AN ABSTRACT OF THE THESIS OF

Donald Igwegbe Anadu for the degree of Doctor of Philosophy

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Richard A. Tubb

Acclimation of juvenile steelhead (S. gairdneri gairdneri) and rainbow (Salmo gairdneri) trout to sublethal concentrations of zinc resulted in significant increases in tolerance to zinc. When steelhead were acclimated to 50 $\mu g\ell^{-1}$ zinc (0.37 of the 96-hr LC50 and 0.80 of the 96-hr LC10), for 1, 2 and 3 weeks, up to 4-fold increase in tolerance was developed. Treatment of rainbow trout in a similar manner at 80 $\mu g\ell^{-1}$ zinc (0.38 of the 96-hr LC50 and 0.60 of the 96-hr LC10) for 3, 7, 21 and 28 days resulted in approximately 3-fold increase. Acclimation to sequentially increasing non-lethal concentrations of zinc increased the 96-hr LC50 by a factor of 5. Zinc exposure of rainbow trout at 100 $\mu g\ell^{-1}$ (0.42 of the 96-hr LC50 and 0.86 96-hr LC10) for 17 days developed cross tolerance to cadmium and copper in addition to zinc. Cadmium and copper tolerances increased by factors of approximately 4 and 5, respectively.

Development of tolerance to zinc was dependent on the time and concentrations of metal used during acclimation. The greatest increase in the steelhead acclimation occurred by the 7th day and no further increases occurred as a result of the extension of duration of acclimation. Acclimation of rainbow trout for periods less than 3 days indicated that acclimation had not occurred in the first 3 days, while by the 7th day, increases above 2-fold had occurred. Significantly greater increases in the lethal levels did not occur with further increases in acclimation time to 21 and 28 days. Rainbow trout acclimated to 100 $\mu g\ell^{-1}$ of zinc for 10 days had increased

tolerance to zinc of approximately equal magnitude to those acclimated sequentially to 300 and 500 $\mu g \ell^{-1}$ of zinc.

Zinc tolerance in rainbow trout was lost soon following cessation of zinc exposure. Deacclimation of the sequentially acclimated rainbow trout resulted in a nearly total loss of acclimation by the 7th day. By the 3rd day of deacclimation tolerance was still retained to the order of 4-fold.

The low molecular weight metal binding soluble hepatoprotein (metallothionein: MT) is known to be involved in metal detoxification. Estimation of MT induction during acclimation of rainbow trout showed a 22 percent increase over the unacclimated fish. A 67 percent average increase occurred in the sequentially acclimated trout. At cessation of acclimation, MT levels gradually decreased and by the 7th day were the same as the controls. Views on the possible involvement of MT in metal cotolerance are expressed, while results strongly suggest the non-metal specificity of MT.

FISH ACCLIMATION AND THE DEVELOPMENT OF TOLERANCE TO ZINC AS A MODIFYING FACTOR IN TOXICITY

by

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INTRODUCTION

There is perhaps an inexhaustible list of factors in nature that modify the response of organisms to toxicants. Shephard (1955) reported that trout acclimated to low but non-lethal oxygen levels increased their tolerance for low dissolved oxygen by up to 5-fold. Fry (1971) reported that the upper incipient lethal temperature changes approximately 1°C for a 3°C change in acclimating temperature in fish. Acclimation is a physiological adaptation by fish to accomodate an applied environmental stress. Acclimation of fish to toxicants has received relatively little attention.

In a review paper, Sprague (1969) covered previous work done in acclimation. Some of the highlights in his review included work done by Neil (1957), who showed that when fish were acclimated to cyanide, short-term resistance to 8 toxicants increased. Ferguson and Boyd (1964) showed that mosquito fish (Gambusia affinis) pre-exposed to the organophosphate insecticide methyl parathion had more resistance to a second exposure. After acclimation of bluegills (Lepomis macrochirus) to detergents for one month, Lemke and Mount (1963) reported a 63 percent increase in tolerance to detergents. Lloyd and Orr (1969) showed increased resistance of rainbow trout (Salmo gairdneri) to lethal ammonia levels after a one-day exposure at half the 96-hr LC50 (concentration causing 50 percent mortality). Lloyd (1960) demonstrated increased survival times of trout exposed to zinc following two weeks of zinc acclimation. Edwards and Brown (1966) reported a 40 percent increase in the 96-hr LC50 following acclimation of trout to 0.5 toxic units of zinc. One toxic unit is equivalent to the LC50. Sprague (1969) indicated that for the Atlantic salmon (Salmo salar), the incipient LC50 of zinc may be raised by a factor of 2 or 3 for maximum acclimation.

A greater amount of work on acclimation of fish to toxicants has appeared since 1969, Chapman (1978b) acclimated juvenile sockeye

salmon (Oncorhynchus nerka) to 242 $\mu g \ell^{-1}$ of zinc. This markedly decreased acute mortality at zinc levels lethal to unacclimated sockeye salmon juveniles. Pascoe and Beatie (1979) demonstrated that pretreatment of rainbow trout eggs with cadmium increased the survival of the resulting alevins when exposed to cadmium. Pre-exposure of rainbow trout to low levels of cadmium produced increased survival times in cadmium concentrations up to 10 $\mu g \ell^{-1}$ (Pascoe and Beatie, 1979).

Dixon and Sprague (1981) demonstrated that juvenile rainbow trout exposed for 3 weeks to 40 percent and 59 percent of the 144-hr copper LC50 showed 105 and 100 percent increases in tolerance to lethal levels of copper, respectively. This work was a systematic study of the degree of adaptation in rainbow trout exposed to sublethal concentrations. Most earlier studies were either offshoots of other major investigations or the time given for acclimation was too short to convincingly argue that complete or maximum acclimation occurred before acute toxicity tests were conducted.

Once acclimation occurs, it is equally important to determine the duration of retention of tolerance to higher doses of the toxicant retained. The acclimation retention time affects temporal changes in toxicant concentrations which produce mortality. Dixon and Sprague (1981) reported that when rainbow trout were returned to control water after a 3-week exposure to 131 $\mu g \ell^{-1}$ copper and bioassays carried out after 7, 14, and 21 days, a continuing decrease in the incipient lethal level relative to controls was observed. Most of the acquired tolerance was lost by the 7th day following cessation of the copper acclimation.

Conclusive agreement as to how acclimation of test organisms to one metal influences toxicity of other metals has not yet been reached. At best there seem to be contradictory findings among studies. Dixon and Sprague (1981) concluded from studies on copper acclimation of rainbow trout that pretreatment with this metal induced no tolerance towards zinc. On the other hand, Hall (1980) found copper-tolerant populations of marine fouling algae had high tolerance to cobalt and zinc as well as copper.

The problem of acclimation of organisms to environmental metal toxicants is important in the evaluation of sublethal effects of toxicants, in the estimation of 'safe' levels of toxicants and in setting water quality criteria. The induction of low-molecular-weight, metal-binding, soluble hepatoproteins is becoming accepted as the mechanism by which fish adapt to metal toxicants in the environment.

The ability to cope with environments containing metal toxicants encompasses adaptive changes. The formation of metallothionein (MT) a low molecular weight protein in livers and other organs of vertebrates has been consistently implicated in metal acclimation and metal toxicity. Historically, the discovery of metallothionein came as a result of a search for the biological role of cadmium in humans, buffered by the interest of Vallee (1979) in zinc metabolism and zinc metalloenzymes in 1955. Mammalian MT have been studied to a greater extent than those of any other groups of organisms. Many researchers have contributed to the knowledge of this ubiquitous protein. Metallothionein has been found in human liver (Kägi, 1970; Kissling and Kägi, 1979); in human kidney (Shaikh and Lucis, 1972), in the human heart and testis (Wisniewska-Knypl et al., 1971), and gastro-intestinal tracts (Kägi and Nordberg, 1979). Metallothionein has been isolated in phyla other than mammals; Maclean et al. (1972) reported a Cd and Zn binding protein in a blue green algae (Anacystus nidulans) and copper containing protein was purified from the fungi (Saccharomyces cerevisiae) by Prinz and Weser (1975). Olafson and Thompson (1974) isolated MT from teleost liver. The list of workers who recently isolated this protein from fish is increasing: Noel-Lambot et al. (1978), Yamamoto et al (1977), Kito et al., (1980), Bouquengneau (1979), Overnell and Coombs (1979), Brown and Parsons (1978), Ridlington et al. (1981), Pierson (1981), McCarter et al. (1982), Roch et al (1982), and Buckley, et al. 1982.

The following is a summary of the properties of mammalian MT (Kagi and Nordberg, 1979):

1) Synthesis is induced by certain metals - Cd, Zn, Cu and Hg,
2) Low molecular weight (6,000 to 10,000),

3) High cysteine content (30 percent) in its amino acid composition,
4) Contains no aromatic amino acid or histidine,
5) Has unique amino acid sequence as a result of fixed distribution of cysteinyl residues,
6) The optical features are characteristic of metal thiolates; the Cd-thionein has an absorption maximum at 250 nm,
7) Heat stable (not denatured at 80°C for 10 minutes),
8) Has great affinity for metal binding because of its net negative charge,
9) Present mostly as intracellular protein.

As a result of considerable research effort recently devoted to the study of MT in many branches of life sciences, MT has emerged as a key component in understanding the physiological mechanism regulating the flow of metals through the organism in health and disease, of the molecular pathway of the metals in biosynthesis and degradation of metalloenzymes and of metal toxicity and detoxification (Kägi and Nordberg, 1979). As a result, MT level is regarded as a probable candidate as an indicator of heavy metal pollution in the environment.

The objectives of the study reported in this thesis were:

1) To determine if acclimation of steelhead or rainbow trout to non-lethal levels of zinc significantly changes the incipient lethal level and on that basis accept or reject the generalization that fish can adapt to metal toxicants,
2) To examine the influence of acclimation concentration and time on the development of zinc tolerance,
3) To measure the effect of deacclimation on the lethal levels of zinc,
4) To measure the absence or presence and magnitude of cross tolerance in rainbow trout to copper and cadmium following acclimation to zinc,
5) To examine the dynamics of MT levels in fish liver following acclimation to zinc and subsequent deacclimation as a way of understanding more about MT induction by sublethal

concentrations of zinc and the possible role of MT in metal detoxification,

6) To understand more about the metal specificity of fish liver MT.

TERMINOLOGY

Some of terms employed in this study may lack a generally recognized or accepted definition. The definitions below will clarify the usage of these terms in the context of this thesis; they are consistent with the usage of many environmental toxicologists.

Acclimation: Physiological adaptations of an organism to some experimental conditions including any adverse stimulus which is involved. Acclimatization would differ from acclimation as the former reflects changes in the morphology and physiology of an organism in response to environmental change (Ricklefs, 1973). Acclimation studies usually concern the modifications acquired by an organism in response to experimental manipulation of a single environmental factor (Dixon and Sprague 1981).

Bioassay and toxicity test (interchangeably). A test performed to determine the concentration of a toxicant in water, to which the response is measured (e.g. the percent mortality of the test organisms).

Co-tolerance: The terms cross tolerance and/or transferred tolerance have been used interchangeably here to indicate tolerance acquired by an organism as a result of exposure to a toxicant that also enhances tolerance of the same organism to a different toxicant.

Resistance: The ability of an animal to survive for a limited period in an environment that will eventually exert a lethal effect (Shephard 1955). Tolerance implies that the change is within the normal adaptive range of the organism and can be sustained indefinitely while resistance implies that the magnitude of the factor lies outside the normal range and that detrimental effects will eventually occur.

Tolerance: Ability of an organism to survive exposure to a poison or other stress (e.g. high temperature, low dissolved oxygen), for a specified period of time such as for 96 hr following continuous or repeated exposure.

Sub-lethal and non-lethal: (interchangeably). Applied to toxicity tests in which toxicant concentrations produce no mortality within the experimental period.

METHODS AND MATERIALS

Experimental Animals:

Fish used in the experiments were rainbow trout (Salmo gairderi) and steelhead trout (S. gairdneri gairdneri). Steelhead trout were acquired from the North Fork Alsea Trout Hatchery, Alsea, Oregon, as "eyed" eggs. The eggs were fertilized in early February 1981, and held in the hatchery where they were prophylactically treated at the eyed stage against fungal infection and other epizootics with Malachite green at 0.1 $\mu g \ell^{-1}$ daily. The eyed eggs were wrapped in wet cheese cloth and transported to the Western Fish Toxicology Station (Corvallis Environmental Research Laboratory USEPA). At the Station they were treated with Wescodyne at 25 ppm for 10 minutes. The pH was adjusted by the addition of 0.1 gm $NaHCO_3$. The eggs hatched in late March and several weeks later, the larvae were transferred as swimups from the incubators to rectangular troughs 3.7 m x 0.6 m x 0.3 m with a capacity of 570 litres. The larvae were fed a ration of Oregon Moist Pellets (OMP) daily (2 percent of their body weight). The OMP was kept frozen to prevent deterioration. Starting in mid-September of 1981, these fish were used in the bioassay experiments. Their size at this time was: means (SD) for the wet weights: 4.95 (1.31) gm, N = 52; total length, 8.62 (1.19) cm, N = 52. Experiments with this group of fish lasted through November 1981.

Rainbow trout were acquired as "green" eggs in mid-January of 1982 from Willamette Hatchery, Oakridge, Oregon. The eggs were spawned dry and transported to the Western Fish Toxicology Station (WFTS) in Corvallis. At the laboratory, the eggs were water hardened and put into an incubator where they were held through swimup. Fish culture procedures were those previously described for steelhead except that the larvae were not treated with Wescodyne or other drugs. By mid-June, at the approximate size of 3 gm, the fish were transferred to holding tanks of 1700 litres capacity supplied with a continuous flow of WFTS well water which was used for all bioassays. The size at the beginning of the bioassay series at the age of approximately five months were: wet weight 3.01 (0.92) gm; total length 6.98 (0.55) cm,

N = 52. Guidelines for loading of the holding tanks and the experimental aquaria were adhered to as per EPA (1975).

Water quality:

All acclimation procedures and toxicity tests were conducted in WFTS well water which was aerated and temperature controlled by chilling or heating. Temperatures in one test aquarium were monitored continuously on thermograph charts. Dissolved oxygen, pH, total alkalinity, and total hardness were determined twice per a 96-or 120-hr period from the diluter aquarium receiving the highest concentration of toxicant and from the diluter control aquarium. The chemical analyses of the acclimation and control tanks were done twice weekly. Temperatures were checked periodically for any significant variations from the test water. Methods used for analyses were those recommended by the American Public Health Association (APHA et al., 1980) and the Environmental Protection Agency (1979). Dissolved oxygen concentrations were measured by USEPA full bottle azide modification of the Winkler technique. Phenylarsineoxide (P.A.O.) was used as titrant. The pH was determined by use of an Orion Model 701 pH meter, alkalinity by the potentiometric procedure for low alkalinity samples, and total hardness by the EDTA titrimetric method. During the entire experimental period mean (SD) water chemistry values in the high metal concentration and control aquaria, respectively, were: dissolved oxygen 10.0 (0.3) range (9.3 - 10.5) $mg\ell^{-1}$ and 9.9 (0.3) range (9.1 - 10.3) $mg\ell^{-1}$; pH 6.6 (0.4) range (5.8 - 7.1), and 6.6 (0.4) range (6.0 - 7.4), alkalinity 25 (4.3) range (20 - 38), and 25.2 (4.3) range (18 - 30) $mg\ell^{-1}$ as $CaCO_3$; hardness 33 (5.5) range 26 - 47, and 33 (5.2) range (27 - 48) $mg\ell^{-1}$ as $CaCO_3$ (Appendix 1). A detailed chemical characterization of the WFTS well water is given by Samuelson (1976) and Chapman (1978a).

