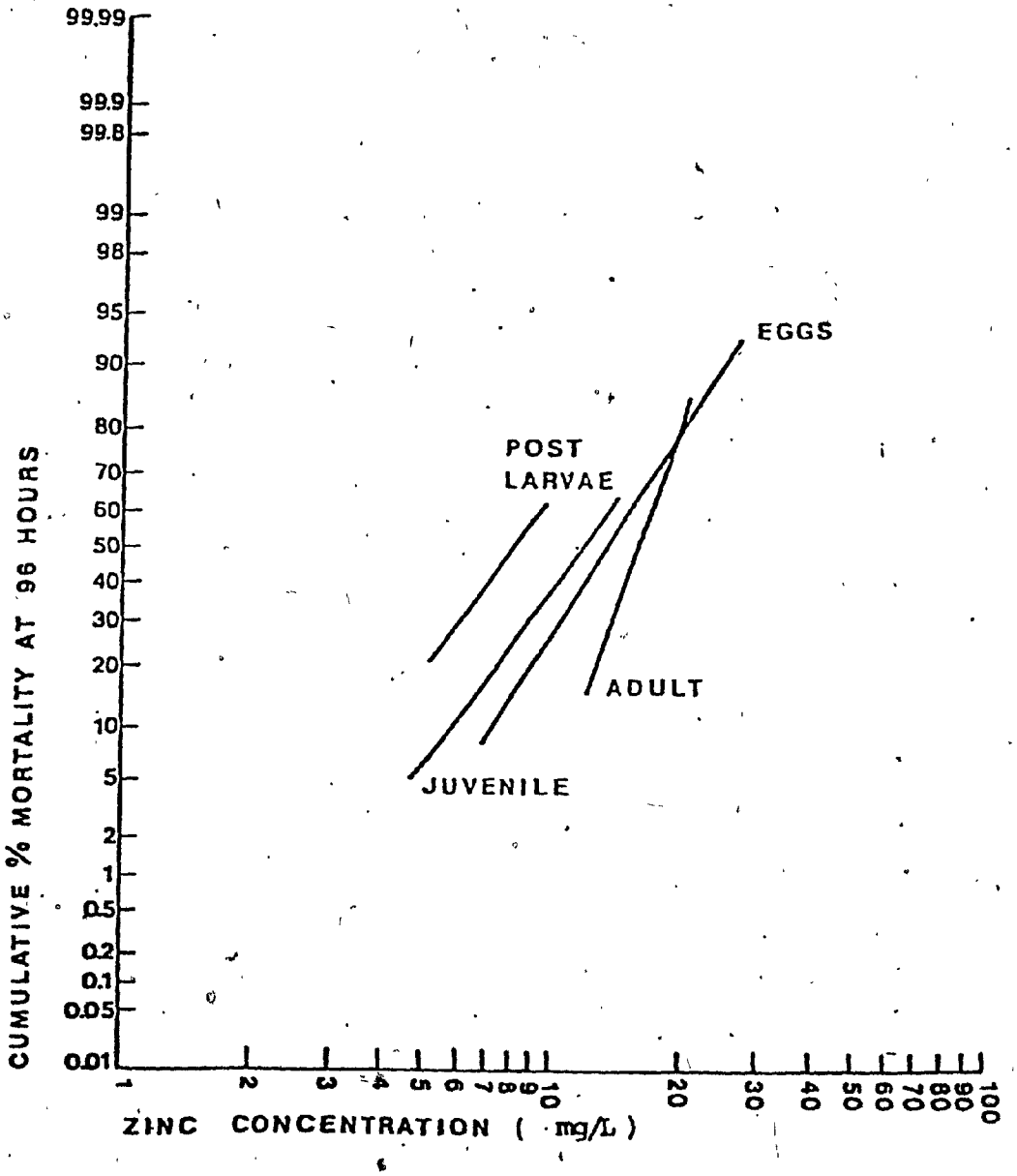


Figure 8. The response of the four test life cycle stagesof the zebrafish exposed to discrete solutions of zinc.

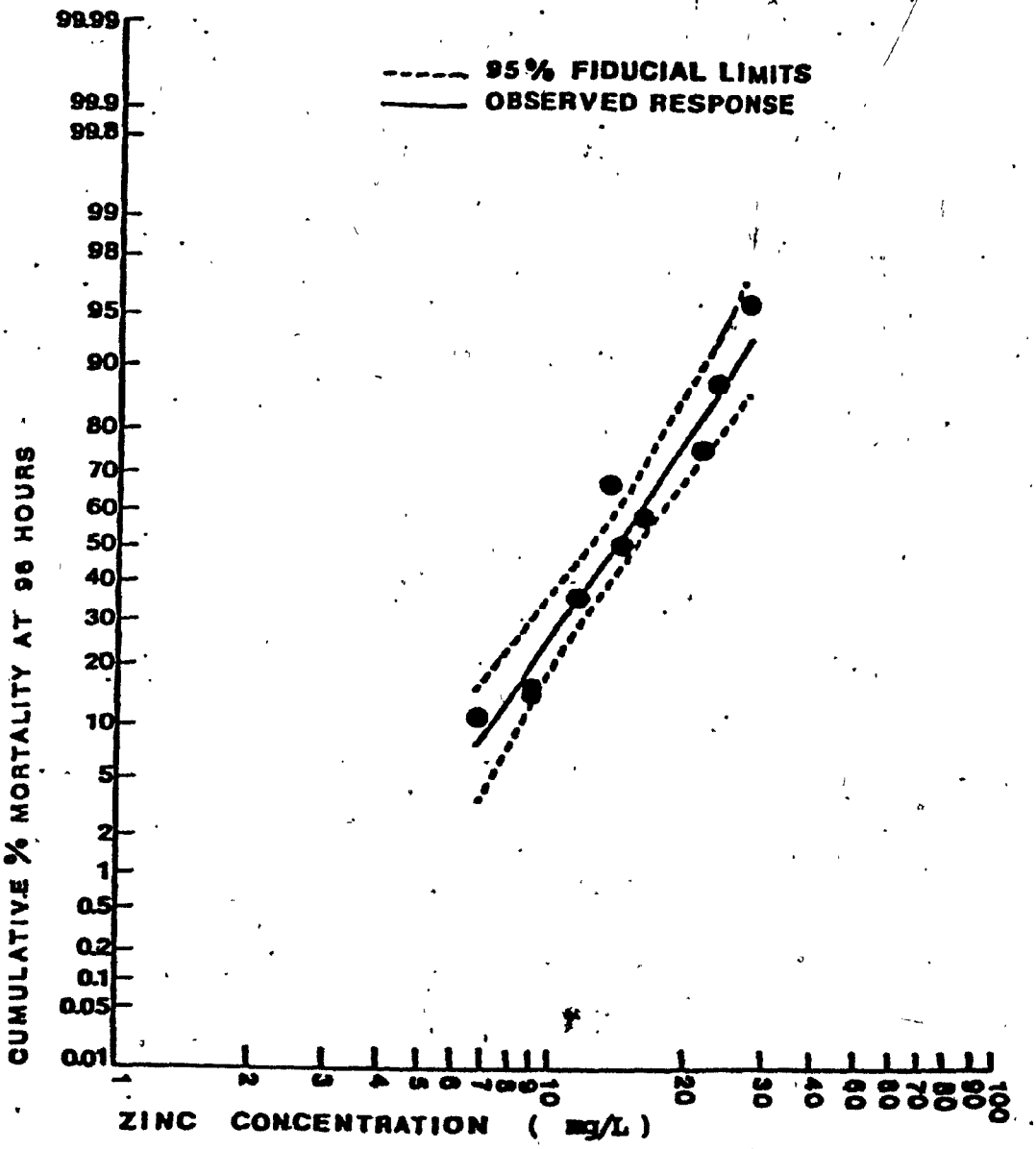


Figure 8a. The response of the zebrafish egg to discrete solutions of zinc.

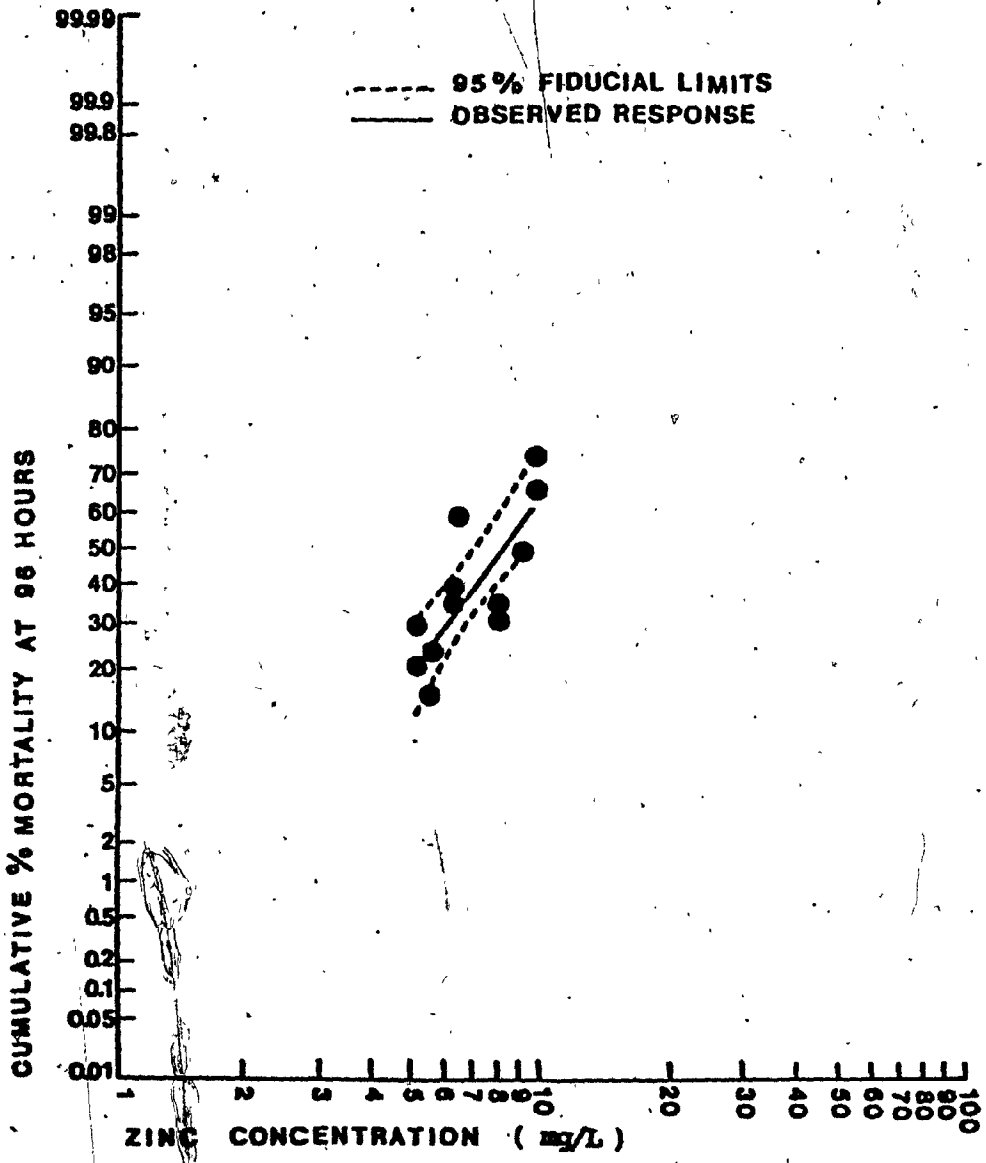


Figure 8b. The response of the post larval zebrafish exposed to discrete solutions of zinc.