Photoperiod and light intensity were controlled by an automatic switching device which allowed for 30-minute dusk and dawn twilight periods and gave a regimen of 12 hours light and 12 hours darkness for all the bioassay experiments. Acclimation tanks were kept outdoors under natural photoperiod. During acclimation, fish were fed OMP at

1.8 percent of body weight. Feeding was terminated one day before fish were transferred to test aquaria. The fish were not fed during the toxicity tests. Bioassays were not performed during December, January, or February because of great variation in water hardness values at this time of the year (Samuelson, 1976; Chapman, 1978a).

Toxicants:

Metal stock solutions were prepared from reagent grade metal chlorides ($ZnCl_2$, $CdCl_2 \cdot 2.5\ H_2O$, or $CuCl_2 \cdot 2\ H_2O$) dissolved in WFTS well water and acidified with concentrated nitric acid at the rate of 0.05 mls ℓ^{-1} of the total stock volume. Chemical analyses were carried out to determine the metal exposure concentrations for calculations of the LC50s. The metal analyses were carried out in the bioassays on the 2nd and 4th days corresponding to one day after each new stock solution or replenishing stock was made. Metal analyses of the acclimation stock solution were done twice weekly. Samples for zinc analysis were taken in 15 or 20 ml tubes and acidified with 1 µℓ of concentrated nitric acid per ml of sample. Analysis was by direct aspiration flame atomic absorption spectrophotometry (Perkin Elmer Model 403). Recovery for zinc added at concentrations ranging from 66 to 740 $\mu g\ell^{-1}$ was 104 (±5) percent, N = 54. Cadmium was analyzed by the furnace technique atomic absorption spectrophotometry (Perkin Elmer Model 305B). Cadmium added at 0.8 to 1 $\mu g\ell^{-1}$ had 103 (±5) percent recovery. Concentrations of copper higher than 50 $\mu g\ell^{-1}$ were analyzed by direct aspiration flame atomic absorption spectrophotometry, while concentrations below 50 $\mu g\ell^{-1}$ were analyzed by the furnace technique. Recoveries at 17 and 21 μgP^{-1} were 100 and 90 percent, respectively. Details of the above methods are described by USEPA (1979).

Acclimation Apparatus:

Metal acclimation was carried out in 2 tanks as shown in Figure 1. There were two 950-litre cylindrical tanks, and two 570-litre cylindrical tanks with semi-conical bases. All the tanks were

Figure 1. Schematic representation of the acclimation apparatus:

1. The 760-litre reservoir from which dilution water used in the entire experiment was obtained. The continuous flow of WFTS well water was aerated and temperature controlled here.

2. A 2-litre toxicant constant head holding container that provided a constant flow rate of the toxicant into the acclimation tanks.

3. A pump which pumped up to 100 ml of toxicant per minute.

4. A 450-litre tank for storing toxicant from which the toxicant was pumped to the holding tank.

5. Two 950-litre tanks, one used for acclimation and the other as control tank.

6. Two 570-litre tanks, one used for acclimation while the other served as control.

7. A 75-litre mixing tank where the toxicant and the dilution water were thoroughly mixed before final delivery to the acclimation tanks.

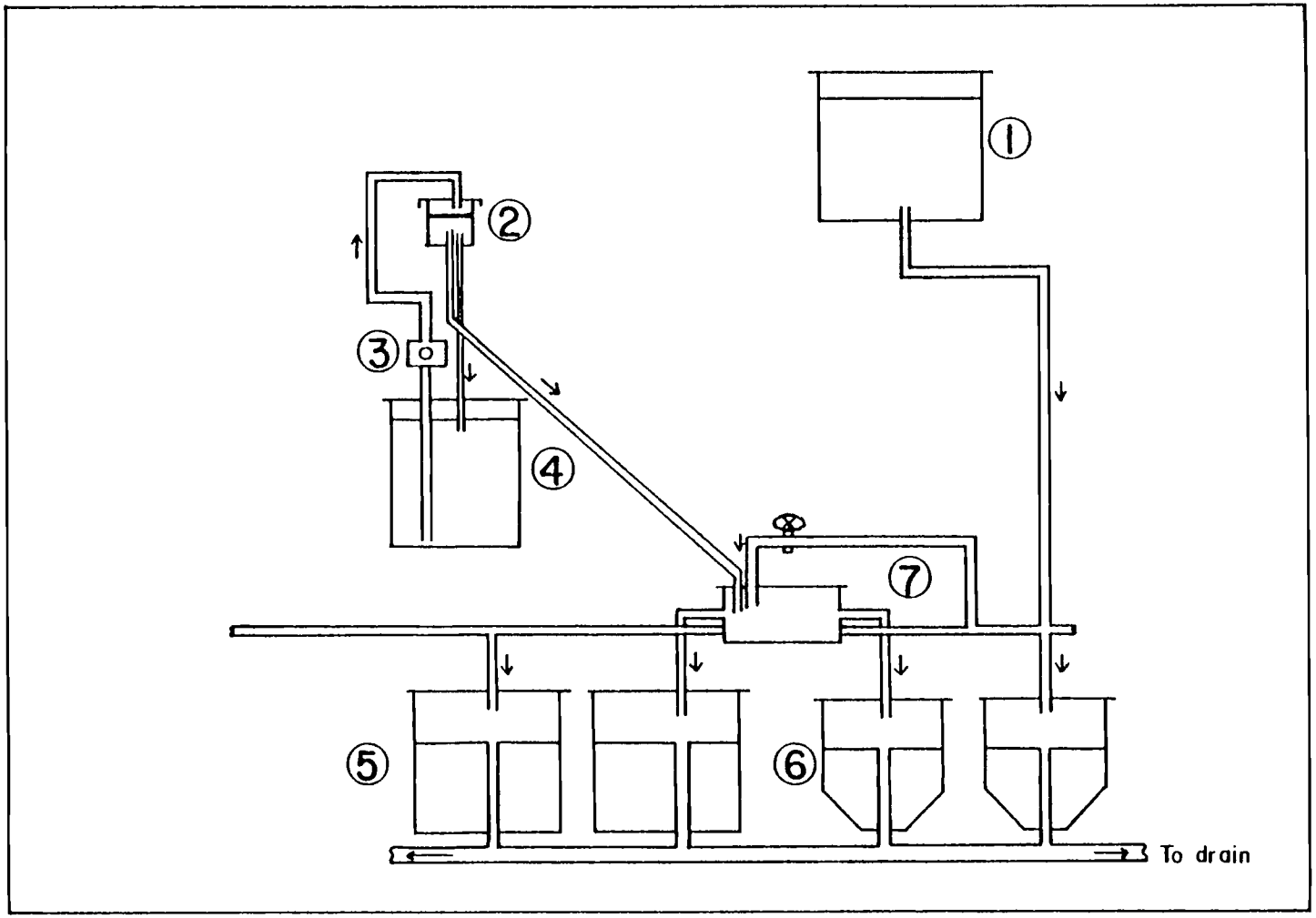


Figure 1.

supplied with a continuous flow of water from an aerated, temperature controlled, 760-litre reservoir with a constant head. The flow rates of the water into each tank were controlled by use of nozzles that delivered water at constant rates. These flow rates were used in calculating the amount of the toxicant solution required to give a desired concentration in the acclimation tanks. Two of the tanks, one of each size, were used for acclimation to the toxicant while the other pair was employed as controls.

Toxicant solutions were pumped from a 450-litre polyethylene tank at 70 ml per minute to a 2-litre constant head chamber to maintain a constant volume delivered per minute. A thorough mixing of the dilution water and the toxicant was assured by introducing the toxicant into a spray of dilution water. A 70 cm diameter stand pipe delivered the diluted toxicant into a 75-litre container. From this container, glass tubing with tygon tubing extensions of equal internal diameter were used to deliver an equal volume of the toxicant solution to each of the two tanks employed for acclimation. The four tanks were drained from the centre by standpipes of 30 cm diameter, maintaining respective volumes of 700-and 400-litres at all times. The zinc solution was delivered from the 2-litre chamber at a rate of 60 ml per minute (Fig. 1). The zinc stock solutions were formulated based on calculations based on the following: the flow rate of the dilution water, the rate of zinc stock solution delivery from the holding tank, and the mole fraction of the toxicant in the chemical in use. For example, the factor of 0.46 was used for zinc which constitutes 46 percent, by weight of $ZnCl_2$.

Choice of Acclimation Concentrations

Acclimation concentrations were chosen based on some fraction of the 96-hr LC10 and were always below 0.50 of the 96-hr LC50 of the initial control bioassays. This ensured that mortalities due to acclimation concentrations never exceeded 10 percent. Acclimation at 50 $\mu g\ell^{-1}$ Zn was 0.37 96-hr LC50 and 0.80 96-hr LC10; at 80 $\mu g\ell^{-1}$ Zn, 0.38 96-hr LC50 and 0.60 96-hr LC10. In the sequential acclimation, the zinc concentrations of 100, 300, 500 $\mu g\ell^{-1}$ (Fig. 2) represented

Figure 2. Zinc exposure profile during the sequential acclimation and deacclimation test series. The squares (A-F) indicate times when acute toxicity tests were started.

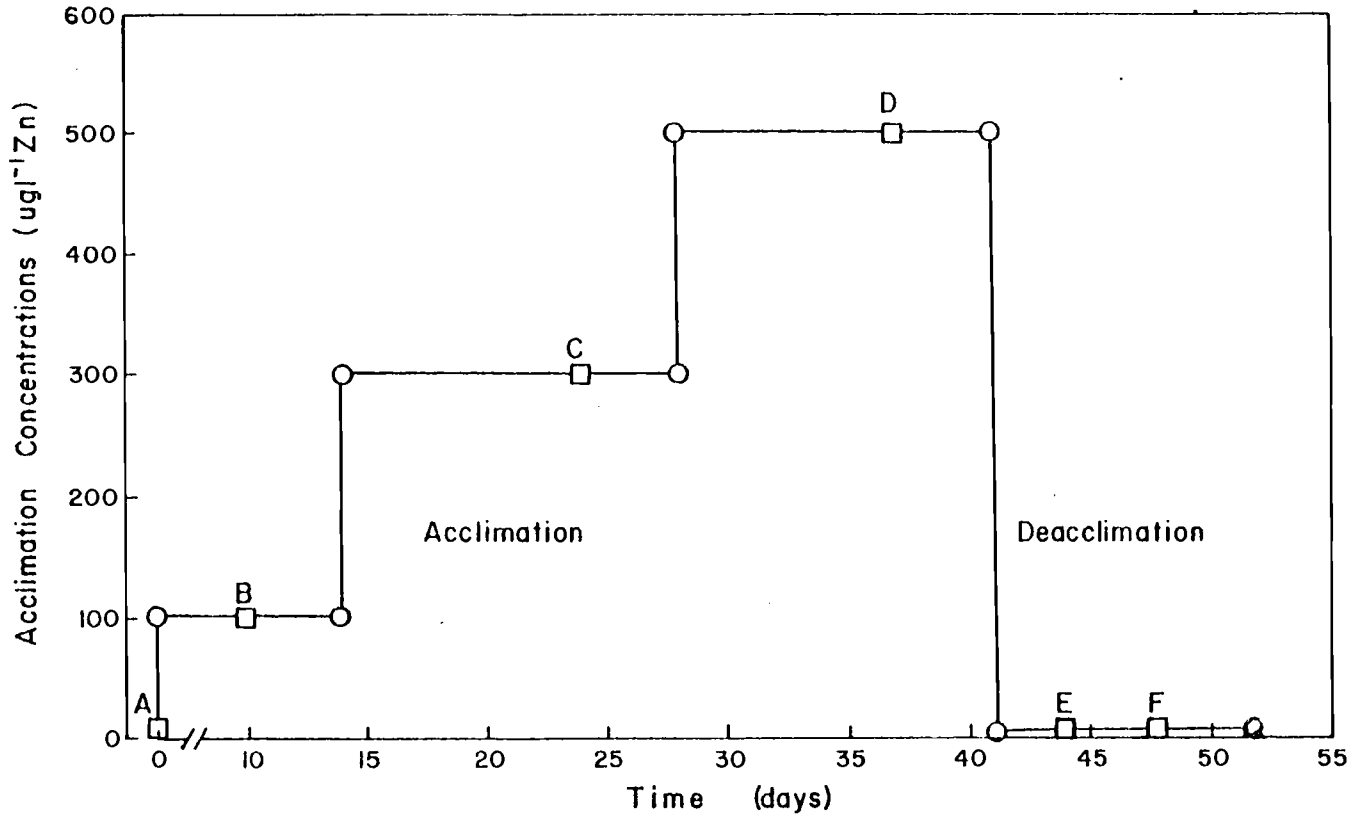

Acclimation Concentrations (ug l⁻¹ Zn)
600
500
400
300
200
100
0
A
B
C
D
E
F
Acclimation
Deacclimation
0
10
15
20
25
30
35
40
45
50
55
Time (days)

Figure 2.

0.40 96-hr LC50 and 1.1 96-hr LC10; 0.30 96-hr LC50 and 0.51 96-hr LC10; and 0.40 96-hr LC50 and 0.60 96-hr LC10, respectively, of the bioassay preceding each phase of acclimation. Acclimation at 100 $\mu g \ell^{-1}$ Zn for the cross tolerance toxicity tests was 0.42 96-hr LC50 and 0.86 96-hr LC10 of the initial control. The highest acclimation mortality observed were during the acclimation at 100 $\mu g \ell^{-1}$ Zn in the sequential acclimation experiment when a 3 percent mortality occurred. All other acclimation mortalities were below 1 percent.

Bioassay apparatus, methods and conditions:

The lethality bioassays were carried out with a simple continuous-flow toxicant dilution system described by Garton (1980). The system uses the principle of serial dilution whereby one source of toxicant is diluted by successive additions of water to produce a series of increasingly dilute concentrations. This system is very reliable as evidenced by the successful operation at WFTS for over 8 years. It delivers accurate concentrations, has only one control valve, only one stock solution is required, there are few small constrictions to be clogged, and has high capacity for flow rate (Garton, 1980; Mount and Warner, 1965). This delivery system was used throughout the bioassay experiments. Three identical diluter units of the continuous flow delivery system were employed. Each of the 3 units dosed 6 pairs of aquaria. Two of these differed in their aquaria size from the third. Two units dosed aquaria of 38 litre capacity and continuously drained through a bottom standpipe which maintained a contant volume of approximately 30 litres. The third unit dosed aquaria of 98 litres with a side drain that maintained a constant volume of 78 litres. This third unit was used only during the cross tolerance bioassays when zinc, cadmium and copper bioassays were carried out simultaneously.

Tanks were covered with black polyethylene on all four sides to minimize visual disturbances. The glass and tygon delivery tubes were also shaded from the light with black polyethylene to minimize algal growth which might present clogging problems. Ten fish per aquaria were distributed by stratified random methods, and the loading density

was in accordance with USEPA (1975) recommendations. Each concentration was split between duplicate aquaria, thereby having 20 fish undergoing each concentration treatment; in the cross-tolerance bioassays only 10 fish were used per concentration treatment as there was only one aquarium per concentration.

In all the bioassays except the cross-tolerance experiments the fish were transferred from control or acclimation tanks to the test aquaria 3 days before the actual bioassay dosing was started. This time was considered necessary for the fish to recover from handling stress before the appropriate toxicant exposures were begun.

In the case of the acclimated stock, the acclimation concentration of zinc was delivered to all the aquaria from the diluter head box until the lethality tests were started. The fish were not fed following transfer to the diluter aquaria. The three diluters delivered their toxicant solutions at the rate of 60, 50 and 60 ml per minute, respectively. The flow rates were checked regularly to avoid unreasonable fluctuations in the toxicant concentrations.

Fish mortalities were checked and recorded several times each day, but dead fish were removed only every 24 hours to avoid physically stressing the non-moribund fish. At the end of the 96-hr exposure period for all other bioassays and the 120-hr exposure period for the cross-tolerance bioassays, percent mortalities were calculated, and the LC50s determined. The extension of the 96-hr exposure period to 120 hr in the cross-tolerance bioassay became necessary when 96-hr mortality in the cadmium exposure was not enough to provide a good estimate of the LC50s.

Quantification of metallothionein-like metal binding soluble hepatoproteins:

Rainbow trout from two treatment groups were used to study hepatic concentrations of metal binding protein associated with zinc acclimation. Measurement of ^{203}Hg binding to a soluble protein fraction represented a generally accepted method to estimate tissue MT content (Piotrowski et al., 1973; Kotsonis and Klaassen, 1977). The first group of fish were those acclimated to 80 $\mu g \ell^{-1}$ of zinc for 3,

7, 14, and 21 days. The second group was sequentially acclimated to 100 $\mu g\ell^{-1}$ of zinc for 10 days, 300 $\mu g\ell^{-1}$ for 14 days and 500 $\mu g\ell^{-1}$ zinc for 10 days. Following the 500 $\mu g\ell^{-1}$ acclimation, they were deacclimated for 3, 7 and 14 days. For convenience, all bioassays have been coded. Details concerning the codes used are given in Appendix 2.

Five fish from the first treatment group, and 10 from each series of the second group were taken from acclimation tank or aquarium, anaesthetized with 2-phenoxyethanol, wrapped in polyethylene bags and stored in a freezer until ready for processing. No fish were kept in the freezer for more than 3 months before dissection of the livers. The livers were subsequently dissected out, taking extra care to remove the intact gall bladder. The excised livers were rinsed in physiological saline (0.7 percent NaCl). Metallothionein-like proteins were then determined by the method described for rat livers [Piotrowski et al (1973) as modified by Kotsonis and Klaasen (1977)]. Gant (personal communication) has successfully employed this method in isolating metallothionein from the marine teleost (Enophrys bison). A schematic representation of this method (slightly modified) is presented in Figure 3.

All solutions were made with distilled deionized water. Liver samples were allowed to thaw in the glass tube containers and were homogenized in approximately 7 volumes of 1.15% KCl using a glass Potter Elvehjem homogenizer fitted with a motorized teflon pestle. The homogenate was centrifuged at 9000 g for 20 minutes in a Sorval refrigerated centrifuge, Model RC-5-B.

Labelled mercury ($^{203}HgCl_2$) obtained from New England Nuclear was added to $HgCl_2$ to make up the required concentration. The final stock solution had 15 μg $HgCl/\mu\ell$ of solution. 750 μg (50 $\mu\ell$ of $^{203}HgCl_2$) were added to 2 ml of liver homogenate from each treatment group. Two mililitres of homogenate was equivalent to 0.25 gm wet weight of liver.

To optimize the mercury binding method the concentration of ^{203}Hg added to the homogenate is critical (Kotonis and Klaassen, 1977). If insufficient ^{203}Hg is added to the liver homogentate, all the MT and

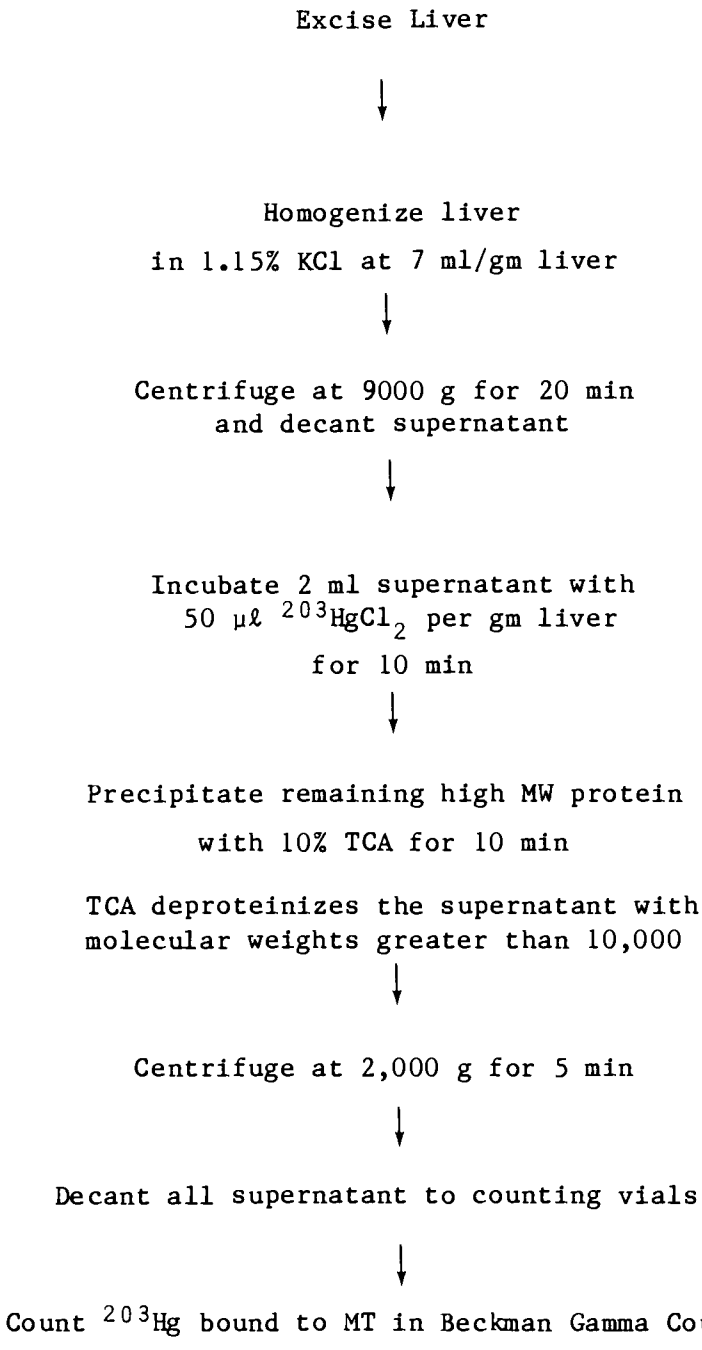


Figure 3. Scheme for quantification of metallothionein HG saturation method (Modified after Piotrowski, J. et al., 1973, and Kotsonis and Klaassen, 1977).

competing proteins will not be bound and a low estimate will result. If too much ^{203}Hg has been added, all binding sites will be saturated and the metal will occur in free solution giving an exaggerated estimate of MT. When an optimal concentation of ^{203}Hg is added all MT is bound and excess ^{203}Hg binds to other proteins which are subsequently precipitated by TCA. To determine this optimum concentration, one group of fish was acclimated to 90 $\mu g \ell^{-1}$ Zn for 3 weeks. Livers from 66 fish were dissected out. Livers from 72 control fish (unacclimated) were treated similarly. ^{203}Hg was added to the homogenates at concentrations ranging from 200-4800 μg Hg gm^{-1} liver. The results as counts per minute obtained from Beckman Gamma Counter were plotted graphically and the appropriate concentration chosen from the plateau region of the graph (Fig. 4). Changes in the quantity of MT at the plateau regions were best estimates of the quanitites of metallothionein present in each treatment. From the plots of ^{203}Hg binding versus ^{203}Hg added, 750 μg ^{203}Hg equivalent to 50 $\mu \ell$ stock solution was selected as optimum.

Data Analysis

The LC10, LC50, and LC90 values (concentrations estimated to kill 10 percent and 50 percent of the test fish, respectively) were calculated for 96-or 120-hr exposure periods. At the end of each bioassay experiment, the LC10, LC50 and LC90 values were calculated from percent mortality values and mean measured toxicant concentrations by probit analysis (Finney, 1971) using the Corvallis USEPA computer program "REGRESS." This program linearly regressed percent mortalities expressed as logits against the logs of the mean measured toxicant concentrations (logit = $\ln \frac{P}{100-P}$; where ℓ = percent mortality). The 96-hr LC10, LC50 and LC90 values were obtained together with the 67, 90 and 95 percent confidence interval estimates. In one case however, the LC50 was obtained by using the Spearman-Karber method as the number of mortalities was inappropriate for the "REGRESS" program. All exposure concentration/mortality data were also plotted to observe the nature of the regression lines.

Figure 4. The amount of ^{203}Hg in the TCA supernatants following addition of increasing amounts of ^{203}Hg to fish liver homogenates. Acclimated and control situations are shown.
Acclimated: livers from rainbow trout acclimated to 90 $\mu g \ell^{-1}$ zinc for 3 weeks.
Control: non-acclimated fish.

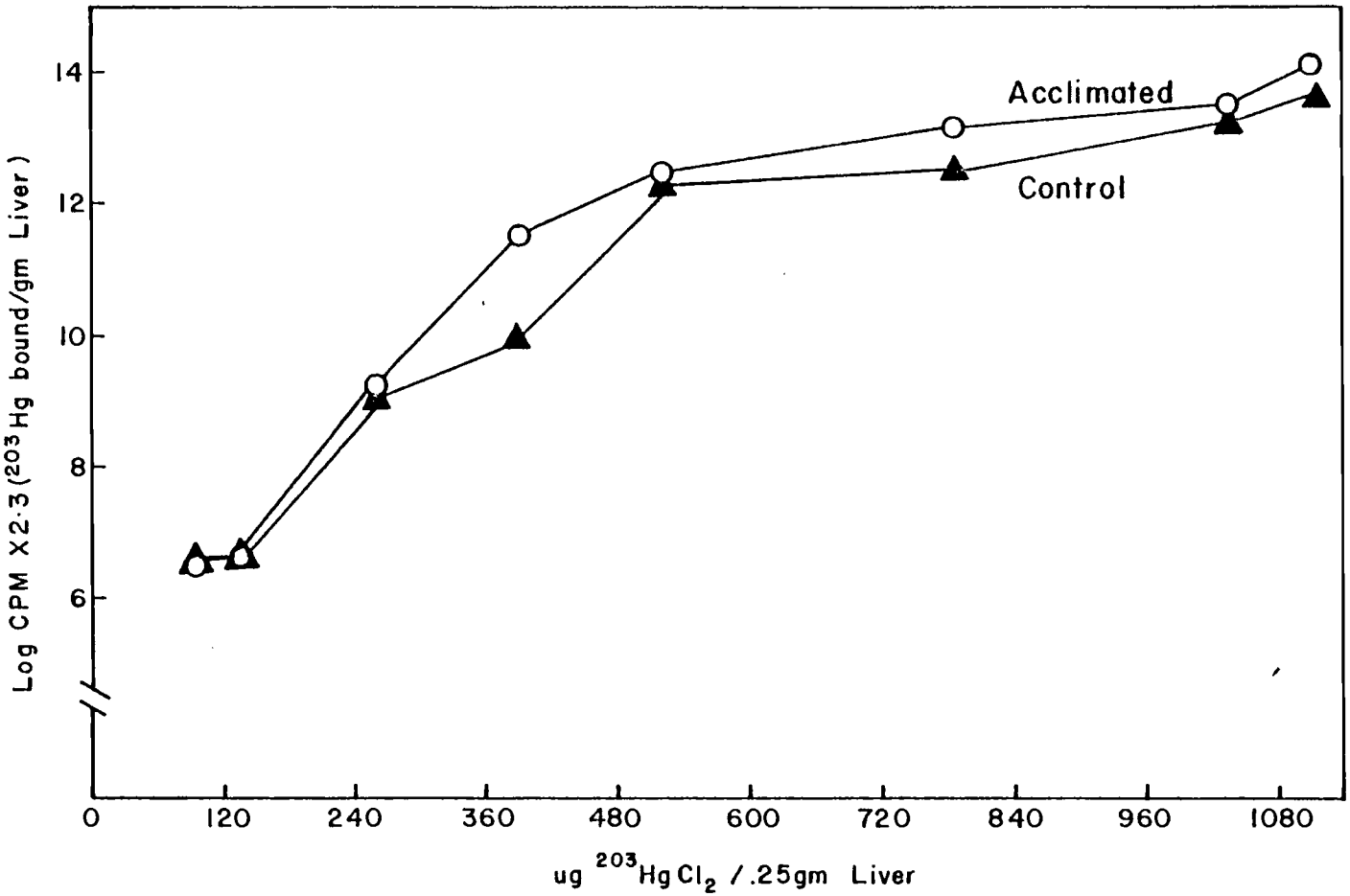

Acclimated
Control
14
12
10
8
6
0
120
240
360
480
600
720
840
960
1080
Log CPM X2·3 (^{203}Hg bound/gm Liver)
ug $^{203}HgCl_2$ / .25gm Liver

Figure 4.

Significant differences between the variously acclimated (duration and concentration) fish and their controls were calculated based on the "Standard Error of the Difference" Finney (1971), Zar (1974) and re-emphasized by Sprague (1977). This method makes use of the ratio ("f" ratio) of the LC50 to its upper 95 percent fiducial limit (Litchfield and Wilcoxon, 1948)

$$\text{i.e.} \qquad f = \frac{\text{upper fiducial limit of LC50}}{\text{LC50}}$$

Then the product $f_{1\ 2} = \text{Antilog} \sqrt{(\log f_1)^2 + (\log f_2)^2}$

If the ratio: $\frac{\text{Greater LC50}}{\text{Lesser LC50}}$, is greater than $f_{1\ 2}$, then the two LC50's are significantly different at ($P < 0.05$). Data on MT levels and their dynamics were compared as a function of acclimation period and zinc concentration using regular scaled and normalized plots.

RESULTS

Steelhead trout acclimated to constant non-lethal zinc concentration

The individual 96-hr LC50s of steelhead trout acclimated to 50 $\mu g \ell^{-1}$ Zn for four different acclimation times were significantly higher than their respective controls ($P < 0.05$) (Table 1 and Appendix 2). The illustrated trend (Fig. 5) shows a rather steep rise in the LC50 values within one week of the acclimation. There was no further significant increase at longer periods of acclimation. The trend at times below 7 days was not determined in this experiment which makes the line joining 0 to 7 days in Figure 5 a hypothetical one. This shows that nearly all acclimation occurred in less than a week of exposure. No mortality occurred during the 21-day acclimation to 50 $\mu g \ell^{-1}$.