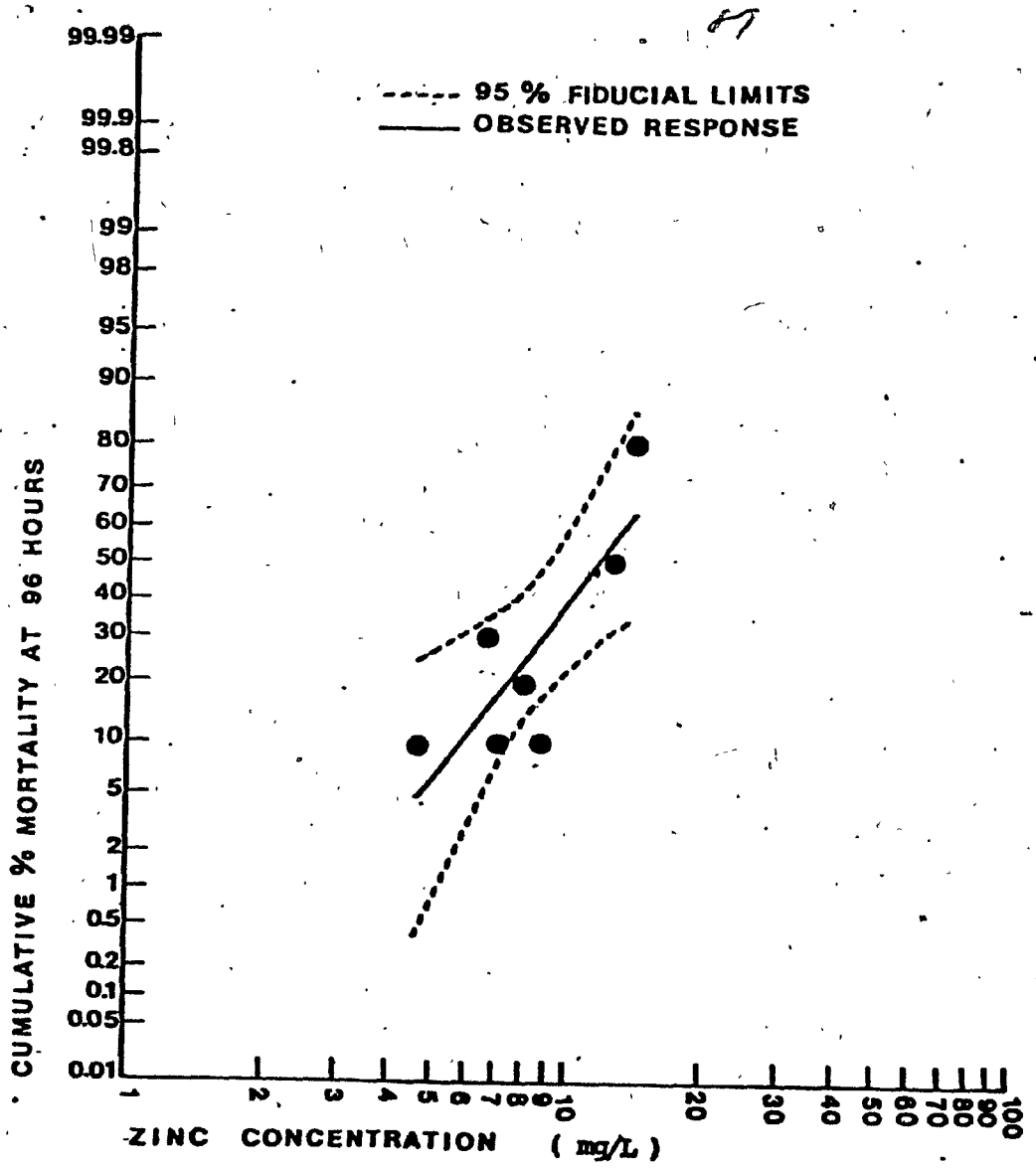


Figure 8c. The response of the juvenile zebrafish exposed to discrete solutions of zinc.

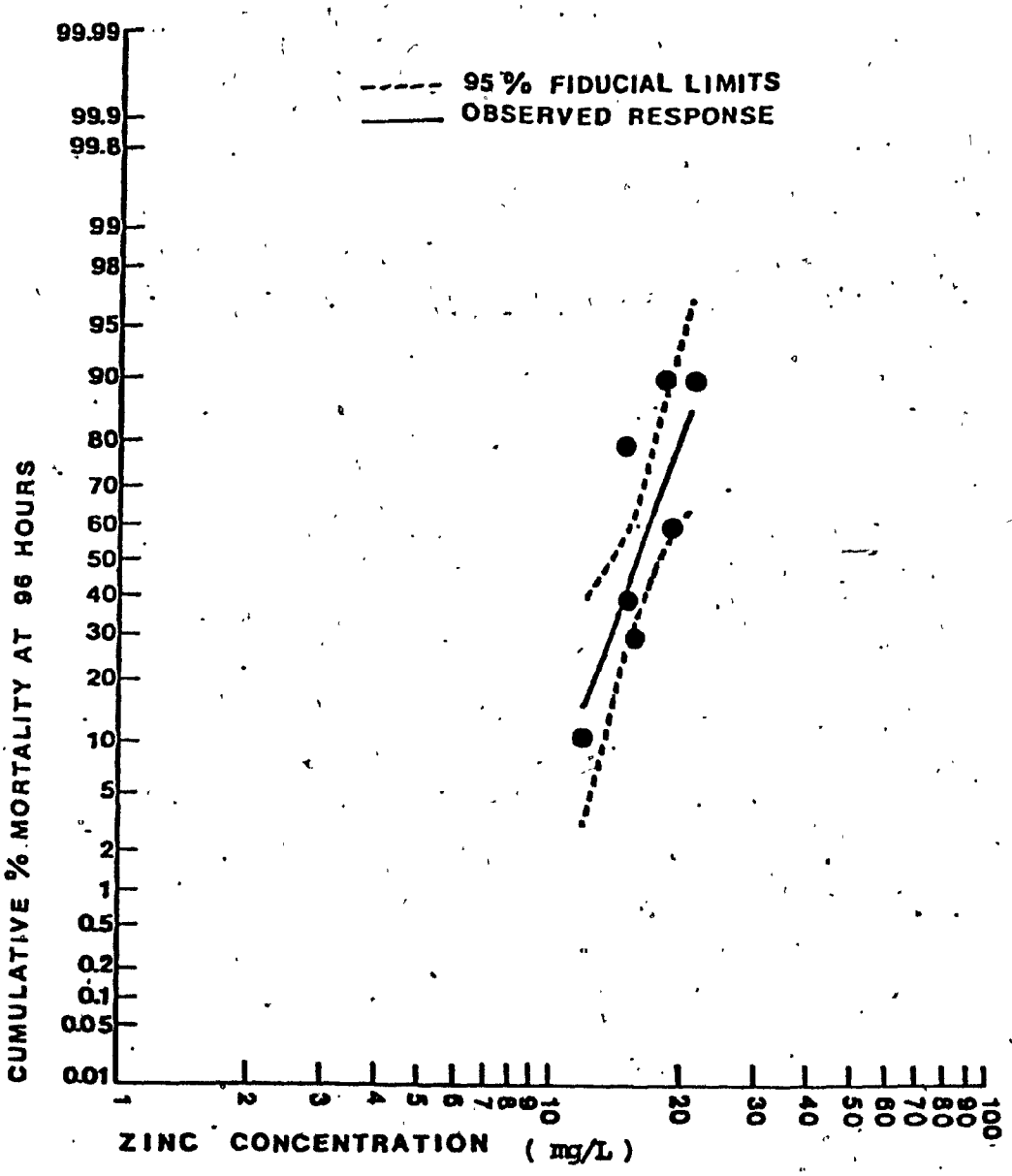


Figure 8d. The response of the adult zebrafish exposed to discrete solutions of zinc.

Table IV. Regression analysis of the lethal response data of the four test life cycle stages of the zebrafish exposed to discrete solutions of cadmium and zinc.

Regression Equation: $Y = a + b \log x$

Toxicant:	CADMIUM				ZINC			
Life Cycle Stage:	Adult	Juvenile	Post Larvae	Egg	Adult	Juvenile	Post Larvae	Egg
a	-2.726	2.622	4.762	-1.662	-5.043	0.633	1.297	-.272
b	8.111	3.517	3.053	4.609	8.360	4.053	4.088	4.664
r = correlation coefficient	0.908	0.917	0.930	0.826	0.735	0.822	0.837	0.958
n = number of test concentrations generating 0.1 - 99.9% response	6	8	10	8	8	7	12	11
computed students "t" for r	4.33	5.63	7.16	3.59	2.66	3.23	4.84	10.02
level of significance of r (n-2 = df)	$p \leq .02$	$p \leq .01$	$p \leq .001$	$p \leq .02$	$p \leq .05$	$p \leq .05$	$p \leq .001$	$p \leq .001$
estimated LC_{50} (mg/L)	8.96	4.74	1.20	27.89	15.90	11.95	8.05	13.50

generally applied to the response of the exposed embryos.

The sample sizes of the lots tested life cycle stages was not constant for each stanza; i.e adult test lots consisted of ten to twelve individuals while the egg test lots contained twenty to twenty-five. Variance in susceptibility between the four life cycle stages tested was examined and was not found to be significantly different at $p = 0.01$.

Data recorded during the larval, juvenile and adult discrete bioassays were pooled and again analyzed by computer. The program used for this analysis was designed to quantitate the influence of the mean wet body weight of the lots of exposed fish on the magnitude of their response to the toxicants (Weinstein, 1978). Regression equations describing the relationship between body weight of the lots of test organisms and their response to a range of fixed concentrations of cadmium and zinc were generated (Equation 8). The pool larval, juvenile and adult quantal response data was also analyzed with the aid of the computer program designed for Finney's method of probit analysis (Weinstein, 1978).

The respective correlation coefficients of the concentration response (Finney, 1971 - Equation 1) and effective concentration response (Anderson & Weber, 1975 - Equation 8) regression equations were compared (Table V). The incorporation of the weight correlated factor greatly reduced the departure of empirical data points from a linear representation. The weight correlated factors which generated the best correlation coefficients for the regressions of the discrete cadmium and zinc quantal response relationships were 0.31, and 0.10 respectively (Figures 9 & 10).

Table V. Concentration response and effective concentration response regression analysis of the pooled quantal response data of three life cycle stages of the zebrafish exposed to discrete solutions of cadmium and zinc

Regression Equation	Concentration Response $Y = a + b \log x$	Effective Concentration Response $Y = a + b \log x/w^h$
Cadmium		
a	4.50	4.01
b	0.71	3.20
h	-	0.31
r=correlation coefficient	0.328	0.879
computed students "t" for r	1.665	8.841
level of significance of r (n=25)	$p \leq 0.2$	$p \leq 0.001$
Zinc		
a	2.60	0.86
b	2.19	4.25
h	-	0.10
r= correlation coefficient	0.597	0.788
computed students "t" for r	3.72	6.41
level of significance of r (n=27)	$p \leq 0.01$	$p \leq 0.001$

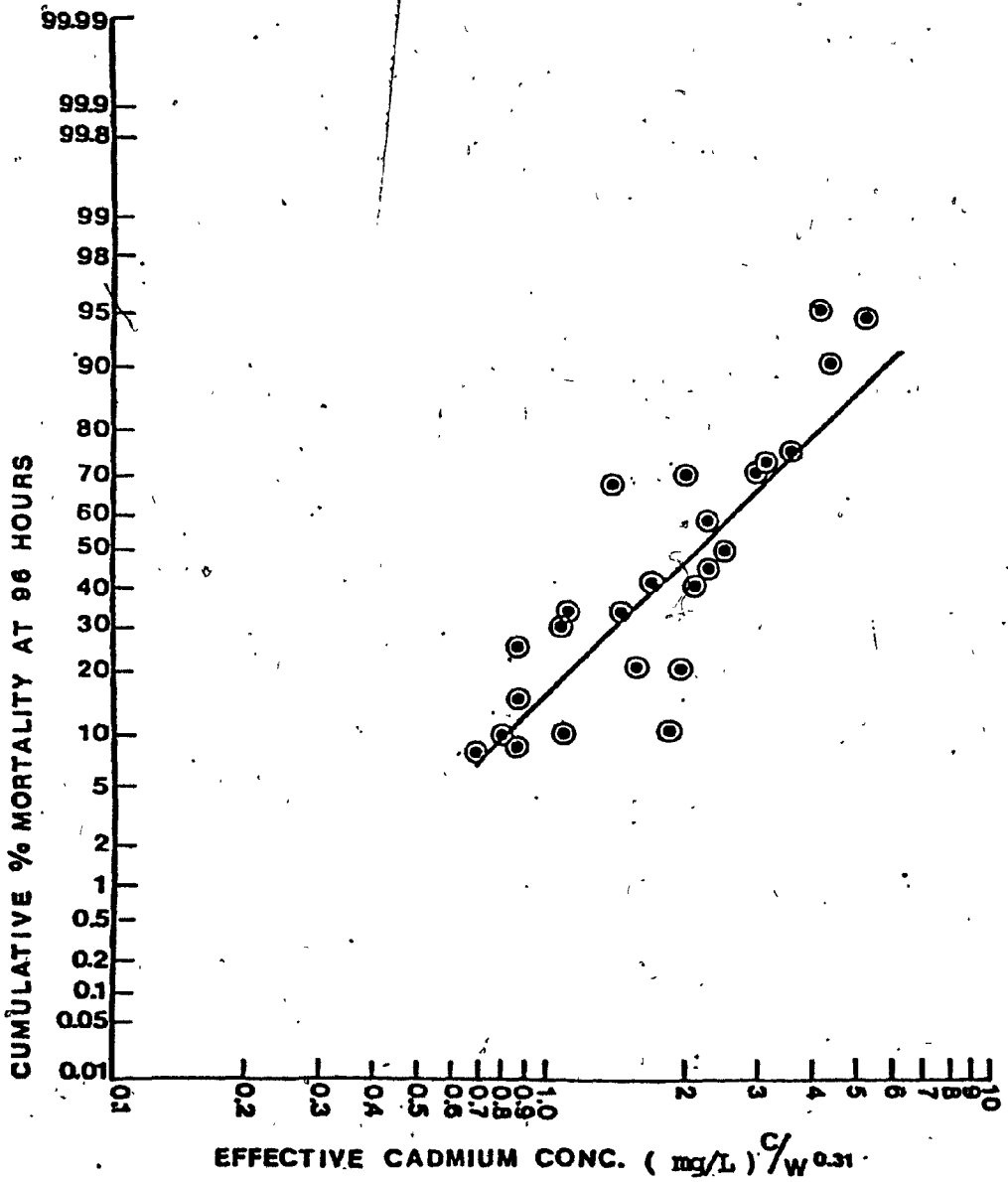


Figure 9. The effective concentration response relationship of the zebrafish adult, juvenile and post larvae exposed to discrete solutions of cadmium.