The mean (±SD) 96-hr LC50 for steelhead trout acclimated to 50 $\mu g \ell^{-1}$ of zinc for 7, 14, 17 and 21 days was 507.25 ± 74.240 $\mu g \ell^{-1}$ of zinc. The mean ± SD of the controls was 164.4 ± 61.985 $\mu g \ell^{-1}$. This acclimation resulted in a 3-fold increase over control LC50s.

The dose-response curves are shown in Figure 6 and the regression equations describing the curves are given in Table 2. The probit transformation used in the plots adjusted the percent mortality to an assumed normal population distribution (Klaassen and Doull, 1980).

The non-parallel regression lines indicate greater effect of acclimation at lower lethality levels, where the LC10 concentration differences are rather great. However, at higher lethality levels, for example the LC90, the concentration differences are smaller. The other group of fish exposed to 50 $\mu g \ell^{-1}$ of zinc for 7 days and then to 200 $\mu g \ell^{-1}$ of zinc for 10 days showed similar response. The 96-hr LC50 for the acclimated fish in the latter test was 634 while the control was 280 $\mu g \ell^{-1}$ Zn. The regression plot showed a common LC95 (i.e. 5 percent could survive higher zinc concentrations with or without acclimation).

Table 1. Summary of results showing the effect of acclimation of steelhead trout to zinc on the 96-hr LC50.

Bioassay termination date	Bioassay description	Acclimation time (days)	96-hr LC50 ($\mu g \ell^{-1}$ zinc)	Change in tolerance expressed as Acclimated: Control
9/25/81	Initial bioassy	0	134	--
10/16/81	Acclimated[a]	7	468*	
	Control		98	4.8
10/23/81	Acclimated[a]	14	481*	
	Control		168	2.9
10/30/81	Acclimated[a]	21	446*	
	Control		142	4.2
11/27/81	Acclimated[b]	17	634*	
	Control		280	2.3

[a] Acclimation to 50 $\mu g \ell^{-1}$ Zn.

[b] This group acclimated to 50 $\mu g \ell^{-1}$ for 7 days, then to 200 $\mu g \ell^{-1}$ for 10 days. Not used in the plot of Figure 5.

* Significant difference based on the "Standard error of the difference." F values, ($P < 0.05$).

Figure 5. The effect of acclimation on the 96-hr LC50s of steelhead trout acclimated to constant non-lethal zinc concentration for 7, 14, and 21 days. The 95 percent fiducial limits are given.

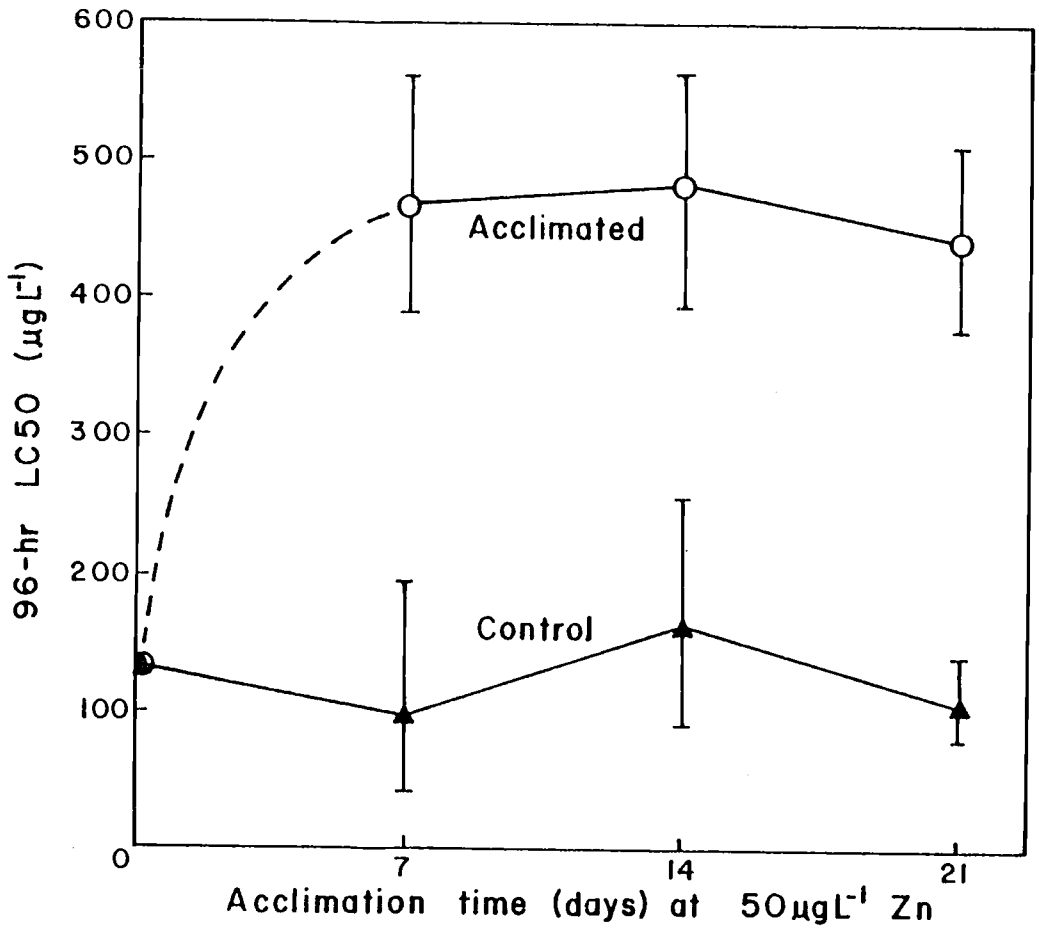
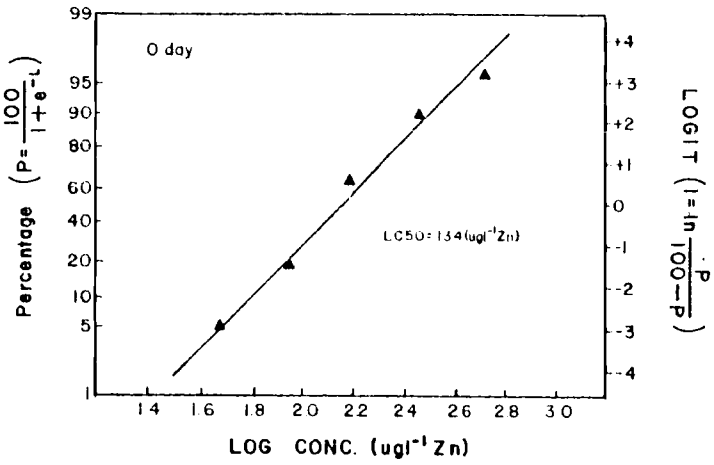
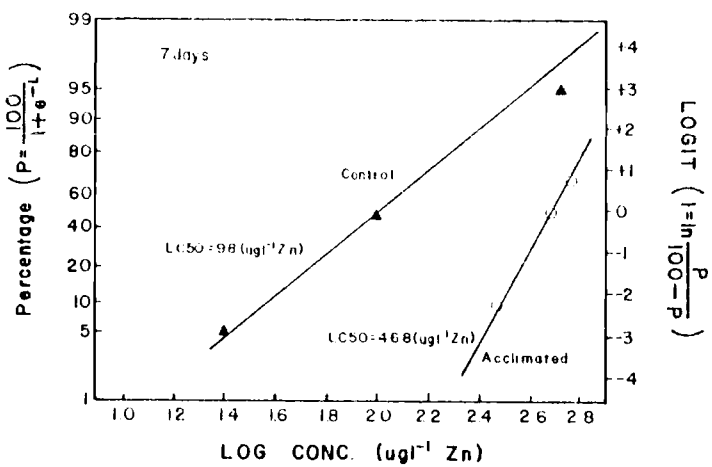
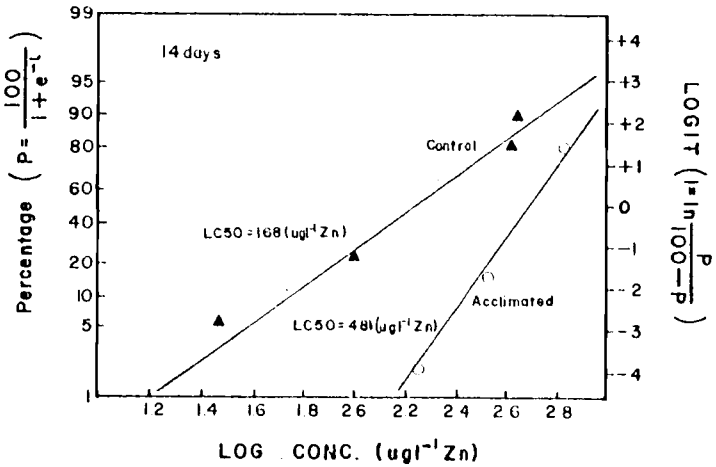

600
500
400
300
200
100
0
96-hr LC50 (µgL⁻¹)
Acclimated
Control
7
14
21
Acclimation time (days) at 50µgL⁻¹ Zn

Figure 5.

Figure 6. Dose-response curves showing effects of acclimation of steelhead trout to constant non-lethal zinc concentrations for 0, 7, 14 and 21 days on the 96-hr LC50s.


0 day
LC50=134(ugl⁻¹Zn)
Percentage (P=100/1+e⁻ᴸ)
LOGIT (l=ln P/100−P)
LOG CONC. (ugl⁻¹ Zn)


7 days
Control
LC50=98(ugl⁻¹Zn)
LC50=468(ugl⁻¹Zn)
Acclimated
Percentage (P=100/1+e⁻ᴸ)
LOGIT (l=ln P/100−P)
LOG CONC. (ugl⁻¹ Zn)


14 days
Control
LC50=168(ugl⁻¹Zn)
LC50=481(ugl⁻¹Zn)
Acclimated
Percentage (P=100/1+e⁻ᴸ)
LOGIT (l=ln P/100−P)
LOG CONC. (ugl⁻¹ Zn)

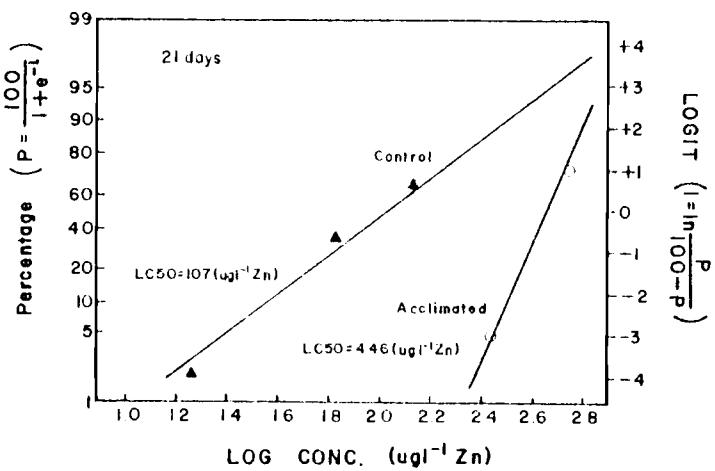

21 days
Control
LC50=107(ugl⁻¹Zn)
Acclimated
LC50=446(ugl⁻¹Zn)
Percentage (P=100/1+e⁻ᴸ)
LOGIT (l=ln P/100−P)
LOG CONC. (ugl⁻¹ Zn)

Figure 6.

Table 2. Regression equations for zinc bioassay response curves, their respective t-values and standard error of the slope for steelhead trout acclimated to 50 $\mu g \ell^{-1}$ zinc for 7, 14, 21, and 17 days.

Description	Regression Equation	96-hr LC50 $\mu g \ell^{-1}$ Zn	Standard error of slope	T-values for slope
Un-Zn-INIT-Zn†	Logit P = -13.875 + 6.521 log Conc.	134	1.13	5.78
Ac-Zn-50-7d-Zn	Logit P = -30.026 + 11.246 log Conc.	468	2.66	4.24
Un-Zn-50-7d-Zn	Logit P = -10.075 + 5.065 log Conc.	98	0.98	5.18
Ac-Zn-50-14d-Zn	Logit P = -23.463 + 8.747 log Conc.	481	1.94	4.50
Un-Zn-50-14d-Zn	Logit P = -9.251 + 4.560 log Conc.	107	0.76	5.76
Ac-Zn-50-21d-Zn	Logit P = -39.620 + 14.953 log Conc.	446	3.51	4.26
Un-ZN-50-21d-Zn	Logit P = -9.251 + 4.560 log Conc.	107	0.76	5.97
Ac-Zn-50-7d-200-10d-Zn	Logit P = -9.251 + 4.560 log Conc.	634	2.20	4.56
Un-Zn-50-7d-200-10d-Zn	Logit P = -10.785 + 4.408 log Conc.	280	0.080	5.50

†Bioassays coded as in Appendix 2.

Rainbow trout acclimated to constant non-lethal zinc concentration:

Rainbow trout acclimated to constant non-lethal zinc concentration for 1, 2, 3, 7, 21, and 28 days had 96-hr LC50s determined concurrently with controls. Acclimation did not occur in the first 2 days because there was little or no difference between the 96-hr LC50s of the two groups. By the 3rd day, significant differences existed between the 96-hr LC50s of the acclimated trout and the controls. At longer acclimation periods, there were no further significant increases in the 96-hr LC50s. The mean (± SD) 96-hr LC50 for this group of rainbow trout was 960 ± 65 $\mu g \ell^{-1}$ zinc for fish acclimated for 3, 7, 21, and 28 days, while the control mean was 372 ± 65 $\mu g \ell^{-1}$ Zn (excluding the 21d value). This represents approximately a 2.5-fold increase in the zinc concentrations required to cause 50 percent mortality in the acclimated fish over the controls. The controls did not show a wide variation in their 96-hr LC50s with the unexplained exception of one case where the LC50 (>793 $\mu g \ell^{-1}$) could not be determined due to insufficient mortality (Fig. 7 and Table 3).

Exposure for short periods (1 and 2 days) to 100 $\mu g \ell^{-1}$ zinc showed that in both cases, the 96-hr LC50s of the control fish were higher than those for the acclimated fish (Fig. 7). This may be due to "sensitization" (Dixon and Sprague, 1981) immediately following initial exposure. However, in an earlier test following three days of acclimation, a greater than 2-fold increase in the LC50 of the acclimated fish resulted. No further significant increases of LC50s occurred after 7 to 28 days of acclimation at the same concentration. Rather a levelling off was observed. The dose-response curves are plotted in Figure 8 and described in detail in Table 4.

Rainbow trout acclimated to increasing non-lethal concentrations of zinc and deacclimated for 3 and 7 days:

Rainbow trout showed no definite increase in the tolerance level with an increasing sequential exposure to zinc concentrations greater than 100 $\mu g \ell^{-1}$ (Table 5, Figure 9). A mean (± SD) 96-hr LC50 of 1113 ± 93 $\mu g \ell^{-1}$ Zn was found for the acclimated fish as compared to the

Figure 7. The effect of acclimation of rainbow trout to a constant non-lethal zinc concentration (80 $\mu g \ell^{-1}$) for 0, 1, 2, 3, 7, 21, and 28 days.

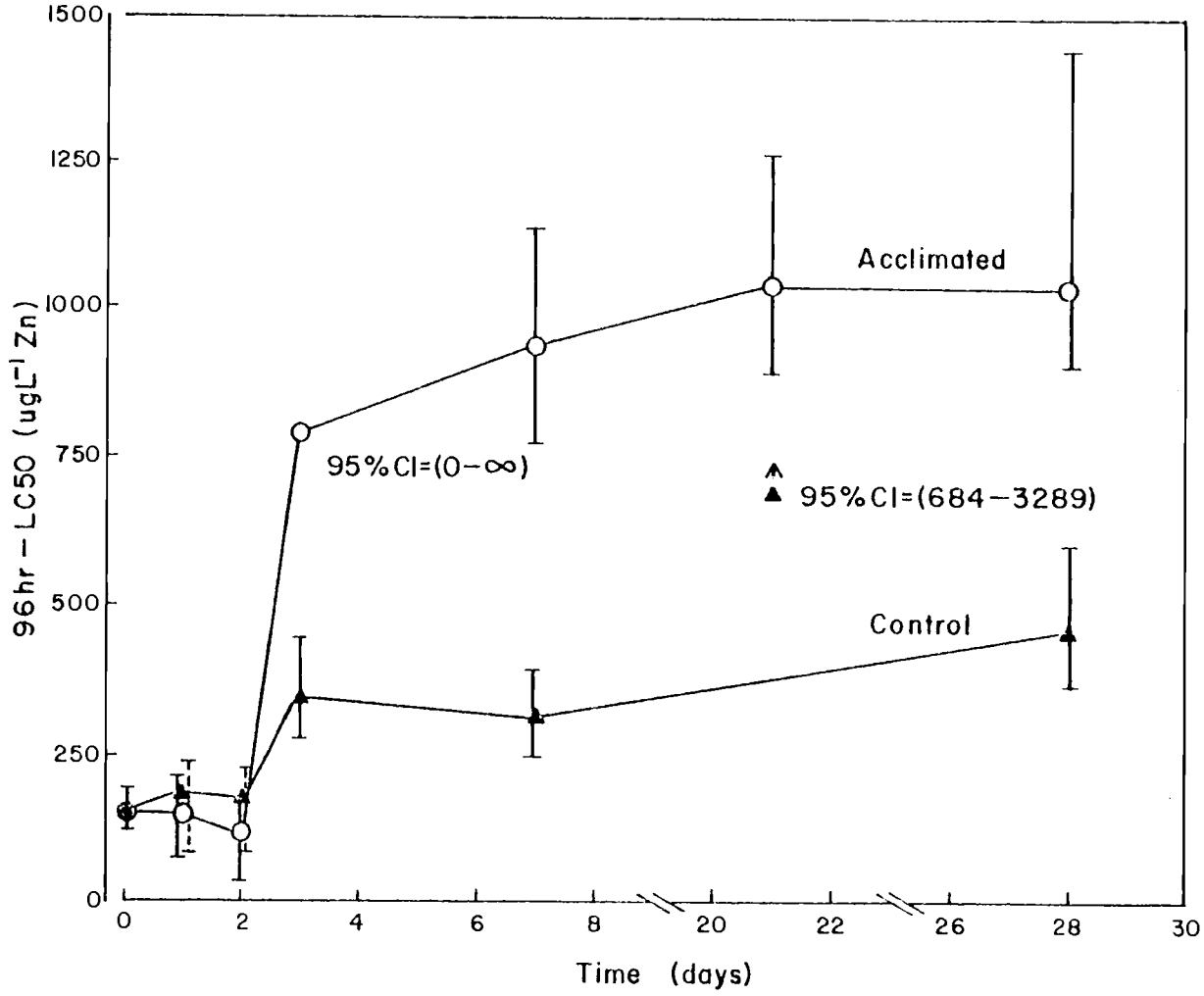

1500
1250
1000
750
500
250
0
96hr – LC50 (ugL⁻¹ Zn)
Acclimated
95%CI=(0–∞)
95%CI=(684–3289)
Control
0
2
4
6
8
20
22
26
28
30
Time (days)

Figure 7.

Table 3. The effect of acclimation of rainbow trout to 80 $\mu g\ell^{-1}$ of zinc on the 96-hr LC50s.

Bioassay termination date	Bioassay descriptions	Acclimation time (days)	96-hr LC50 $\mu g\ell^{-1}$	Change in Tolerance
11/4/82	INITIAL BIOASSAY	0	181	--
11/4/82	Ac-Zn-100-1d-Zn(c)†	1	152	0.84
	Control		181	
11/4/82	Ac-Zn-100-2d-Zn(c)	2	112	0.62
	Control		181	
5/14/82	INITIAL BIOASSAY	0	211	
6/11/82	Ac-Zn-80-3d-Zn	3	>798*	2.32
	Control		344	
6/18/82	Ac-Zn-80-7d-Zn	7	938*	2.88
	Control		311	
7/2/82	Ac-Zn-80-21d-Zn	21	1049	ND
	Control	ND	>793	
7/9/82	Ac-Zn-80-28d-Zn	28	894*	1.94
	Control		462	

(c) bioassay performed at different times with different sub-population of fish and results from 100 $\mu g\ell^{-1}$ acclimation group.

* Significant difference based on the "Standard error of the difference" 'f' values ($P < 0.05$).

† Bioassay coded as in Appendix 2.

ND Not determined.

Figure 8. Dose response curves showing the effect of acclimation of rainbow trout to constant non-lethal zinc concentrations for 1, 2, 7, and 28 days.

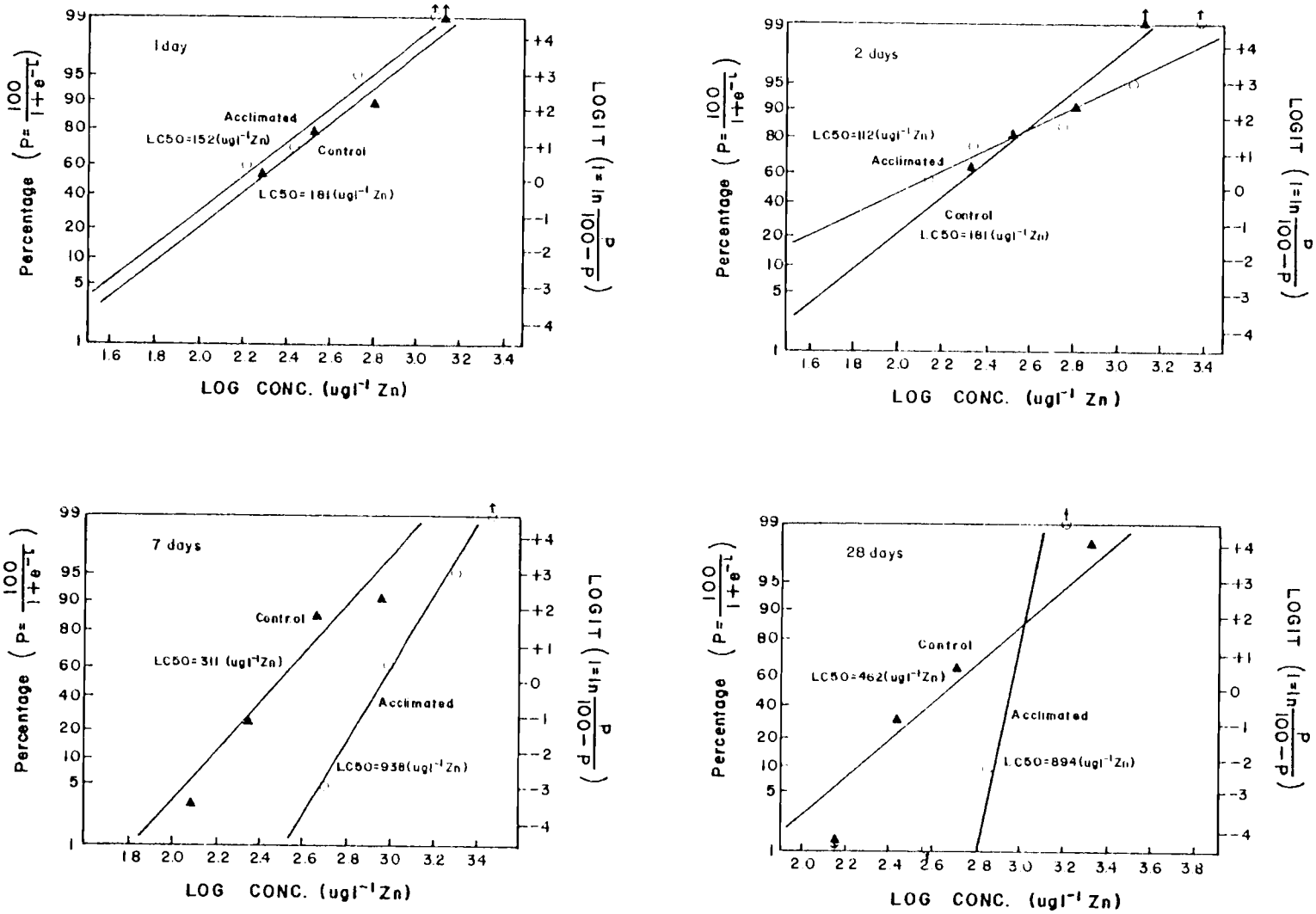

1 day
Acclimated
LC50=152(ugl⁻¹Zn)
Control
LC50=181(ugl⁻¹Zn)
2 days
LC50=112(ugl⁻¹Zn)
Acclimated
Control
LC50=181(ugl⁻¹Zn)
7 days
Control
LC50=311 (ugl⁻¹Zn)
Acclimated
LC50=938(ugl⁻¹Zn)
28 days
Control
LC50=462(ugl⁻¹Zn)
Acclimated
LC50=894(ugl⁻¹Zn)
Percentage (P= 100/(1+e^-I))
LOGIT (I=ln P/(100−P))
LOG CONC. (ugl⁻¹ Zn)

Figure 8.

Table 4. Regression equations for dose-response curves, the respective t-values and the standard error of the slope from zinc bioassays with rainbow trout acclimated to zinc for 0, 1, 2, 3, 7, 21, and 28 days. 96-hr LC50s are given.

Description	Regression Equation	96-hr LC50 $\mu g \ell^{-1}$ Zn	Standard error of slope	t-values for slope
Un-Zn-INIT-Zn†	Logit P = -29.020 + 12.485 log Conc.	211	3.28	3.81
Ac-Zn-100-1d-Zn	Logit P = -10.725 + 4.913 log Conc.	152	1.49	3.30
Un-Zn-100-1d-Zn	Logit P = -10.958 + 4.853 log Conc.	181	1.40	3.46
Ac-Zn-100-2d-Zn	Logit P = -6.093 + 2.973 log Conc.	112	0.86	3.44
Un-Zn-100-2d-Zn	Logit P = -10.958 + 7.853 log Conc.	181	1.40	3.46
Ac-Zn-80-3d-Zn	not plotted	>798	--	--
Un-Zn-80-3d-Zn	Logit P = -15.732 + 6.201 log Conc.	344	1.23	5.05
Ac-Zn-80-7d-Zn	Logit P = -29.567 + 9.948 log Conc.	938	2.28	4.36
Un-Zn-80-7d-Zn	Logit P = -16.692 + 6.697 log Conc.	311	1.26	5.32
Ac-Zn-80-21d-Zn	Logit P = -37.699 + 12.479 log Conc.	1050	3.22	3.88
Un-Zn-80-21d-Zn	not plotted	>793	-	-
Ac-Zn-80-28d-Zn	Logit P = -94.382 + 31.981 log Conc.	894	12.08	2.65
Un-Zn-80-28d-Zn	Logit P = -13.985 + 5.248 log Conc.	462	0.98	5.38

† Bioassays coded as in Appendix 2.

Table 5. The effect of zinc acclimation and deacclimation on the 96-hr LC50s of zinc to rainbow trout.

Bioassay termination date	Bioassay description	Total Acclimation Time in days	96-hr LC50	Increase in tolerance Acclimated: Control
9/2/82	Initial control	0	247	--
9/10/82	Ac-Zn-100-10d-Zn†	10	1097*	4.570
	Control		240	
9/30/82	Ac-Zn-100,300-14d-Zn	24	1234*	6.02
	Control		205	
10/14/82	Ac-Zn-100,300,500-10d-Zn	37	1009*	5.02
	Control		201	
10/18/82	Ac-Zn...500.de.3d-Zn	41	872*	4.152
	Control		210	
10/26/82	Ac-Zn...500.de.7d-Zn	41	306	1.302
	Control		235	

† Bioassays coded as in Appendix 2.

* Significant difference ($P < 0.05$).

Figure 9. The effect of acclimation to increasing concentrations of zinc and subsequent deacclimation on toxicity of zinc to rainbow trout.

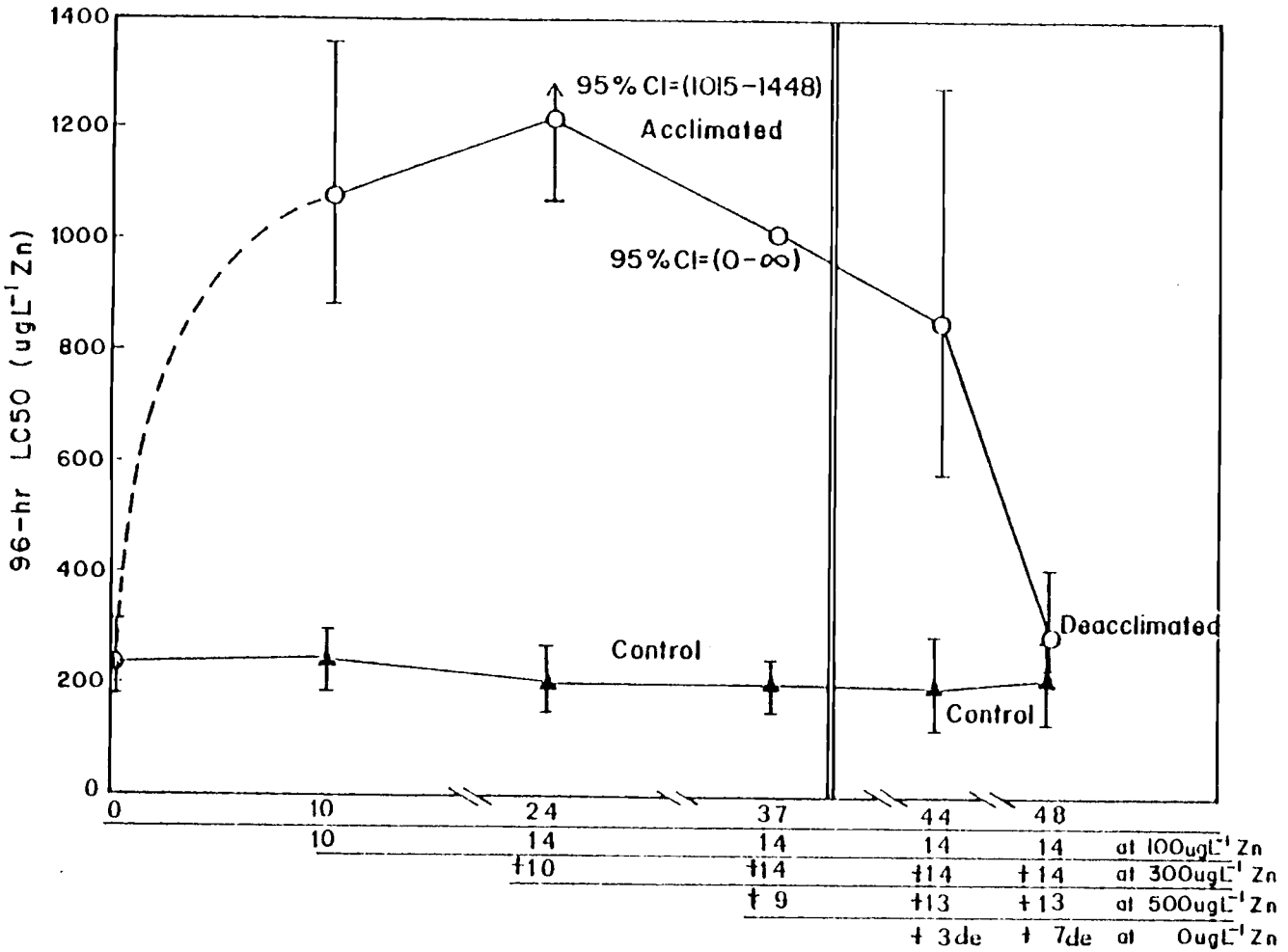

1400
1200
1000
800
600
400
200
0
96-hr LC50 (ugL⁻¹ Zn)
95% CI=(1015-1448)
Acclimated
95% CI=(0-∞)
Deacclimated
Control
Control
0
10
24
37
44
48
10
14
14
14
14
at 100ugL⁻¹ Zn
+10
+14
+14
+14
at 300ugL⁻¹ Zn
+ 9
+13
+13
at 500ugL⁻¹ Zn
+ 3de
+ 7de
at 0ugL⁻¹ Zn
Acclimation Time (days)