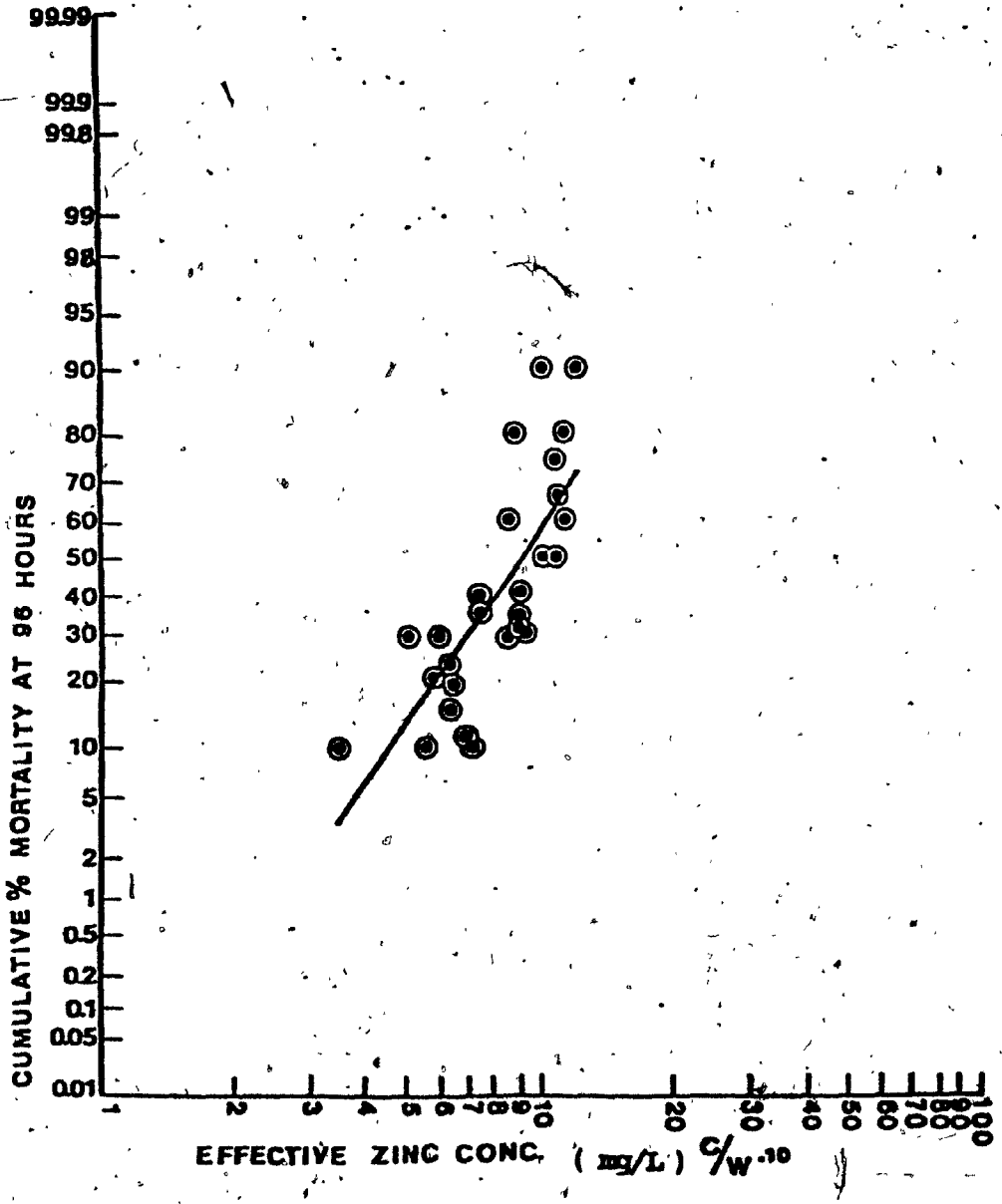


Figure 10. The effective concentration response relationship of the zebrafish adult, juvenile and post larvae exposed to discrete solutions of zinc.

Multiple Toxicity Patterns

The slopes of the regressions describing the response of test organisms to cadmium and zinc discretely were compared statistically. For all life cycle stages tested there was no significant difference at $p = 0.05$ between the slopes of the cadmium and zinc concentration response regression lines. Similarity in the slopes of the discrete regressions may suggest the concentration addition of cadmium and zinc mixtures (Bliss, 1939; Anderson & d'Apollonia,1978). In accordance with this model Finney's method of probit analysis was applied to the quantal response data of the four test life cycle stages of the zebrafish exposed to mixtures of cadmium and zinc (Tables VI - IX). The characteristics of the respective regression equations and "t" values for the corresponding correlation coefficients are compiled in Table X.

The predicted response of the zebrafish to similarly acting mixtures of cadmium and zinc is the concentration response regression of the discrete reference toxicant (i.e. cadmium). The slopes of the observed and predicted concentration response regressions for the four life cycle stages were compared statistically and found to be significantly similar at $p=0.05$. Each of the observed quantal response curves fell within the 95% fiducial limits of their respective predictions (Figures 11 - 14).

The model of response addition was also considered in examining the response of the four tested life cycle stages of the zebrafish to mixtures of cadmium and zinc (Tables XI - XIV). Non linearity of the

Table VI. Lethal response data of the egg stage of the zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Observed response at 96 hrs. (probits)	Predicted response at 96 hrs. (probits)
0	0	0	25	3.25	-
0.7 ± .08	3.43 ± .37	7.41	23	3.29	2.34
1.64 ± .12	5.48 ± .49	12.59	24	3.61	3.41
1.64 ± .12	5.48 ± .49	12.59	26	3.98	3.41
1.11 ± .13	8.08 ± .5	17.38	24	3.61	4.05
3.31 ± .42	10.73 ± .62	25.12	24	4.33	4.79
3.31 ± .42	10.73 ± .62	25.12	24	4.57	4.79
4.6 ± .40	14.8 ± 1.42	34.67	22	5.35	5.44
4.6 ± .40	14.8 ± 1.42	34.67	27	5.77	5.44

Table VII. Lethal response data of the post larval zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Mean wet weight of test organisms (mg.± S.D.)	Observed response at 96 hrs. (probits)	Predicted response at 96 hrs. (probits)
0.16 ± .03	1.28 ± 0.40	0.26	20	0.21± 0.13	3.36	2.96
0.30 ± 0	2.40 ± 0.45	0.54	19	0.21± 0.13	3.75	3.94
0.30 ± 0	2.40 ± 0.45	0.54	18	0.21± 0.13	3.78	3.94
0.75 ± .10	3.25 ± 0.37	1.10	20	0.21± 0.13	4.16	4.88
0.72 ± .13	3.98 ± 0.49	1.17	20	0.21± 0.13	4.33	4.97
0.72 ± .13	3.98 ± 0.49	1.17	17	0.21± 0.13	5.72	4.97
1.68 ± .17	0.85 ± 0.58	1.74	18	0.21± 0.13	5.28	5.49
1.23 ± .22	8.30 ± 0.40	2.45	19	0.21± 0.13	6.64	5.95
1.23 ± .22	8.30 ± 0.40	2.45	20	0.21± 0.13	6.64	5.95

Table VIII. Lethal response data of the juvenile zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition

Mean assayed cadmium concentration (mg/L ± S.D.).	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Mean wet weight of test organisms (mg. ± S.D.)	Observed response at 96 hrs. (probits)	Predicted response at 96 hrs. (probits)
0.75 ± 0.11	2.95 ± 0.19	1.69	10	12.60 ± 3.30	3.72	3.43
0.70 ± 0.08	3.43 ± 0.37	1.82	10	18.25 ± 5.20	4.16	3.54
0.70 ± 0.08	3.43 ± 0.37	1.82	10	5.63 ± 3.10	4.48	3.54
1.70 ± 0.39	5.09 ± 2.20	3.47	10	21.58 ± 4.30	4.16	4.52
1.29 ± 0.34	6.59 ± 2.70	3.72	10	34.89 ± 10.60	3.72	4.63
1.16 ± 0.21	7.64 ± 3.80	3.98	10	3.71 ± 1.90	5.25	4.73
1.11 ± 0.13	8.08 ± 0.50	4.17	10	9.90 ± 2.40	3.72	4.80
4.09 ± 0.57	4.90 ± 0.60	5.75	10	3.63 ± 0.40	5.52	5.30
5.98 ± 0.87	3.38 ± 1.57	7.08	10	62.45 ± 8.40	5.0	5.61
3.31 ± 0.42	10.73± 0.62	7.59	10	8.52 ± 2.50	5.0	5.72
3.31 ± 0.42	10.73± 0.62	7.59	10	9.28 ± 4.70	4.75	5.72
4.61 ± 0.40	14.80± 1.42	10.72	10	9.64 ± 1.80	5.52	6.25
4.61 ± 0.40	14.80± 1.42	10.72	10	10.60 ± 3.00	5.52	6.25

Table IX. Lethal response data of the adult zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Mean wet weight of test organisms (mg. ± S.D.)	Observed response at 96 hrs. (probits)	Predicted response at 96 hrs. (probits)
1.67 ± 0.08	3.43 ± 0.37	3.63	11	200 ± 100	4.09	1.81
1.70 ± 0.39	5.09 ± 2.20	4.57	10	915 ± 115	3.72	2.62
1.64 ± 0.39	5.50 ± 2.20	4.79	10	200 ± 100	3.72	2.78
1.11 ± 0.13	8.08 ± 0.50	5.75	10	200 ± 100	4.16	3.43
6.70 ± 0.90	3.13 ± 0.71	8.51	10	549.5 ±49.5	3.72	4.81
3.31 ± 0.42	10.73± 0.62	9.55	9	200 ± 100	5.43	5.22
7.29 ± 1.01	5.08 ± 1.29	10.23	10	449.5 ±49.5	5.84	5.46

Table X. Regression analysis of the lethal response data of the four test life cycle stages of the zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition

Regression Equation	$Y = a + b \log (A + B)$			
Life Cycle Stage:	Egg	Post Larvae	Juvenile	Adult
a	-0.529	4.687	3.544	1.486
b	3.761	3.579	1.757	3.670
r = correlation coefficient	0.77	0.83	0.72	0.73
n = number of test concentrations generating 0.1 - 99.9% response	8	9	13	7
computed students "t" for r	2.96	3.94	3.44	2.39
level of significance of r	$p \leq .05$	$p \leq .01$	$p \leq .01$	$p \leq .10$

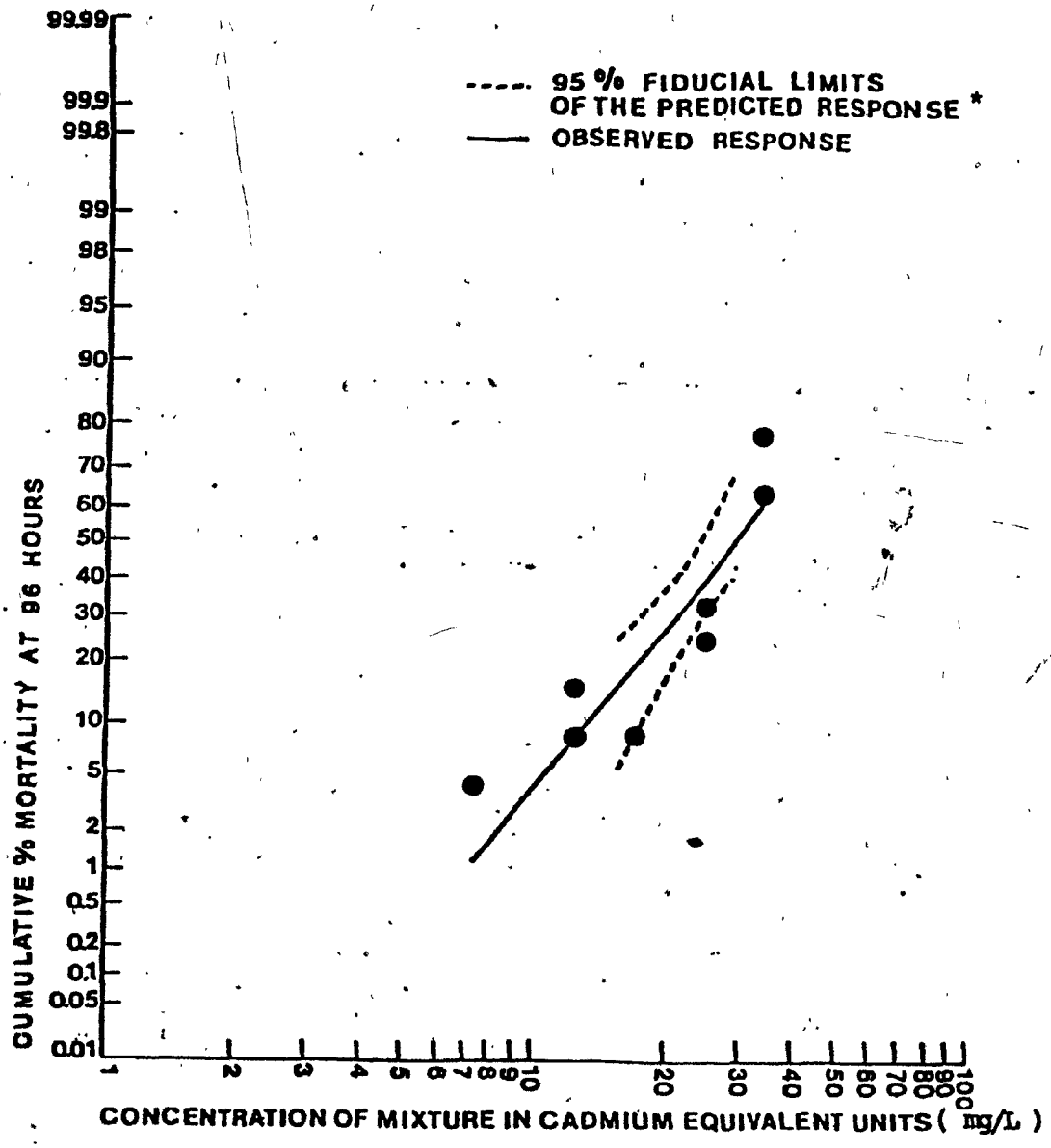


Figure 11. The response of the zebrafish egg exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition.....

* Note: The 95% fiducial limits are those of the response predicted in accordance with the model of concentration addition, ie. those of the cadmium regression.

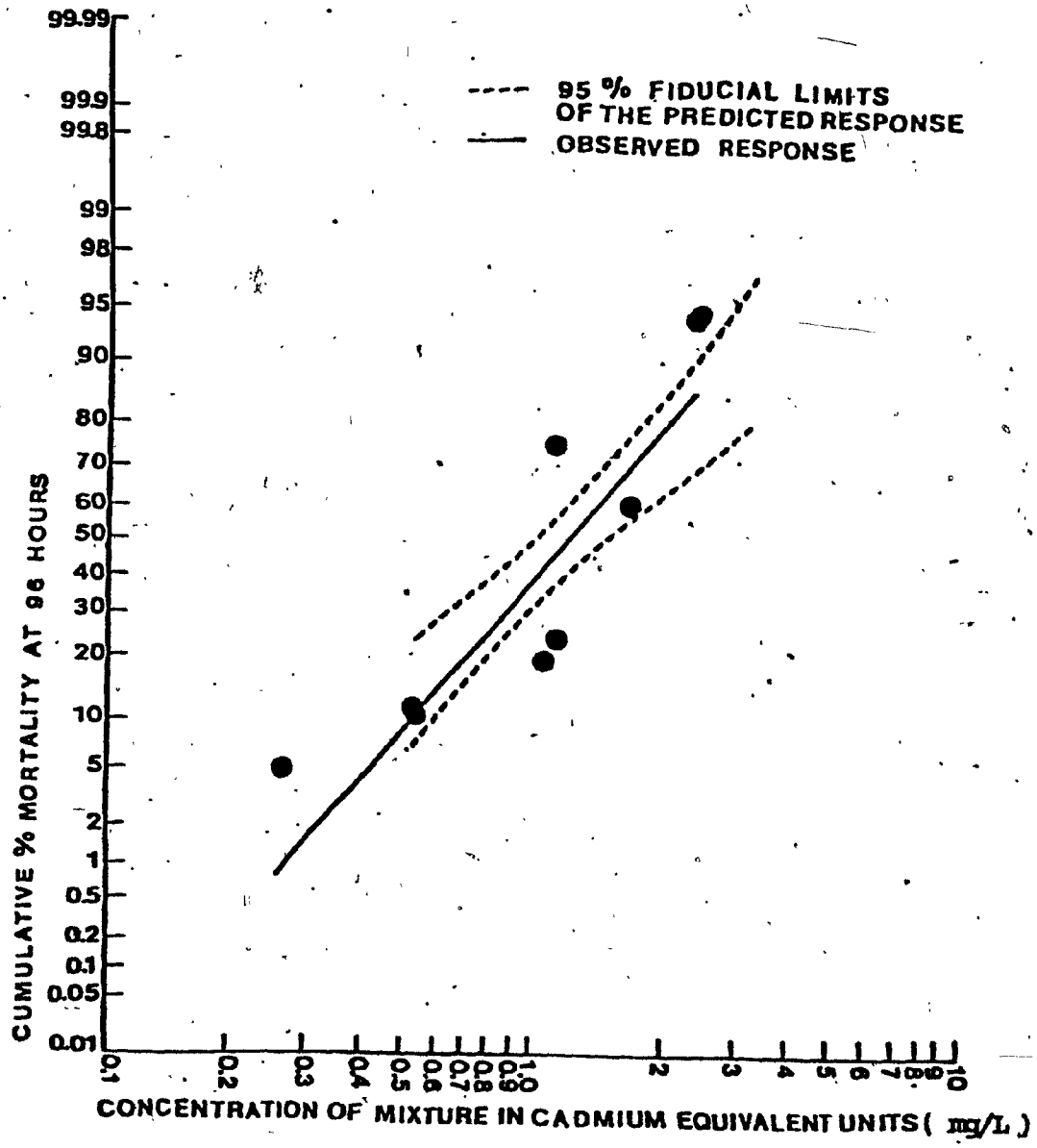


Figure 12. The response of the post larval zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition.

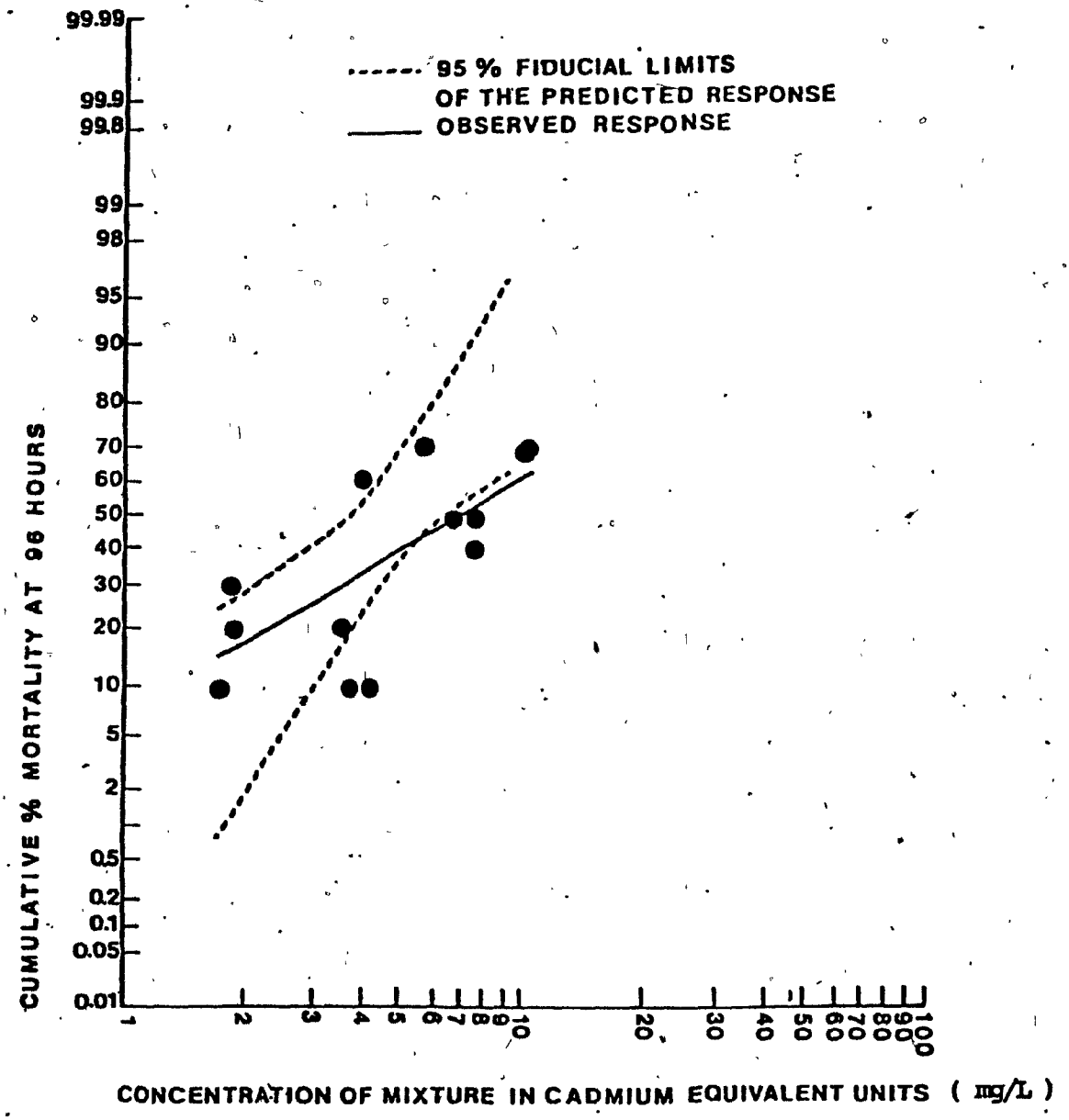


Figure 13. The response of the juvenile zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition.

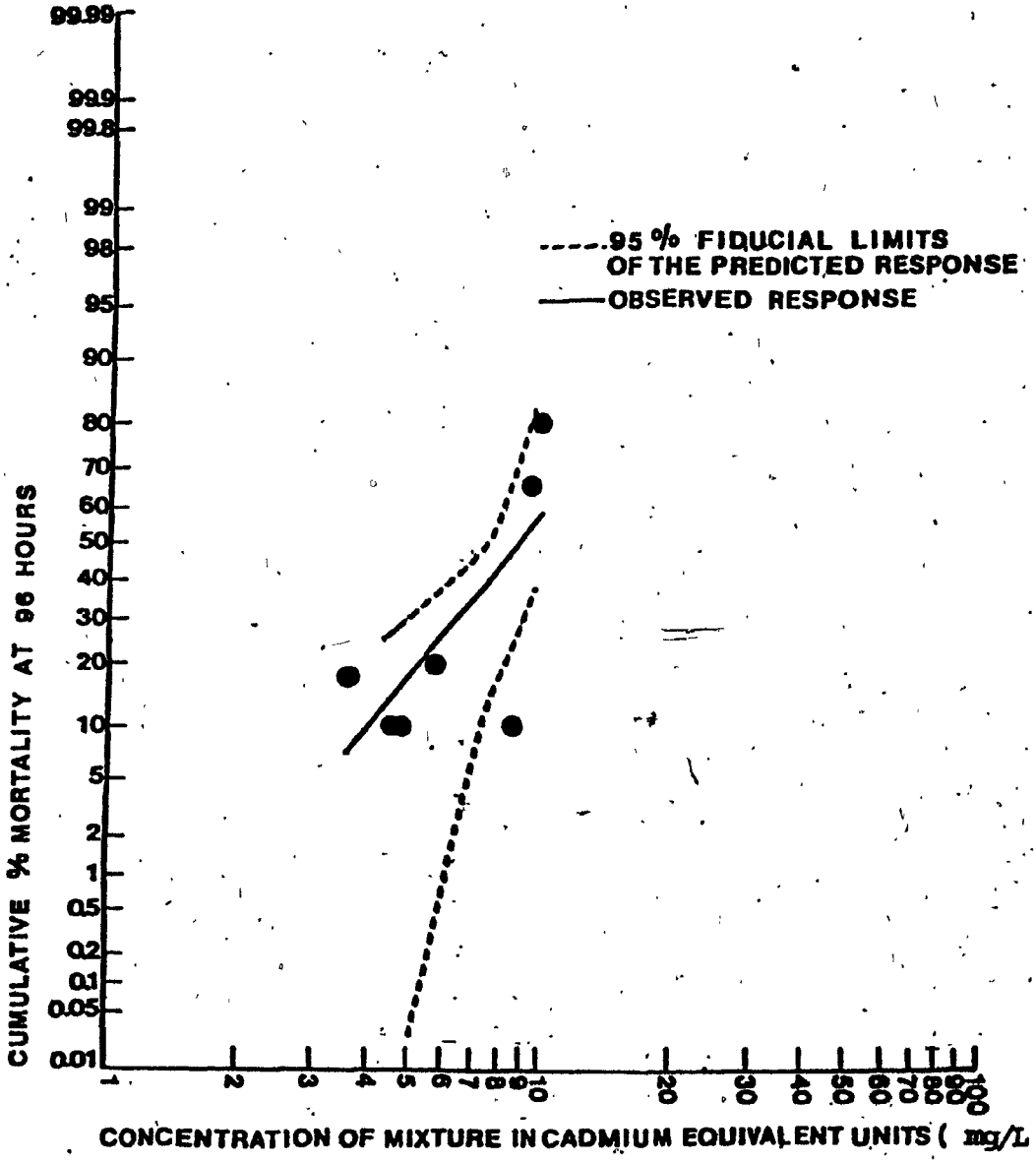


Figure 14. The response of the adult zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition.