Figure 9.

control mean (± SD) 96-hr LC50 of 215 ± 18 $\mu g \ell^{-1}$. This is a 5-fold increase. The fluctuation from 1097 to 1234 and back to 1009 $\mu g \ell^{-1}$ at 100, 300, and 500 $\mu g \ell^{-1}$ Zn does not seem significant. A description of the dose-response curves is given in Table 6. Most of the acclimation apparently occurred during the first 10 days, confirming the results of the previous experiment which showed that acclimation occurred after 3 days and became nearly maximum within the first week. No significant increase or decrease in the LC50 values after the first week or 10 days of acclimation occurred in the bioassay results.

Following the acclimation to 500 $\mu g \ell^{-1}$ Zn, the acclimation exposure was terminated and normal well water introduced into the tanks for periods referred to as deacclimation. The 96-hr LC50s for the 3-and 7-days deacclimated fish were determined. After 3 days the 96-hr LC50 value remained 4-fold above the control. This was still significantly greater tolerance ($p < 0.05$); however, there was an apparent downward trend from the 96-hr LC50 values of acclimated fish. After 7 days, however, an obvious loss of acclimation occurred since there was only a 1.3-fold increase in the 96-hr LC50 over the controls, and the difference between the absolute values was not significant. The 7-day deacclimated fish had 96-hr LC50 values significantly lower than the acclimated and 3 day deacclimated values but not lower than the controls. A summary graph shows the acclimation and deacclimation conditions (Fig. 9) as a continuing response; the significant differences are shown more clearly in the dose-response curves (Fig. 10). Less tolerance was developed in fish exposed to 80 $\mu g \ell^{-1}$ of zinc than by those sequentially acclimated to 100, 300, and 500 $\mu g \ell^{-1}$ of zinc.

CROSS TOLERANCE

Cadmium and Copper

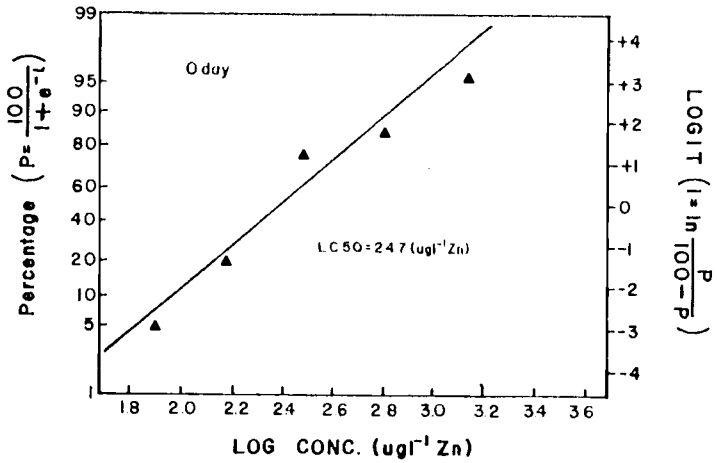
Rainbow trout exposed to 100 $\mu g \ell^{-1}$ of zinc for 17 days showed a 3.2-fold increase in zinc tolerance over the controls. Acute toxicity bioassays with this group of fish were also conducted using cadmium ($CdCl_2$) and copper ($CuCl_2$). Zinc acclimation resulted in 4.3-and

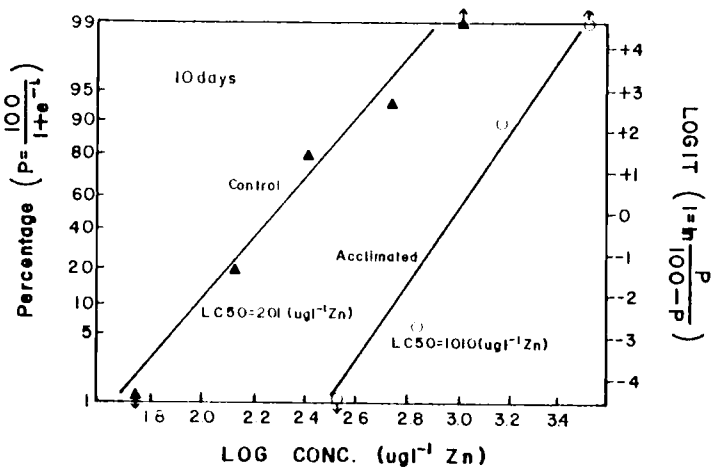
Table 6. Regression equations for dose-response curves, the respective t-values and standard error of the slope from zinc bioassays with rainbow trout acclimated to various concentrations of zinc for various periods and subsequently deacclimated for one week. 96-hr LC50s are given.

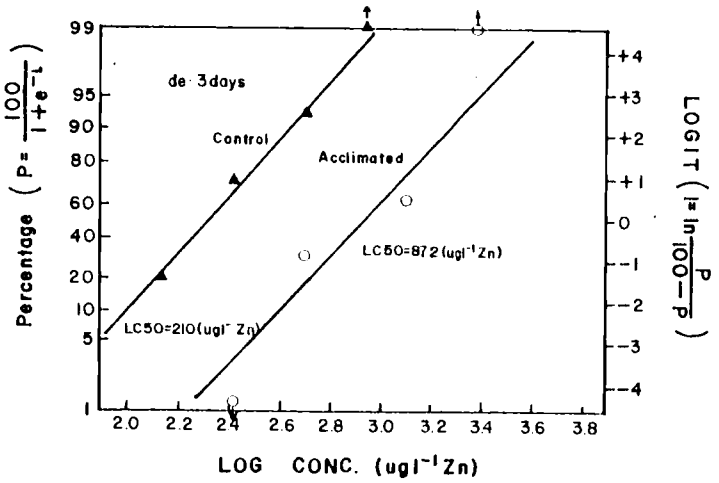
Description	Regression Equation	96-hr LC50 $\mu g \ell^{-1}$	Standard error of slope	t-values for slope
Un-Zn-INIT-Zn†	Logit P = -12.054 + 5.39 log Conc.	247	0.93	5.43
Ac-Zn-100-10d-Zn	Logit P = -24.573 + 8.083 log Conc.	1097	1.58	5.13
Un-Zn-100-10d-Zn	Logit P = -16.977 + 7.130 log Conc.	240	1.41	5.07
Ac-Zn-300-10d-Zn	Logit P = -39.887 + 12.902 log Conc.	1234	3.27	3.94
Un-Zn-300-10d-Zn	Logit P = -10.403 + 4.499 log Conc.	205	0.87	5.16
Ac-Zn-500-10d-Zn	Logit P = -27.090 + 9.018 log Conc.	1009	4.23	2.13
Un-Zn-500-10d-Zn	Logit P = -16.598 + 7.206 log Conc.	201	1.49	4.84
Ac-Zn-500-10d-de.3d-Zn	Logit P = -18.850 + 6.411 log Conc.	872	1.67	3.85
Un-Zn-500-10d-de.3d-Zn	Logit P = -15.792 + 6.798 log Conc.	210	2.15	3.46
Ac-Zn-500-de.7d-Zn	Logit P = -24.801 + 9.980 log Conc.	306	3.28	3.05
Un-Zn-500-de.7d-Zn	Logit P = -16.967 + 7.154 log Conc.	235	2.39	2.99

† Bioassays coded as in Appendix 2.

Figure 10. Dose response curves for rainbow trout acclimated at increasing non-lethal zinc concentration, and then deacclimated for 3 and 7 days.


0 day
LC50=247 (ugl⁻¹Zn)
Percentage (P= 100/(1+e^-t))
LOGIT (l=ln P/(100−P))
LOG CONC. (ugl⁻¹ Zn)


10 days
Control
Acclimated
LC50=201 (ugl⁻¹Zn)
LC50=1010(ugl⁻¹Zn)
Percentage (P= 100/(1+e^-t))
LOGIT (l=ln P/(100−P))
LOG CONC. (ugl⁻¹ Zn)


de 3days
Control
Acclimated
LC50=210(ugl⁻ Zn)
LC50=872(ugl⁻¹Zn)
Percentage (P= 100/(1+e^-t))
LOGIT (l=ln P/(100−P))
LOG CONC. (ugl⁻¹Zn)

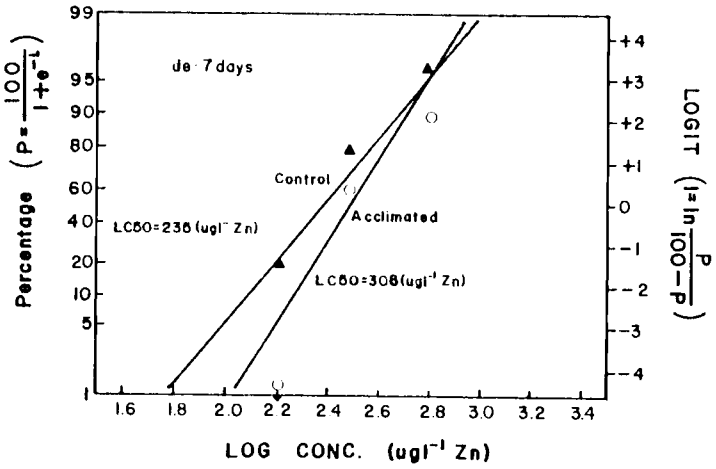

de 7days
Control
Acclimated
LC50=238(ugl⁻ Zn)
LC50=308(ugl⁻¹ Zn)
Percentage (P= 100/(1+e^-t))
LOGIT (l=ln P/(100−P))
LOG CONC. (ugl⁻¹ Zn)

Figure 10.

3.2-fold increases in tolerance to copper and cadmium, respectively. The durations of these bioassays were extended to 120-hr instead of the conventional 96-hr period because of the initially slow mortality rate in the cadmium test. The 120-hr LC50 values and the regressions are given in Table 7 and the statistically analyzed data in Appendix 3.

Low molecular weight metal binding soluble hepatoprotein (metallothionein):

The levels of metallothionein-like proteins (MT) were determined following acclimation of rainbow trout to Zn at 100, 100+300, 100+300+(375), 100+300+(750), 100+300+500, 100+300+500+(189), 100+300+500+(720) $\mu g \ell^{-1}$ for 10, 10+14, 10+14+14, 10+14+4, 10+14+10, 10+14+10+4 and 10+14+10+4 days, respectively, in two different experiments. The plus symbols (+) indicate that the test fish were acclimated at the first concentration for the first period, then at the second concentration for the second period, etc.

Results showed that livers from acclimated fish had higher concentrations of MT than livers from control fish. Following 10 days acclimation at 100 $\mu g \ell^{-1}$ MT increased 26 percent over control levels. Neither higher acclimation concentration levels nor longer periods of acclimation produced levels of MT higher than those observed following 10 days acclimation at 100 $\mu g \ell^{-1}$. Fluctuations of the MT levels occurred in both acclimated and control fish over the period of the experiment. Acclimation produced a mean (± SD) percent increase in MT over the controls of 22 (± 8.2) percent as illustrated in Table 8 and Figure 11.

Another group of rainbow trout (from the same stock) were acclimated to 80 $\mu g \ell^{-1}$ Zn for 3, 7, 14 and 21 days and the MT levels determined. A gradual increase in the MT levels was seen in acclimated fish over the entire 21-day period of the test. The rate of increase appeared greatest between day 7 and day 14. The increase in MT content of livers seemed to level off after 14 days as the difference between the 14th and 21st days was not appreciable. The mean (± SD) percent increase in MT (all acclimated groups) over the controls was

Table 7. Regression equations for zinc, copper and cadmium bioassay dose-response curves, the 120-hr LC50s, the respective t-values, and standard error of the slope for rainbow trout after acclimation to 100 $\mu g \ell^{-1}$ zinc for 17 days.

Bioassay Description	Regression Equation	120-hr LC50	Standard error of slope	t-values for slope
Ac-Zn-100-17d-Zn†	Logit P = -75.250 + 26.960 log Conc.	618	16.77	1.61
Un-Zn-100-17d-Zn	Logit P = -18.888 + 8.472 log Conc.	170	5.01	1.69
Ac-Zn-100-17d-Cu	Logit P = -7.398 + 4.851 log Conc.	33	1.20	4.05
Un-Zn-100-17d-Cu	Logit P = -2.316 + 2.719 log Conc.	7	0.89	3.07
Ac-Zn-100-17d-Cd	Logit P = -4.422 + 7.206 log Conc.	4.1	1.96	3.68
Un-Zn-100-17d-Cd	Logit P = -0.748 + 25.644 log Conc.	1.1	9.27	2.77

† Bioassays coded as in Appendix 2.

Table 8. Metallothionein levels in livers of juvenile rainbow trout acclimated to various zinc concentrations for various times.

Bioassay Condition	MT (μmoles ^{203}Hg per gm liver)	Ratio of Acclimation to Control (Expressed as % Control)
Ac-Zn-100-10d-Zn†	7.832	1.262 (126.2)
Control	6.202	
Ac-Zn-100,300-14d-Zn	6.206	1.105 (110.5)
Control	5.615	
Ac-Zn-100,300,500-10d-Zn	6.206	1.235 (123.5)
Control	5.024	
Ac-Zn-100,300,(375)-4d-Zn	7.684	1.182 (118.2)
Control	6.498	
Ac-Zn-100,300,(750)-4d-Zn	7.241	1.114 (111.2)
Control	6.498	
Ac-Zn-100,300,500,(189)-4d-Zn	7.182	1.313 (131.3)
Control	5.467	
Ac-Zn-100,300,500,(720-4d-Zn	7.241	1.324 (32.4)
Control	5.467	

(---) Indicate acute bioassay test exposure concentration and time.