Table XI: Lethal response data of the egg stage of the zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of response addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Observed % response at 96 hrs.	Predicted % response at 96 hrs.		
					r = 0	r = 1	r = -1
0	0	-	25	4.0	-	-	-
0.7 ± 0.08	3.43 ± 0.37	6.92	23	4.35	0.3	0.3	0.3
0.7 ± 0.08	3.43 ± 0.37	6.92	25	0	0.3	0.3	0.3
1.64 ±0.12	5.48 ± 0.49	11.22	24	8.33	3.4	3.4	3.4
1.64 ±0.12	5.48 ± 0.49	11.22	26	15.40	3.4	3.4	3.4
1.11 ±0.13	8.08 ± 0.50	16.60	24	8.33	14.9	14.9	14.9
1.11 ±0.13	8.08 ± 0.50	16.60	25	0	14.9	14.9	14.9
3.31 ±0.42	10.73± 0.62	21.88	24	25.0	32.0	32.0	32.0
3.31 ±0.42	10.73± 0.62	21.88	24	33.3	32.0	32.0	32.0
4.61 ±0.40	14.8 ± 1.42	33.88	22	63.6	57.2	57.2	57.2
4.61 ±0.40	14.8 ± 1.42	33.88	27	77.8	57.2	57.2	57.2

Table XII. Lethal response data of the post larval zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of response addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Observed percent response at 96 hrs.	Predicted percent response at 96 hrs.		
					r = 0	r = 1	r = -1
0.16 ± 0.03	1.28 ± 0.40	0.24	20	5.00	0.40	0.40	0.40
0.16 ± 0.03	1.28 ± 0.40	0.24	20	0.00	0.40	0.40	0.40
0.30 ± 0.00	2.40 ± 0.45	0.80	19	10.53	5.85	3.30	4.90
0.30 ± 0.00	2.40 ± 0.45	0.80	18	11.11	4.85	3.30	4.90
1.68 ± 0.17	0.85 ± 0.58	1.63	18	61.10	70.90	70.90	70.90
0.75 ± 0.10	3.25 ± 0.37	2.02	20	20.00	30.68	26.80	32.10
0.72 ± 0.13	3.98 ± 0.49	2.31	17	76.47	32.89	25.00	35.50
0.72 ± 0.13	3.98 ± 0.49	2.31	20	25.00	32.89	25.00	35.50
1.79 ± 0.24	5.85 ± 0.57	5.29	19	100.00	78.88	70.30	99.20
1.79 ± 0.24	5.85 ± 0.57	5.29	21	100.00	78.88	70.30	99.20
1.23 ± 0.22	8.30 ± 0.48	5.37	20	95.00	76.77	52.20	100.00
1.23 ± 0.22	8.30 ± 0.48	5.37	19	94.74	76.77	52.20	100.00

Table XIII. Lethal response data of the juvenile zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of response addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of test organisms	Observed percent response at 96 hrs.	Predicted percent response at 96 hrs.		
					r = 0	r = 1	r = -1
0.75 ± 0.11	2.95 ± 0.19	0.17	10	10	0.90	0.70	0.90
0.70 ± 0.08	3.43 ± 0.37	0.20	10	30	1.50	1.40	1.50
0.70 ± 0.08	3.43 ± 0.37	0.20	10	20	1.50	1.40	1.50
1.29 ± 0.34	6.59 ± 2.70	1.11	10	10	16.80	14.70	17.10
1.11 ± 0.13	8.08 ± 0.50	1.28	10	0	25.50	24.50	25.80
1.11 ± 0.13	8.08 ± 0.50	1.28	10	10	25.50	24.50	25.80
1.70 ± 0.39	5.09 ± 2.20	1.76	10	20	12.10	6.60	12.50
4.09 ± 0.57	4.90 ± 0.60	2.72	10	70	43.90	41.0	45.90
5.98 ± 0.87	3.38 ± 1.57	3.05	10	50	64.30	63.80	65.10
1.64 ± 0.39	5.48 ± 2.20	3.72	10	0	13.30	8.50	13.70
1.16 ± 0.21	7.64 ± 3.80	4.30	10	60	22.90	21.60	23.20
3.31 ± 0.42	10.73 ± 0.62	4.98	10	40	59.20	42.50	71.60
3.31 ± 0.42	10.73 ± 0.62	4.98	10	50	59.20	42.50	71.60
4.61 ± 0.40	14.80 ± 1.42	8.99	10	70	81.60	64.50	100.0
4.61 ± 0.40	14.80 ± 1.42	8.99	10	70	81.60	64.50	100.0

TABLE XIV. Lethal response data of the adult zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of response addition

Mean assayed cadmium concentration (mg/L ± S.D.)	Mean assayed zinc concentration (mg/L ± S.D.)	Concentration of mixture (cadmium equivalent units)	Number of organisms tested	Observed percent response at 96 hrs.	Predicted percent response at 96 hrs.		
					r = 0	r = 1	r = -1
0	0	0	10	0	0	0	0
1.7 ±0.42	5.09 ± 0.28	0	10	0	0	0	0
1.67 ±0.98	3.43 ± 0.37	0	11	18.2	0	0	0
0.75 ±0.11	2.95 ± 0.19	0	10	0	0	0	0
1.64 ±0.39	5.50 ± 2.20	3.09	10	10	<0.1	<0.1	<0.1
4.09 ±0.59	4.90 ± 1.07	4.09	10	0	0.3	0.3	0.3
1.11 ±0.13	8.08 ± 0.59	4.68	10	20	0.7	0.7	0.7
5.[illegible] ±0.89	3.38 ± 0.56	5.98	10	0	7.6	7.6	7.6
1.29 ±0.34	6.59 ± 1.03	6.59	10	0	<.1	<.1	<.1
6.70 ±0.78	3.13 ± 1.32	6.70	10	10	15.1	15.1	15.1
7.29 ±0.99	5.08 ± 0.52	7.29	10	80	23.2	23.2	23.2
1.16 ±0.71	7.64 ± 0.80	7.64	10	0	0.4	0.4	0.4
1.20 ±0.23	8.65 ± 0.06	8.65	10	10	1.4	1.4	1.4
3.31 ±0.42	10.73 ± 0.62	9.86	9	67	7.7	7.7	7.7
4.61 ±0.40	14.80 ± 1.42	16.70	10	100	40.0	39.9	40.9

of the predicted concentration response relationship is inherent to this model of additivity. Thus, a Pearson chi square test was employed to compare the observed and predicted mortality rate. The similarity between the expected and observed response was not supported statistically ($p = .05$).

The difficulties of applying a Pearson chi square test to the results of the aquatic bioassays reported here have been previously discussed. Since the models of concentration and response addition are theoretically mutually exclusive, empirical data describing the additive interactions of binary mixtures of toxicants can conform to only one of these two models. In the absence of empirical data that conforms to the model of concentration addition, conclusive statistical support of the alternative model of response addition may only be obtained when the sample size of each exposure lot of fish is increased. The results of the bioassays reported here did conform to the concentration addition model and, therefore, may not be representative of the alternative model of additivity (i.e. response addition).

The influence of wet body weight of the lots of exposed adults, juveniles and post larvae on their response to the mixture, in accordance with the model of concentration addition, was examined (Weinstein, 1978). The pooled multiple toxicity data of these three test stages was also subjected to Finney's method of probit analysis (Weinstein, 1978). The incorporation of a weight related factor greatly reduced the departure of the empirical multiple toxicity data points from a linear representation. The weight related factor that generated the best correlation coefficient for the effective concentration response regression was 0.31 (Table XV).

Table XV. Concentration response and effective concentration response regression analysis of the pooled quantal response data of three life cycle stages of the zebrafish exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition

Regression Equation	Concentration Response $Y = a + b \log (A + B)$	Effective Concentration Response $Y = a + b \log x/w^h$
a	4.28	4.16
b	0.76	2.36
h	-	0.32
r = correlation coefficient	0.35	0.78
computed students "t" for r	1.94	6.47
level of significance of r (n = 29)	$p \leq .1$	$p \leq 0.005$

the effective concentration response regression was 0.31 (Table XV).

The observed effective concentration response for the similarly acting mixture almost coincides with that of the predicted effective concentration response regression for cadmium (Figure 15). The slopes of the observed and predicted effective concentration response regressions were compared statistically and found to be significantly similar ($p = 0.05$).

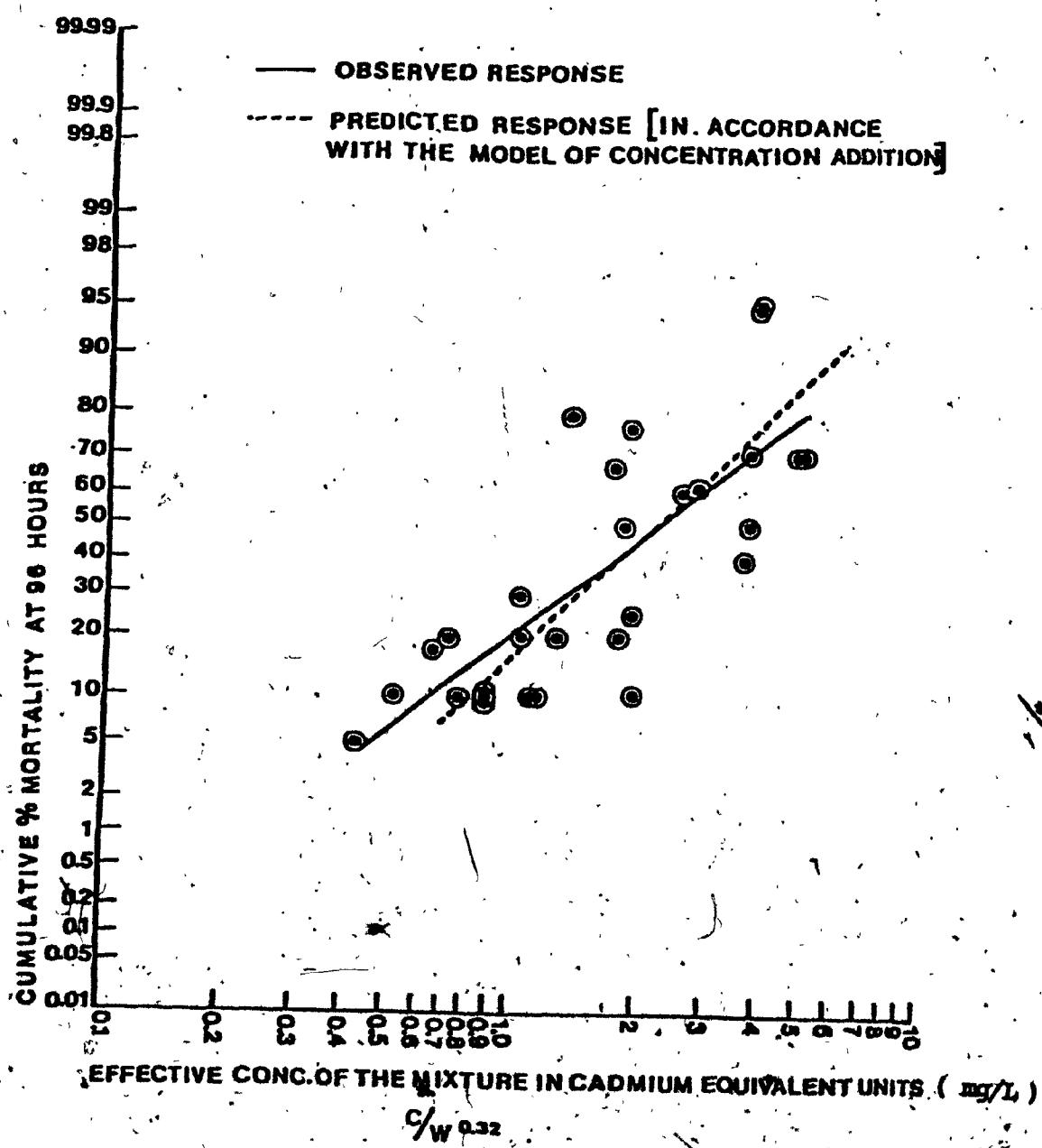


Figure 15. The effective concentration response relationship of the zebrafish adult, juvenile and post larvae exposed to mixtures of cadmium and zinc in accordance with the model of concentration addition.