† Bioassays coded as in Appendix 2.

Figure 11. Metallothionein levels in livers of juvenile rainbow trout acclimated to sequentially higher zinc concentrations for various times. A = regular plot, and B = normalized plot (as percent of controls) vs zinc concentrations.

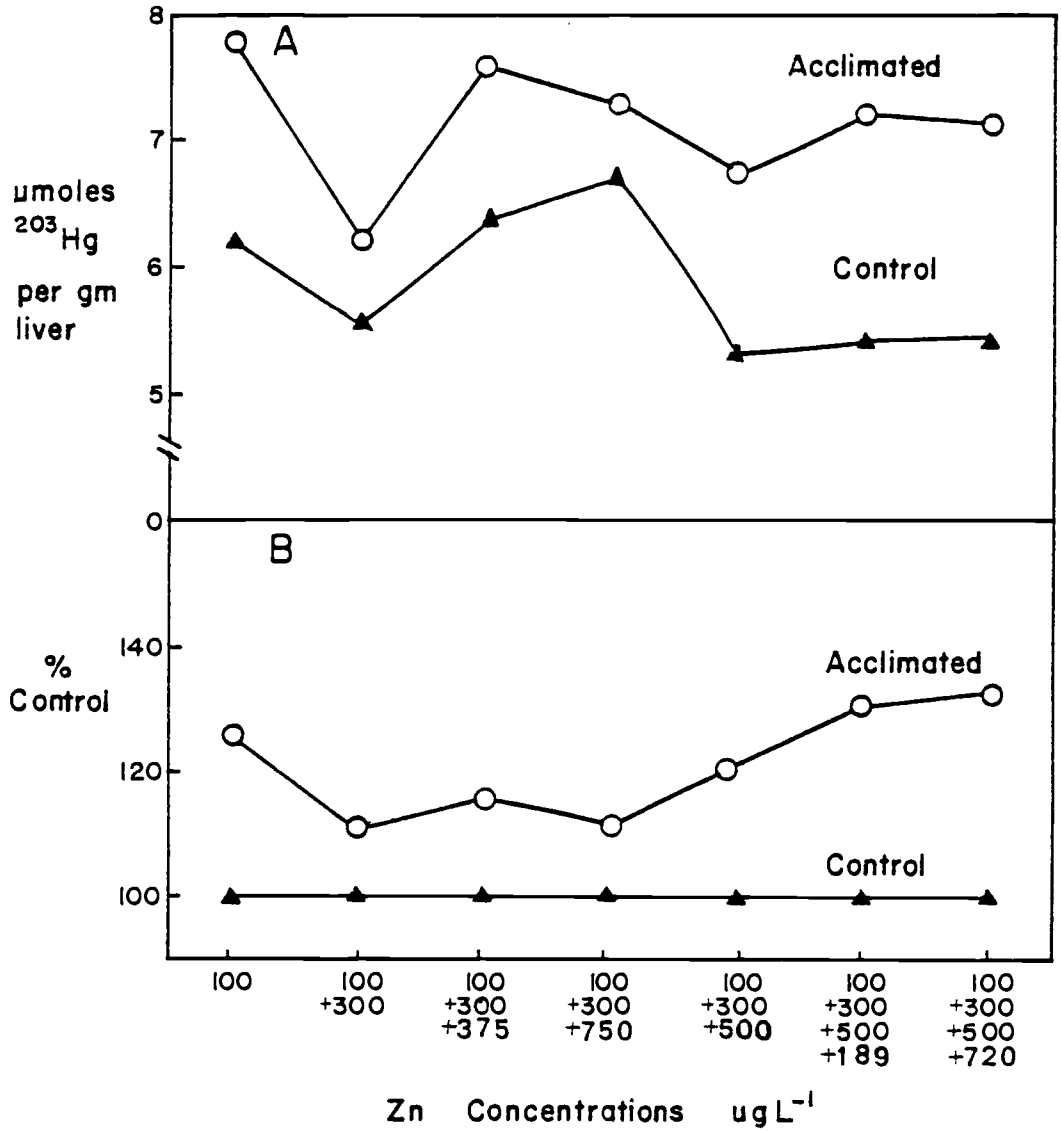

A
Acclimated
Control
µmoles
203Hg
per gm
liver
8
7
6
5
0
B
%
Control
140
120
100
Acclimated
Control
100
100 +300
100 +300 +375
100 +300 +750
100 +300 +500
100 +300 +500 +189
100 +300 +500 +720
Zn Concentrations ug L-1

Figure 11.

66.5 (± 39.83 percent). More than a 2-fold increase over the control MT levels resulted from acclimation for 14 and 21 days (2.0615 ± 0.0185) as illustrated in Table 9 and Figure 12.

The group of fish acclimated to 100, 300, 500 $\mu g \ell^{-1}$ for 14, 14, and 9 days, respectively, was deacclimated and the MT levels determined following 3, 7, and 14 days of deacclimation. Levels of MT showed no decrease after the fish had been in dilution water for 3 days, but after 7 days, the MT levels had returned to control levels. On the third day, results show that the MT levels were still over 30 percent higher than the controls, while at 7 and 14 days the MT levels had essentially returned to control values as shown on Table 10 and Figure 13.

There was no quantitative correlation between the increase in the 96-hr LC50s and the mt levels of acclimated fish. Qualitatively, acclimated fish always had elevated MT levels and higher LC50 values. The zinc tolerance of deacclimated fish was reflected in a similar fashion to MT levels; when MT dropped to control values, the LC50 concentrations did likewise.

Table 9. Metallothionein levels of juvenile rainbow trout acclimated to a constant non-lethal zinc concentration for 3, 7, 14 and 21 days.

Bioassay Description	Acclimated time (days)	μ moles ^{203}Hg bound per gm fish liver	Ratio of Acclimated to Controls (expressed as % Controls)
Acclimated	3	4.286	1.208 (120.8)
Control		3.547	
Acclimated	7	6.502	1.343 (134.3)
Control		4.847	
Acclimated	14	6.945	2.043 (204)
Control		3.399	
Acclimated	21	7.684	2.08 (208)
Control		3.694	

Figure 12. Metallothionein levels of juvenile rainbow trout acclimated to a constant non-lethal zinc concentrations for 3, 7, 14, and 21 days. A = regular plot, and B = normalized plot (as percent of controls) versus acclimation time.

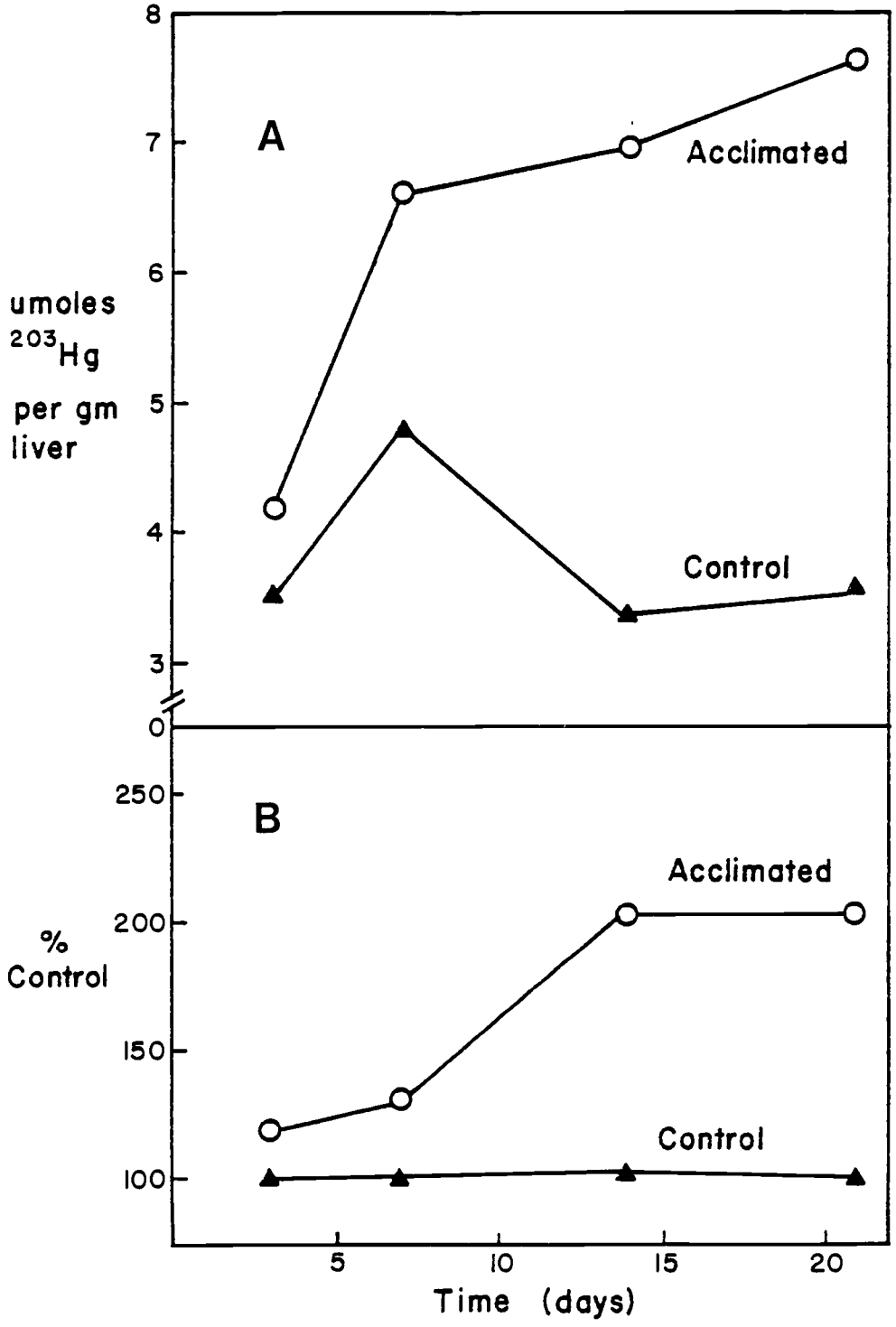

A
Acclimated
Control
umoles
^{203}Hg
per gm
liver
8
7
6
5
4
3
0
B
Acclimated
Control
%
Control
250
200
150
100
5
10
15
20
Time (days)

Figure 12.

Table 10. Metallothionein levels of juvenile rainbow trout acclimated to zinc then deacclimated for 3, 7 and 14 days.

Acclimation History	MT bound µmoles ^{203}Hg	Ratio of Acclimated to Control (Expressed as % of Control	No. of days acclimated /deacclimated
Ac-500*-Zn†	6.206	1.235 (123.5)	10
Control	5.024		
Ac-500(189-4d)*-Zn	7.182	1.313 (131.3)	14
Control	5.467		
Ac-500*-Zn-de.3d-Zn	5.773	1.302 (130.2)	3
Control	4.433		
Ac-500*-Zn-de.7d-Zn	5.615	1.000 (100)	7
Control	5.615		
Ac-500*-Zn-de.14d-Zn	6.206	1.049 (100.5)	14
Control	5.9113		

* Last concentration to which acclimation took place. Further explanations in methods and material.

† Bioassays coded as in Appendix 2.

Figure 13. Metallothionein levels of juvenile rainbow trout acclimated to zinc and then deacclimated for 3, 7 and 14 days. A = scaled regular plot. B = normalized plot.

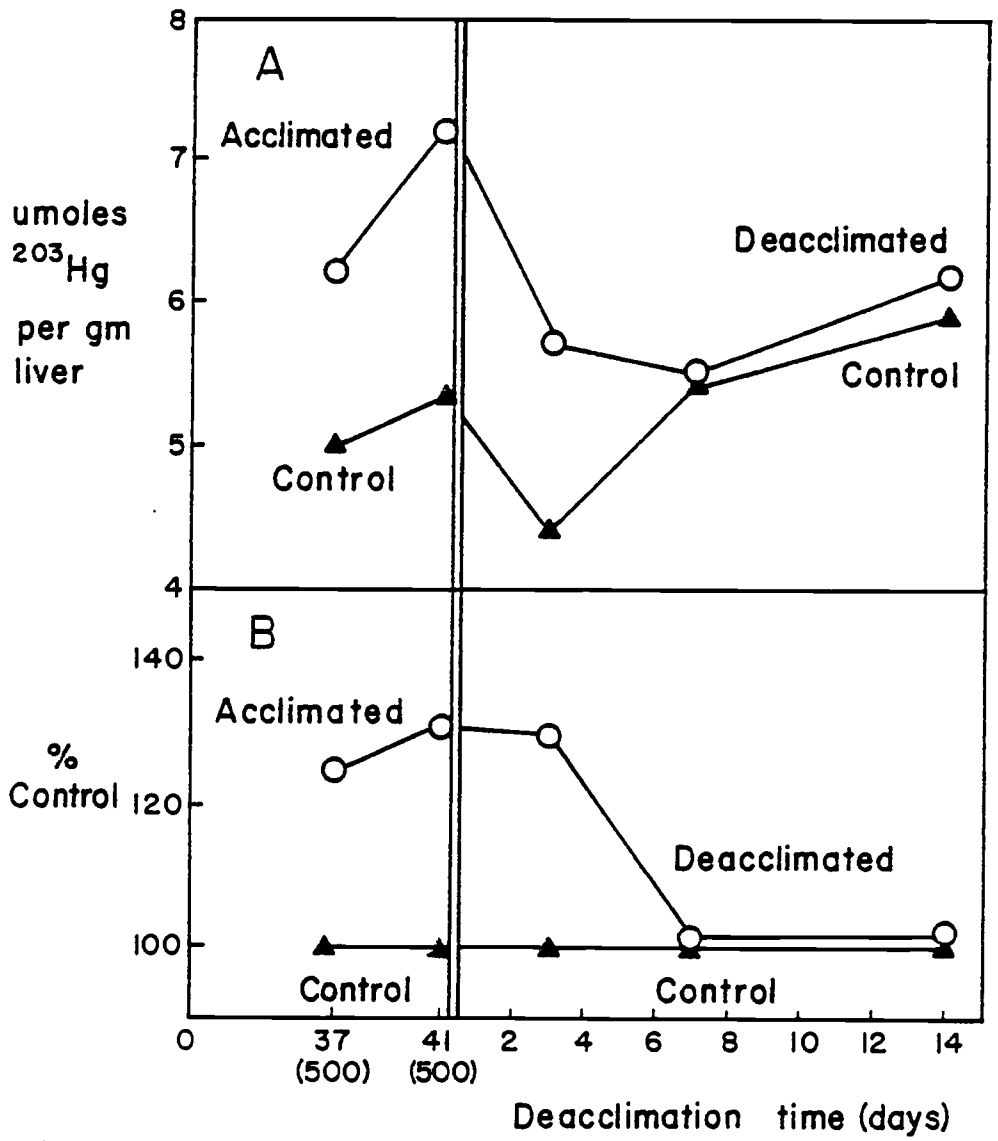

A
Acclimated
Deacclimated
Control
Control
umoles
^{203}Hg
per gm
liver
8
7
6
5
4
B
Acclimated
Deacclimated
Control
Control
%
Control
140
120
100
0
37
(500)
41
(500)
2
4
6
8
10
12
14
Deacclimation time (days)

Figure 13.