Table XVI. Lethal tolerance, as measured by the ninety-six hour LC_{50}'s, of the four test life cycle stages of the zebrafish exposed to cadmium, zinc and their mixtures

Toxicant	Life cycle stanza tested	Age (days) at onset of bioassay	96 hour LC_{50} (mg/L)	95% fiducial limits of 96 hour LC_{50} (mg/L)
Cadmium	egg	0	27.89	25.17 - 34.16
"	post larvae	8	1.20	1.02 - 1.42
"	juvenile	22 - 40	4.74	3.84 - 5.97
"	adult	77 - 105	8.96	8.17 - 10.49
Zinc	egg	0	13.50	12.39 - 14.68
"	post larvae	8	8.05	7.29 - 9.49
"	juvenile	36 - 49	11.95	9.86 - 18.57
"	adult	87 - 116	15.9	14.28 - 17.35
Mixture (in accordance with the model of concentration addition)	egg	0	29.52	25.9 - 36.22
"	post larvae	8	1.22	1.05 - 1.42
"	juvenile	28 - 48	6.74	5.0 - 11.41
"	adult	88 - 101	9.07	7.33 - 14.96

DISCUSSION

Life Stanza Patterns in Tolerance of Fish

Larval zebrafish were consistently more susceptible to the lethal action of cadmium and zinc than any other life cycle stage tested (Table XVI, Figures 7,8, 11-14). With the exception of the prehatch embryo, the tolerance of the test organisms to the discrete metals and their mixtures progressively increased with post embryonic age (Table XVI). This pattern of tolerance between life cycle stages is in agreement with the reports of other authors.

McKim, 1977, reviewed fifty-six life cycle bioassays in which the toxicities of thirty-four organic and inorganic aquatic pollutants to a variety of fish species were tested. In forty-six of the reports reviewed the larval and or juvenile life cycle stanza was less tolerant than the prehatch embryo or adult of the same species. Certain characteristics of these early post hatch life cycle stages would appear to render these organisms especially sensitive to a variety of toxicants.

Skidmore, 1965, described the resistance to zinc sulphate of the zebrafish (Brachydanio rerio) at different phases in its life history. Larval zebrafish between four and twelve days post fertilization were more susceptible to the lethal action of zinc than were the prehatch embryo and adult. With the exclusion of the prehatch embryo, zinc tolerance increased with the age of the test organism. A similar profile of tolerance of the mummichog (Fundulus heteroclitus) exposed to cadmium at selected life cycle stages has been reported by Middaugh & Dean, 1977. The seven and fourteen day post hatch mummichog were more susceptible to the lethal action of cadmium than were the one day post hatch individuals or adults.

Since the life cycle patterns in tolerance that are reported in the literature are common to a variety of toxicants and fish species, it seems probable that the factor(s) responsible for the increased susceptibility of the early life cycle stages is not specific to the action of any one contaminant or to any one fish species.

On the basis of the ninety-six hour lethal bioassays, the threshold concentration of the discrete cadmium and zinc solutions which just failed to elicit a response was different for each of the life cycle stanzas tested. However, once this threshold concentration had been exceeded, the increase in the response of the test organism per unit increase in the ambient toxicant concentration (i.e. slope) was similar for all life cycle stanzas tested ($p = .05$). The similarity in the slopes of the concentration response regressions of all life cycle stages may describe a common mode of toxicant action which is consistent throughout the life cycle stages tested (Anderson & d'Apollonia,1978; Bliss, 1939).With physico-chemical characteristics similar to those of the bioassays reported here, the ninety-six hour LC_{50} of cadmium and zinc for the adult zebrafish were found to be 5.82 mg/L (95% fiducial limits:5.39-6.58 mg/L) and 17.68 mg/L (95% fiducial limits:16.2-19.3 mg/L) (Hewitt, personal communication; Horovitch, 1979). The ninety-six hour LC_{50}'s of 8.96 mg/L (95% fiducial limits:8.17-10.49mg/L) cadmium and 15.9 mg/L (95% fiducial limits:14.28-17.35) zinc for the adult zebrafish were determined in this study. These results compare favorably with those previously reported. On the basis of the ninety-six hour LC_{50}'s, cadmium was more toxic to the adult, juvenile and larval zebrafish than was zinc. However, the prehatch zebrafish embryo was more resistant to cadmium than to zinc (Table XVI).

Multiple Toxicity Patterns

The multiple toxicity data for cadmium and zinc mixtures conforms to the model of concentration addition for all life cycle stanzas tested (Figures 11 - 14). The response of the test organisms exposed to the mixtures could be predicted from a knowledge of the discrete toxicities of cadmium and zinc and the assumption that each, as constituents in the mixture would act similarly (Anderson & d'Apollonia, 1978; Finney, 1971; Bliss, 1939). Although dramatic differences in structure and physiology exist between the various life cycle stages tested, interactions in either the kinetic or dynamic phase of each of the toxicants, that would cause a less than or greater than additive effect, were absent.

Inherent to the model of concentration addition is the existence of a critical target or receptor system within the test organism which is common to both constituents in the mixture (Bliss, 1939). The observed consistency of this multiple toxicity pattern from egg through to the adult test stage of the zebrafish, therefore, suggests the existence of a critical target which is common to both cadmium and zinc and to all life cycle stanzas tested.

The multiple toxicity pattern of cadmium and zinc mixtures has been investigated by other authors. Eisler, 1967, 1971, & 1973, described the cadmium and zinc tolerance pattern of the marine teleost, Fundulus heteroclitus, exposed to these heavy metal mixtures as supra-additive. However these data, analyzed in accordance with the methodology of Anderson & d'Apollonia, 1978, supported the concentration additive multiple toxicity model. According to this model one would expect, as Eisler observed, that subthreshold levels of cadmium and zinc, in combination, could evoke a toxic response.

Spehar, 1976; and Spehar et al.,1978, described the response of the flagfish (Jordanella floridae) exposed to cadmium, zinc and their mixtures. Spehar reported that cadmium and zinc mixtures did not act additively and that the toxicity of the mixture was little if any greater than the toxicity of zinc alone. However, the relative proportion of zinc in the mixture greatly exceeded that of cadmium such that the potency of the mixture was largely attributable to the presence of zinc. The author's analysis of Spehar's multiple toxicity data contends the additivity of cadmium and zinc mixtures. The response of the flagfish to the mixture falls within the ninety-five percent fiducial limits of the prediction of concentration addition. The cadmium and zinc constituents of the mixtures contribute to the toxicity of the mixture in accordance with their relative potency and proportion.

Infra-additive antagonism of cadmium and zinc mixtures has also been reported, but, in studies where the organism was pre-exposed to one or the other metal (Chapman,personal communication; Norberg, 1976; Spehar et al., 1978; Beattie & Pascoe, 1978).Thus previous exposure of the test organism to low levels of cadmium or zinc may affect the mode of toxic interactions of these metals in subsequent exposures. For example, it has been reported that pre-treatment of test flagfish with low levels of these discrete heavy metals, protected the test organism from the harmful effects of a subsequent exposure to mixtures of cadmium and zinc (Spehar et al., 1978; Beattie & Pascoe, 1978). The protective capacity of cadmium or zinc pre-treatment is thought to be due to the induction of the hepatic protein metallothionein (Norberg, 1976). Both cadmium and zinc are bound by metallothionein and may thereby be rendered less

toxic (Cherian et al., 1978). Thus, the induction of metallothionein synthesis by cadmium and zinc pre-treatment may influence the turnover and multiple toxicity patterns of these two heavy metals. This was not observed in the bioassays reported here because the toxicants, as constituents of mixtures, were applied concurrently.

Tolerance of the Egg Stage to Cadmium and Zinc

In the cadmium, zinc and mixture bioassays, the zebrafish egg was equally if not more resistant to the lethal action of the toxicants than the other life cycle stages tested (Table XVI, Figures 7,8 & 11). The resistance of the prehatch embryo of many teleost species, to a variety of water contaminants, has been reported (McKim, 1977) and in some cases has been attributed to the protective capacity of the egg capsule (Rosenthal & Alderdice, 1976).

In contrast to the post larval, juvenile and adult bioassays, the potency of zinc was greater than that of cadmium to the prehatch embryo (Table XVII, Figures 7a & 8a); yet a similar mode of toxicant action, which is consistent throughout the life cycle, has been suggested. Reports in the literature on the relative toxicities of cadmium and zinc to prehatch embryos may provide an explanation for this switch in lethal potency and yet lend support to the hypothesis of similar action.

Investigating cadmium uptake by rainbow trout, Salmo gairdneri eggs, Beattie & Pascoe, 1978, reported that cadmium content of the eggs increased with increasing exposure time and toxicant concentration. However, 98% of the cadmium in the egg was associated with the mucopolysaccharides of the egg capsule. Alevins hatching

from eggs which had been exposed to low levels of cadmium (0.1 to 1.0 mg/L) contained significantly less cadmium than alevins which had been directly exposed to the toxicant. The protective capacity of the egg capsule may therefore reduce the effective concentration of cadmium to which the prehatch embyro is exposed and thereby reduce the relative potency of this toxicant.

Skidmore, 1966, investigated the protective capacity of the egg capsule of the zebrafish prehatch embryo exposed to zinc. No significant decrease in zinc tolerance was reported when the outer egg capsule was removed or ruptured. Zinc also has an affinity for the mucopolysaccharides of the egg capsule, however, Wedemeyer, 1968, has reported that the binding capacity of the egg capsule may be saturated at ten minutes of exposure to a solution of 10mM zinc. The initial physico-chemical sorption onto the egg capsule may then be followed by a passive diffusion of zinc into the perivitelline fluid, yolk and embryo (Wedemeyer, 1968). Thus, the egg capsule may not afford long term protection to the toxicity of zinc in the intact prehatch embryo during the ninety-six hour bioassays.

The lower metabolic rate of the prehatch embryo, relative to the other life cycle stages tested, may also contribute to its comparatively greater tolerance (Skidmore, 1967). This influence may be masked by the protective capacity of the egg capsule to cadmium but not to zinc (Figures 16 & 17). The difference in the potency of cadmium and zinc might therefore be attributed to differences in the affinities of these metals for receptors of the egg capsule.

Modifying Factors: Body Weight

: Metabolic Rate

The model of concentration addition, which appears to define the lethal response patterns of all life cycle stages exposed to mixtures of cadmium and zinc (Figures 11 to 14) assumes that the constituents of the mixture share a common mode of toxic action (Anderson & d'Apollonia, 1978; Bliss, 1939). A plausible explanation for this similarity of lethal action is the tendency of zinc and cadmium, like certain other heavy metals, to bind with ligands of proteins, particularly at cell surfaces (Passow et al., 1961). These authors suggest that through ligand binding, heavy metals may denature membrane protein and thereby adversely affect a protein's structural role and/or enzymatic activity. It follows that those cell surfaces such as in the gill epithelium, that encounter high ambient concentrations of a water borne metal could be a critical target site for the metal's toxic action. Many authors have suggested that gill dysfunction is the cause of death in fish exposed to lethal levels of a heavy metal (Anderson & Spear, 1975; Anderson et al., 1979; Skidmore, 1972). Severe histological damage to the gills' lamellar epithelium has been reported in rainbow trout following exposure to lethal levels of zinc. The morphological damage concurred with toxicological symptoms resembling hypoxia (Skidmore, 1972). Other authors have implied such a causal relationship by showing a strong correlation between ligand binding capacity and the toxicity of the heavy metal (Shaw et al., 1957).

The hypothesis that cadmium and zinc have a non specific yet similar action at boundary membrane surfaces such as the gill, is supported by certain quantitative patterns in the tolerance of test organisms exposed to either cadmium, zinc or their mixtures. For example, the age related susceptibility (Table XVI) of the post hatch zebrafish to either heavy metal or their mixtures, was found to decrease disproportionately with increasing body size, as measured by body weight (Figures 9, 10 & 15). This relationship was quantitatively expressed by an allometric formula (Equation 8) in which "h", the weight related exponent, was empirically determined to be 0.31, 0.1 and 0.32 for cadmium, zinc and their mixtures. When these factors for body weight were included in the function which correlated lethal response with ambient concentration, a linear relationship was obtained for the life cycle from post larvae through to adult (Tables V & XV). The difference in tolerance between the life cycle stages could be explained by a weight related variable.

A similar lethal toxicity pattern to copper, nickel and zinc has been reported for various size classes of adult Poecilia reticulata and Lepomis gibbosus (Anderson & Weber, 1975; Spear & Anderson, 1975). These authors also noted the similarity between their empirically derived weight related factors and that factor which correlated the metabolic rate of the test organism to body size.

The observed weight related trends in tolerance to these heavy metals may accord with the Law of Mass Action in that the number of

toxicant target sites within a test organism increase in proportion to body weight (Anderson & Weber, 1975). According to this law, small fish have lesser body weight and have fewer critical target receptor sites than larger fish having greater body weight. In fact, the respiratory surface area of the gill lamella in a variety of fish species has been expressed as an allometric function of body weight. A weight related factor of 0.8 to 0.9 adequately discribed the change in gill surface area with change in body size (Equation 9 - Muir, 1969). Expressing this relationship on a weight specific basis (Equation 10), the exponent relating unit of body mass and gill surface area becomes -0.1 to -0.2 indicating that smaller fish have a larger gill surface area per unit weight than do larger fish.

Weight Related Surface Area		Weight Specific Surface Area	
$Y = aW^{0.8}$	(9)	$\frac{Y}{W} = a\frac{W}{W}^{0.8} = aW^{-0.2}$	(10)

where Y = Lamellar surface area.

a = Lamellar surface area of a 1 gram fish.

W = Body mass as measured by weight.

0.8 = Exponent relating body weight to the gill lamellar surface area. (Muir, 1969)

The magnitude of this weight specific exponent approximates that of the weight related factors of 0.31, 0.1 and 0.32 describing the disproportionate decrease in susceptibility of the zebrafish exposed to cadmium, zinc and their mixtures, with increasing body weight. Susceptibility increased with decreasing body weight at a rate that was approximately opposite and, therefore, comparable to the reported increase in weight specific gill surface area with decreasing body weight. These opposing trends may suggest a causal relationship between increasing weight specific surface area of the gill lamella and cadmium and zinc susceptibility. Smaller post larval zebrafish would have a greater lamellar surface area per unit body weight with fewer critical target sites available for toxicant interactions (Law of Mass Action) than larger juvenile and adult fish. Thus, lower levels of cadmium and zinc would be required to saturate the critical targets of the smaller individuals rendering them more susceptible to the lethal action of these heavy metals.

The non specific and similar lethal action of cadmium and zinc may be further supported by another quantitative pattern in the lethal tolerance of the test organisms. Tolerance as measured by the ninety-six hour LC_{50}'s for cadmium, zinc and their mixtures, decreased disproportionately with increasing size and weight specific metabolic rate of the post hatch individuals.

The relationship between body size, as measured by weight and metabolic rate of an organism, is well known. Although smaller organisms utilize less oxygen than do larger organisms, the weight specific oxygen consumption of the former is greater than that of the latter (Adolph, 1949).

The potential influence of metabolic rate on the susceptibility of test organisms to certain heavy metal toxicants has been investigated. The susceptibility of rainbow trout exposed to ambient levels of zinc was greater in actively swimming individuals than in quiescent fish (Skidmore, 1967). Susceptibility was related to the metabolic requirement of the test organisms.

Skidmore, 1967, investigated the zinc tolerance of the zebrafish, Brachydanio rerio, from prehatch embryo through to the adult life cycle stanza, in relation to metabolic rate per unit weight of the test organism. Zinc tolerance decreased disproportionately with increasing metabolic rate as measured by the routine rate of oxygen uptake. The metabolic rate and susceptibility to zinc increased concurrently in fish from zero to five days post fertilization while the dry body weight of these individuals remained constant. The rate of oxygen uptake for individuals more than five days old however decreased disproportionately with increasing unit of body weight.

At 25° C

$$X = 4720W^{-0.14} \qquad (11)$$

where X = mg. O /kg/hr.

W = Dry weight (mg.)

(Skidmore, 1967)

The metabolic rates of the larvae, juvenile and adult stages tested in these cadmium, zinc and mixtures bioassays were determined using Equation 11. An approximation of the dry weight of the fish was derived by multiplying the wet weight of the individual by a

predetermined factor of 0.139. This factor represented the ratio of dry to wet body weight and had been empirically determined for twenty laboratory reared juvenile and adult specimens. The metabolic rates of fish prior to yolk sac absorption were drawn directly from Skidmore's 1967 data. Tolerance, as measured by the ninety-six hour LC_{50}'s for cadmium, zinc and their mixtures, decreased disproportionately with decreasing size and increasing weight specific metabolic rates of the post hatch individuals (Figures 16 & 17).

The greater metabolic demands of the smaller post larval zebrafish may be met with a greater opercular ventillatory volume thereby increasing toxicant availability and possibly uptake at the critical target (Spear & Anderson, 1975). The proportional decrease in critical receptor sites, with body size coupled with greater toxicant availability and uptake, may cause smaller fish to have a greater toxicant susceptibility than larger individuals. In fact, a causal relationship between susceptibility of fish to the lethal action of heavy metals and rate of accumulation by the gill has been proposed (Spear & Anderson, 1978).

At first glance, the tolerance of the prehatch embryo exposed to cadmium and cadmium and zinc mixtures does not conform to the pattern established by the other life cycle stages tested (Figures 16 & 17). The metabolic rate of the embryo is only slightly greater than that of the adult, yet the cadmium tolerance of the adult is almost four times less than that of the prehatch embryo. The relationship between metabolic rate and the susceptibility of the embryo to discrete solutions of zinc, however, did comply with that established by the other life cycle stanzas. This apparent

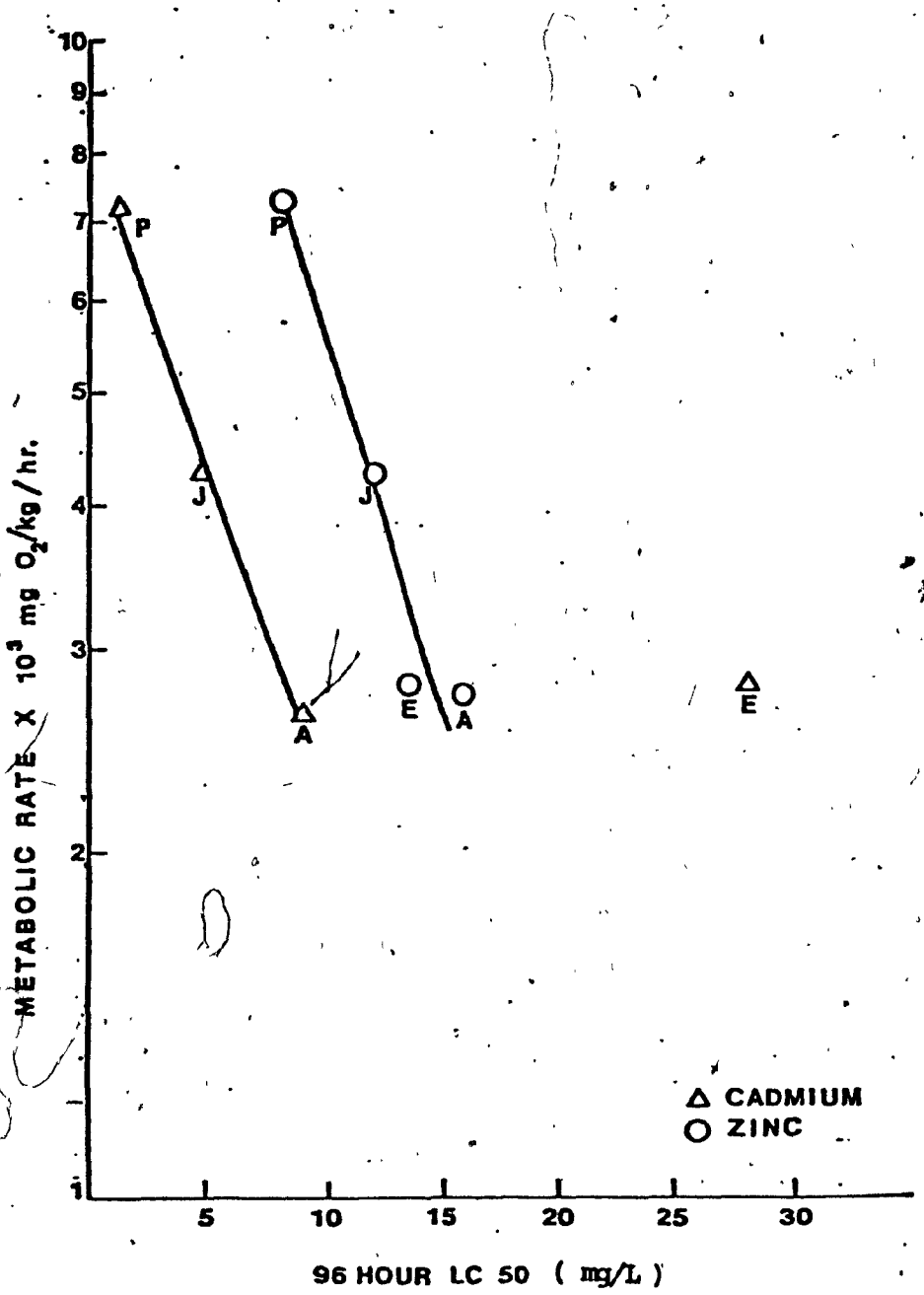


Figure 16. The influence of metabolic rate of the four tested life cycle stages on their lethal tolerance to discrete solutions of cadmium and zinc. (E represents the egg stage; P, post larvae; J,juvenile; A, adult).

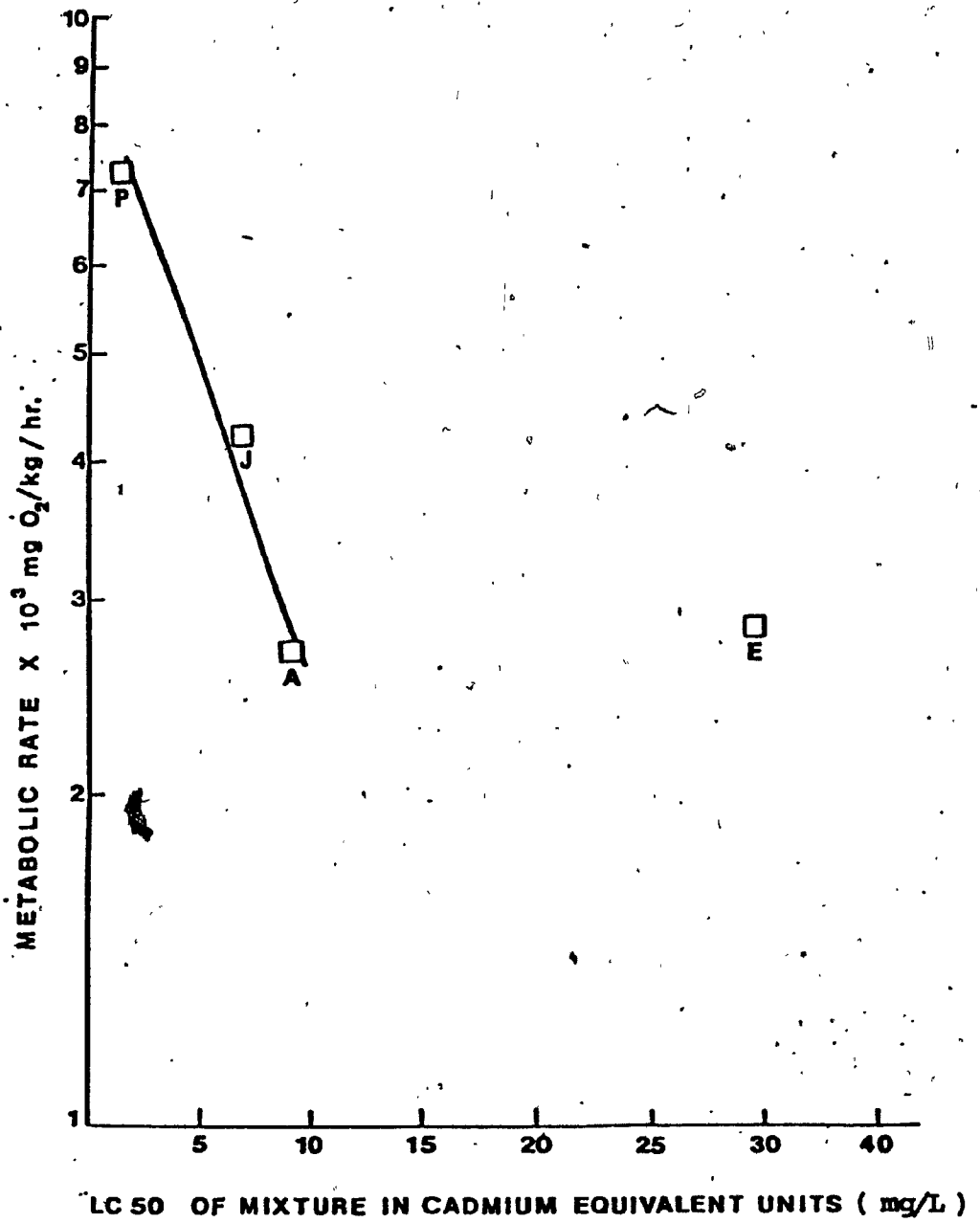


Figure 17. The influence of metabolic rate of the four tested life cycle stages on their lethal tolerance to mixture of cadmium and zinc in accordance with the model of concentration addition. (E represents the egg stage; P, post larvae; J, juvenile; A, adult).

discrepancy may be explained by the protective capacity of the egg capsule against the lethal action of cadmium (Rosenthal & Alderdice, 1976). However, the protection offered by the egg capsule against the toxicity of zinc appears to be negligible (Skidmore, 1966).

In the absence of a functional gill, respiration in the embryo occurs by diffusion across the entire surface area of the developing organism (Blaxter, 1969). The relationship between the surface area and volume (as measured by body weight) of the embryo approximates that of the respiratory lamellar surface area and body weight of post hatch individuals (Muir, 1969). The low metabolic demands of this test stage which allow diffusion to be an efficient method of gas exchange, may therefore influence toxicant availability, uptake and thus embryo susceptibility. Failure to consistently observe this influence may be attributed to factors that are specifically instituted by this test stage. The efficiency with which the egg capsule protects the embryo from cadmium toxicity may mask the modifying influence of metabolic rate of these individuals. Thus, the relationship between tolerance and metabolic rate of the prehatch embryo exposed to cadmium may not be apparent (Figures 16 & 17). Since the removal or rupture of the egg membrane does not influence zinc toxicity (Skidmore, 1966) one may presume that the egg capsule does not offer protection to this toxicant. Thus, the influence of the metabolic rate of the prehatch embryo on zinc tolerance may be apparent (Figure 16).

To further elucidate the relationship between metabolic rate and cadmium and zinc tolerance, a preliminary experiment was designed. Lots of ten to twenty post hatch individuals were exposed

to a pure solution of cadmium or zinc. All zebrafish constituting one lot were of the same age and the test lots ranged in age from four to twelve days post fertilization. At twenty-four hours of exposure, the number of deaths in each lot were recorded. The metabolic rates of the individuals in each test lot were drawn from those described by Skidmore, 1967 (Figure 18). The rapidly changing metabolic rates of these early life cycle stages concurred with changes in their susceptibility to cadmium and zinc (Figures 19 & 20).

In examining the pattern of tolerance of the zebrafish, at various life cycle stages, a disproportionate increase in susceptibility with decreasing body size and increasing metabolic rate has been quantitatively expressed. The concentration addition of these heavy metal mixtures suggests the existence of a non specific critical target which is common to both cadmium and zinc and to all life cycle stages tested. The proposal that in fish, the boundary membranes, such as the gill, that have a respiratory function are the critical targets of certain heavy metal toxicants (Spear & Anderson, 1975; 1978; Skidmore, 1972) is supported by the quantitative relationships of metabolic rate, body size and the multiple toxicity patterns that have been reported here.

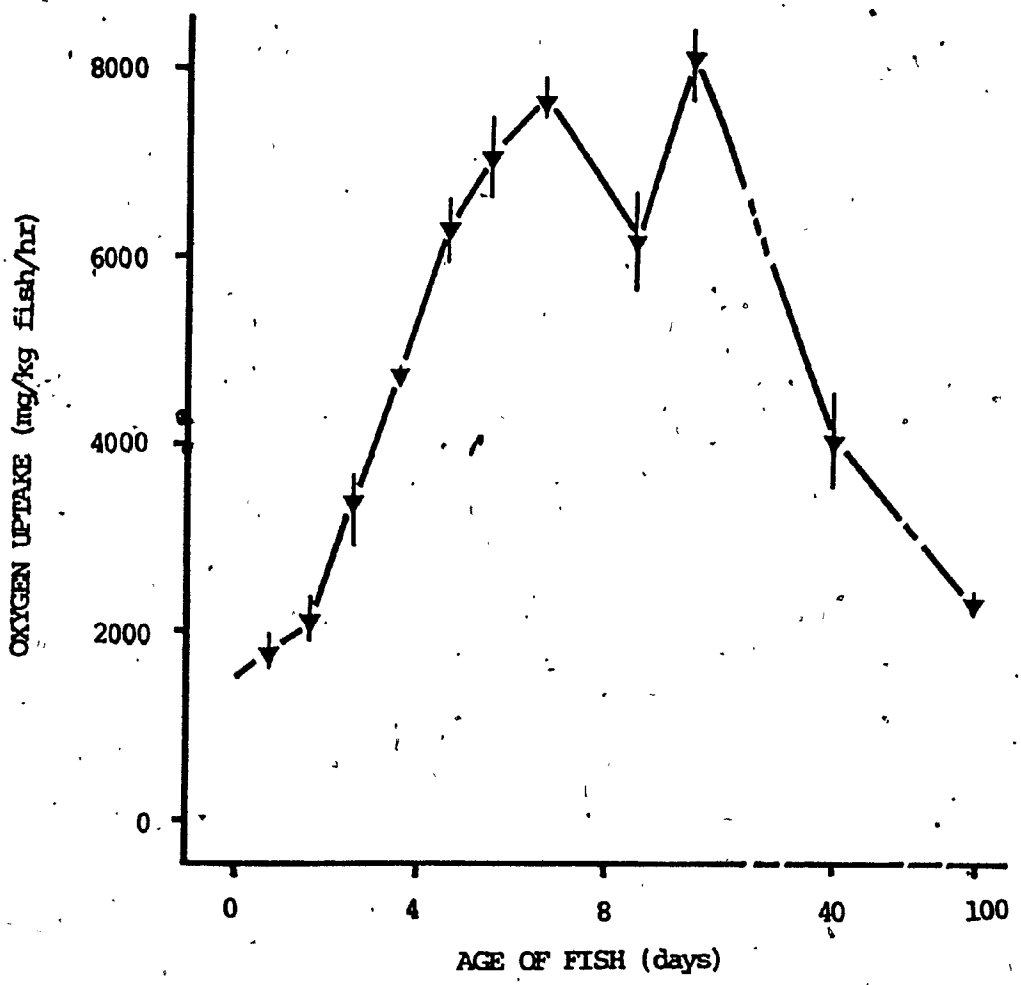


Figure 18. The routine rate of oxygen uptake of zebrafish aged 0 to 100 days, calculated per unit dry weight of fish (From Skidmore, 1967).

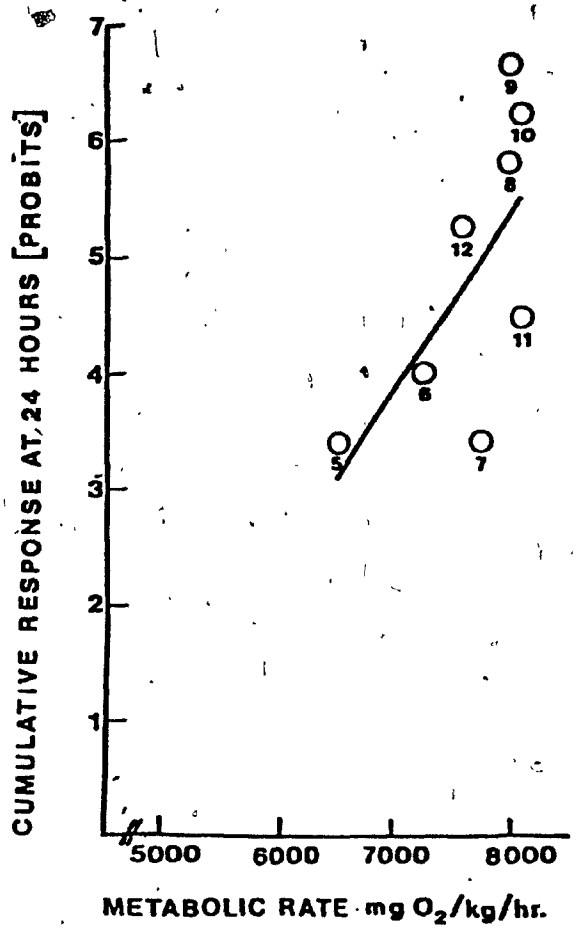


Figure 19. The influence of the rapidly changing metabolic rate of zebrafish aged 5 to 12 days post fertilization on their susceptibility to 3.88 mg/L cadmium.(The subscripts beneath each point represent the age of the test organisms)

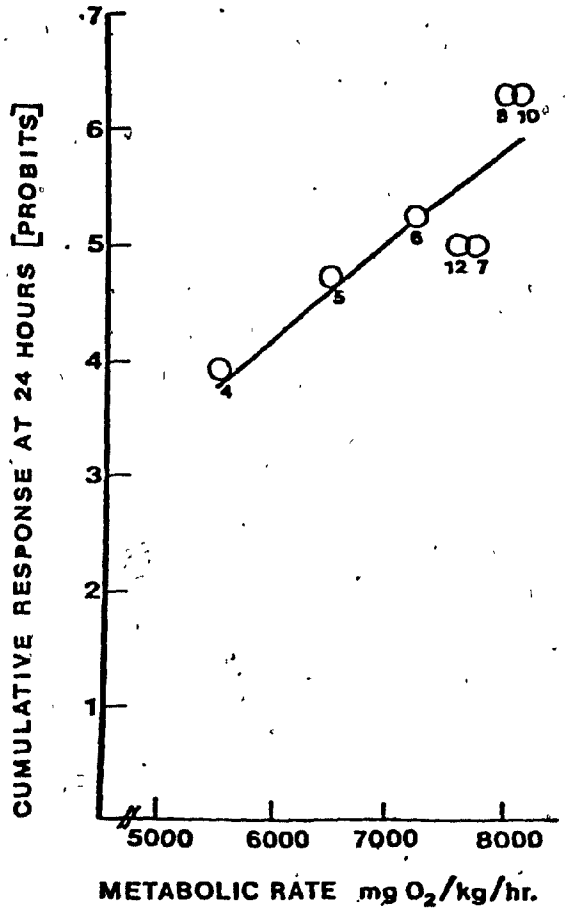


Figure 20. The influence of the rapidly changing metabolic rate of zebrafish aged 4 to 12 days post fertilization on their susceptibility to 9.23 mg/L zinc. (The subscripts beneath eacn point represent the age of the test organism).

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APPENDIX I

The Probit Transformation

The susceptibility of a population to a poison is usually distributed normally (Bliss, 1939). The proportion of individuals responding to a given dose of the poison may be expressed in terms of standard deviation units about the mean of the normal curve. A table of statistical units called probits has been devised in which the zero of the usual statistical table of deviates, corresponding to the mean of the normal curve, has been equated to 5. The deviates of the normal curve have been added algebraically to secure the probit corresponding to each percentage response (Bliss, 1939). Since the normal curve is asymptotic at the extremities, i.e. it approaches ± infinity at the horizontal axis (Ferguson, 1966), a probit value cannot be assigned to the extreme cases of 0 and 100 percent response (Bliss, 1939).

The relationship between the percentage response and the log of the administered dose of a drug or toxicant is graphically represented by a sigmoid curve. However, if the percentage response is converted to the corresponding probit response, this relationship can be linearly represented (Bliss, 1939). The reciprocal of the slope of the resulting dose response regression describes the variance in susceptibility between individuals in the population. A linear dose response relationship enhances the ease with which the results of bioassays can be statistically examined and compared. These characteristics are in contrast to the difficulties in the statistical manipulation of non linear dose response curves.