DISCUSSION

From my perspective and probably many of those in environmental toxicology, acclimation means an adaptation to continued toxicant exposure which results in increased tolerance or resistance to the undesirable consequences of the exposure. From this work and that before it, it is reasonably certain that sublethal exposure to at least some toxicant (in this case zinc) can significantly alter the tolerance of fish to that metal. The question of cross tolerance for metals in fish and other organisms seems an interesting one as there are contradictory findings among studies addressing this issue.

Zinc Exposures and Development of Tolerance: In this work, steelhead trout exposed to 50 $\mu g \ell^{-1}$ zinc and rainbow trout exposed at 80 $\mu g \ell^{-1}$ of zinc for 7 days clearly exhibited significant increases in zinc tolerance. The acquisition of tolerance as a result of sub-lethal exposure of fish to toxicants is of growing interest to many workers and as a result the literature is growing rapidly. Acquisition of tolerance to otherwise lethal levels of several heavy metals following zinc acclimation at sublethal levels was observed in this study and agrees with the results obtained by previous workers.

Rearing of eggs and embryos of the fathead minnow (Pimphales promelas) in water with low level zinc contamination conferred significant protection when they were subsquently exposed to higher zinc levels (Pickering and Vigor 1965). Sinley et al. (1974) demonstrated similar results from studies on the toxicity of zinc to rainbow trout. Studies by Lloyd (1960), Chapman (1978b), Beatie and Pascoe (1978), Spehar (1976), Dixon and Sprague (1981), and Buckley et al. (1982) working with trout and zinc, copper, cadmium, arsenic and cyanide exposures support these findings. The type of tolerance acquisition focused upon in this study is of the short-term physiological. Genetic adaptations undoubtedly occur (Blanc, 1973; Luoma, 1977; Rahel, 1981), but were not investigated here.

Tolerance to otherwise lethal zinc concentrations was developed in rainbow trout only when they were acclimated for 3 days and more. In less than 3 days, the acclimated fish had 96-hr LC50s slightly lower

than the controls, but not statistically different from the controls. Rainbow trout were not acclimated below 80 $\mu g\ell^{-1}$ Zn (0.53 of 96h-LC10) to determine if there was a low threshold concentration for the response. Dixon and Sprague (1981) reported that rainbow trout acquired significant increases in lethal tolerance when acclimated to 94, 131 and 194 $\mu g\ell^{-1}$ Cu each for 7, 14 and 21 days. Acclimation to 58 $\mu g\ell^{-1}$ Cu (0.18 of control incipient LC50) resulted in no significant changes in the incipient lethal levels relative to controls, exposure to 30 $\mu g\ell^{-1}$ Cu however resulted in decreased tolerance. This reduced tolerance was attributed to what they (Dixon and Sprague, 1981) termed 'sensitization' implying that induction of tolerance had not yet occurred and that some deleterious effect of earlier exposure contributed to the impact of the subsequent lethal concentration. "Sensitization" did not occur in the present study; less than 3 days of acclimation produced no significant differences between the exposed and control LC50s. It was obvious from the present work that acclimation occurred between 3 and 7 days of exposure and that the acclimation process was not accomplished more rapidly. Some adaptive mechanism is probably induced by exposure which takes time to proceed and result in protection of the fish. This mechanism operated as long as exposure was maintained (up to 28 days).

The at least 3-fold increase in the LC50 of acclimated fish at 80 $\mu g\ell^{-1}$ of Zn under controlled laboratory conditions represents significant induction of tolerance in fish. Evidence from a series of "round robin" acute tests with Daphnia magna, rainbow trout, and fathead minnows as test organisms with silver and endosulfan as toxicants (Chapman, 1983) indicated that duplicate tests within laboratories produced lethality results that differed by factors of two or less. It is therefore suggested that a factor of about two or less be regarded as general state-of-the-art variability in acute toxicity test. The present tests are clearly outside this range and were performed under closely controlled conditions with dilution water of minimal water quality variation.

Acclimation of rainbow trout at 100 $\mu g\ell^{-1}$ of zinc for 10 days and subsquently higher concentrations of 300 and 500 $\mu g\ell^{-1}$ for times

specified (Fig. 3) resulted in greater tolerance induction than acclimation at 80 $\mu g \ell^{-1}$. This increase does not, however, progress with increasing concentration after acclimation at 100 $\mu g \ell^{-1}$ Zn. This probably indicates that maximum tolerance induction has both concentration and time thresholds. The long term protection of the fish may not be guaranteed at elevated acclimation concentrations. For example, chronic exposure could result in reduced scope for activity (Fry, 1947; Leduc, 1977), or growth (Warren, 1971).

Loss of Acclimation: Rainbow trout lost acquired tolerance to zinc when acclimation level exposure was stopped. Literature is scant concerning the duration of acclimation-induced tolerance once the source of acclimation is removed. Fry (1971) suggests that acclimation is a physiological response elicited by environmental history and which is essentially reversible. Dixon and Sprague (1981) investigated loss of tolerance of rainbow trout which had been previously acclimated to 194 $\mu g \ell^{-1}$ Cu and reported tolerance was quickly lost on return of fish to control water, most of it by 7 days. This investigation shows a similar result for zinc. Loss of tolerance appeared to be underway by the third day of deacclimation although statistically insignificant, and tolerance had returned to the control condition by the end of 7 days following removal of acclimation level exposure.

It seems the physiological mechanisms in fish that induce increase in tolerance during acclimation to sublethal concentrations of zinc is "switched" off once the toxicant is removed from the environment of the fish. The acclimation-induced resistance is analagous to the resistance phase of Selyes General Adaptation Syndrome in mammals (Selye 1950). Selye proposed that adapatability and resistance to stress are fundamental prerequisites for life and that every vital organ and function participates in them. Loss of acclimation by fish after toxicant delivery is stopped would be thought of as a negative feedback mechanism whereby all the organ functions that have been involved in this tolerance reaction are repressed as their operation is no longer required once the source of stress is removed.

Cross Tolerance:

In an independent experiment, with fish from the same stock, rainbow trout exposed to 100 $\mu g\ell^{-1}$ zinc for 17 days showed a 3.2-fold increase in tolerance to zinc over the controls. Acute toxicity tests with this same group of fish conducted with cadmium and copper showed that a 4.3 and 3.2-fold increase in tolerance was developed to copper and cadmium, respectively, following acclimation to zinc.

There have been relatively few reported experiments on cross tolerance with fish. Generally, however, the occurrence of cross-tolerance to metals toxicity seem to have less support than its non-occurrence. This is evidenced by the work of Wu and Antonovics (1975) on the uptake of zinc and copper by the grass (Agrostis stolonifera) Gregory and Brashaw (1965) on heavy metal tolerance in populations of Agrostis tenuis and other grasses, and Bryan 1974 on the adaptation of the marine polycheate (Nereis diversicolor) to sediments containing high concentration of heavy metals. Recently, Dixon and Sprague (1981) acclimated rainbow trout to 194 $\mu g\ell^{-1}$ of copper for 28 days in the laboratory. These fish were significantly less tolerant of lethal levels of zinc than the controls. These findings suggest that the mechanisms responsible for metal tolerance are quite specific.

However, other evidence indicates that organisms that have been acclimated to one metal may acquire protection to exposure to a second metal. Certainly, there are possible differences between environmental contamination and laboratory exposure in relation to both genetic and physiological development of tolerance. These conflicting results cannot be explained and the question of cross tolerance and sensitization will require further investigation.

Cox and Hutchinson (1979) reported the evolution of tolerances to nickel, copper, aluminum in the grass (Deschampsia cespitos) in response to elevated soil levels which "coincidentally" increased tolerance to lead and zinc. Co-tolerance to nickel was reported in a zinc tolerant population of the grass (Agrostis tenuis) despite lack of nickel in the soil (Brashaw et al. 1965); cadmium tolerant bacteria

and yeast were found to be resistant to additional metals (Tatsuyama et al., 1975; Stokes et al., 1973], reported that the unicellular green algae (Scenedesmus acutiformis and Chlorella fusca) collected from nickel-copper polluted lakes showed not only nickel and copper tolerance but also silver tolerance; Allen and Sheppard (1971) showed that populations of the monkey flower (Mimulus guttatus) growing on abandoned copper mines were tolerant to normally toxic levels of copper and co-tolerant to zinc, lead and nickel even when the soil was not rich in these other metals. These results indicate that tolerance to different metals may not be purely specific. These data suggest the possibility of cross tolerance in fish exposed to heavy metals in the laboratory albeit contingent upon the concentrations and times of exposure.

If toxicity tests are carried out over longer periods than the time required for deacclimation to occur, results are likely to be affected seriously. For instance, copper acclimated rainbow trout showed more resistance to zinc during the first 2 days (Dixon and Sprague, 1981). The concentration of copper to which the fish were acclimated might have been too high and possibly too stressful to offer protection against zinc. The overall extension of the test time from the conventional 96 hr to 144 hr in their study might have affected the cross tolerance results since bearing in mind that copper deacclimation occurs during zinc toxicity tests. As the levels of copper drop during deacclimation, it could drop to a level below the threshold when "sensitization" instead of protection occurs. There was no experiment here to determine if zinc had a threshold concentration for the conferment of protection as was reported for copper (Dixon and Sprague, 1981).

In a field exposure (Rahel, 1981), common shiners (Notropis cornutus) inhabiting a zinc polluted stream did not show higher zinc tolerance than conspecifics from a nearby unpolluted stream. Rahel suggested that this phenomenon occurred because the stock from the polluted stream might have been under stress from the chronic exposure to elevated metal levels. It is possible to find explanations for the acclimation of fish to one metal not always offering protection to a second metal or sometimes even of the same metal.

Zinc Metallothionein:

Following acclimation of rainbow trout to zinc at 80 $\mu g \ell^{-1}$ and above for varying periods, the quantities of mercury binding soluble hepatoprotein (MT) were determined. Results suggest the enhanced formation of these metallothionein-like proteins during zinc acclimation. A 22 percent increase in the quantity of metallothionein-like protein occurred in one test, while a 67 percent average occurred in the second test. The maximum was a two-fold increase which occurred after two weeks of acclimation and without further changes after that. The length of acclimation time seemed to have affected the dynamics of this protein more than the acclimation concentration (when the acclimation concentration was never less than 0.6 of the 96-hr LC10 of the control).

Of the many properties of MT identified for mammals, this study focused most on its induction by zinc exposure, its role in alleviating zinc toxicity in rainbow trout, and its possible use as an indicator of heavy metal pollution in the aquatic environment. Although the sine qua non for the characterization of MT is the analysis of the amino acid content no attempt was made to purify the low molecular weight protein obtained in this work. However, Kotsonis and Klaassen's (1977) ^{203}Hg-binding method for the estimation of MT is a generally accepted approach. However, it is impossible to know the proportion of MT in the low molecular weight fraction quantified.

Acclimation of rainbow trout to sublethal concentrations of zinc increased hepatic MT and the tolerance to metals. This latter observation indicates an induction of this metal-binding protein consistent with other work on fish by Ridlington et al. (1981), Yamamoto et al. (1978), Pierson (1981), Overnell and Coombs (1979), Bouquegneau (1979), Roch et al. (1982), McCarter et al. (1982), Buckley et al. (1982), Woodworth and Pascoe (1983), Gant (personal communication), and Noel-Lambot et al. (1978). Thus, independent methods indicate that exposure of fish to metals either through the aquatic environment or injection of sublethal doses of copper, zinc, cadmium, or mercury increase the liver content of MT in fish. This evidence and additional evidence from mammals substantiate the fact that zinc and

certain other metals are capable of inducing the biosynthesis of metallothionein-like proteins in fish during exposure to sublethal concentrations.

The mechanisms by which certain metals induce the synthesis of MT in specific organs are not fully understood. There is a consensus that MT is present in low concentrations in the organs of animals not exposed to heavy metals (Noel-Lambot et al., 1978). As a result of induction by certain metals, MT levels were found to be as much as two-fold (this study and Gant personal communication), 3-fold (Viarengo et al., 1981), and 40-fold higher (Piotrowski et al., 1974a). It has been suggested (Squibb and Cousins, 1977) that because of an increase in the concentration of MT mRNA after zinc or cadmium administration, induction of de-novo-synthesis of MT occurred in organs where metals were accumulated. In another view (Webb, 1972) suggested that the regulation of MT synthesis takes place only in the translational step of protein biosynthesis. The latter assumes that MT mRNA is already present in the cell and these heavy metals can transform the mRNA from an inactive to active form. Following deacclimation for periods longer than 3 days, the levels of metallothionein-like proteins in rainbow trout returned to the control level, another evidence supporting the view that MT biosynthesis was triggered by sublethal zinc exposure.

Metal detoxification.

The apparent role of MT in reducing metal toxicity has consistently been supported in research reports. A consensus has been reached that one mechanism of metal detoxification is binding by MT. Once the induction and biosynthesis of MT has occurred, subsequent metal exposure will result in much of the metal being bound tightly to MT. The MT-bound metal may no longer be available for binding to high molecular weight proteins such as enzymes. Inhibition of enzyme-mediated reactions may produce the toxicity of metals and preferential binding of metals to low molecular weight MT provides a sparing effect on the metabolically active cellular proteins from metal toxicity. Experimental evidence in support of this hypothesis exist with regards to copper bioaccumulation in fish tissue (Dixon and Sprague, 1981).

Fish acclimated to copper had elevated tissue (whole fish) concentrations of copper of 34 μg^{-1} while control fish concentrations were 3.8 μg^{-1}. Subsequent exposure of both groups to higher levels resulted in complete mortality of controls at 7.4 μg^{-1} body copper. Zinc bioaccumulation studies were not successfully carried out in the present investigation, but evidence exists which shows that zinc concentrations in liver of rainbow trout have a direct correlation with zinc concentration in the aquatic environment (Roch et al., 1982). Chapman (unpublished work) reported that zinc acclimated sockeye salmon survived and had higher liver zinc than controls which were killed when both groups were exposed to 960 $\mu g\ell^{-1}$ zinc. Results of this work show that MT levels did increase as a result of sublethal zinc exposure, and also that the 96-hr LC50s of the zinc-exposed fish were always significantly above the controls. The simultaneous increase in zinc tolerance and MT supports the hypothesis that MT has a protective function in zinc toxicity.

Cross Tolerance and Metallothionein

Very little literature is available on the subject of cross tolerance among metals in fish. As has already been pointed out, some data suggest MT is specific and that exposure of fish to sublethal levels of one metal usually confers tolerance only to that specific metal in the wild or in the laboratory. However, this study reports cross tolerance to cadmium and copper in fish acclimated to zinc at 100 $\mu g\ell^{-1}$ for 17 days. Cherian and Goyer (1978) reported increased accumulation of both cadmium and zinc in liver of rats repeatedly treated with cadmium. They suggested that the induced biosynthesis of MT provides unsaturated metal binding sites on the newly synthesized MT for binding of both metals. Leber and Miya (1976) suggested that the tolerance to cadmium that was induced by pretreatment with cadmium was the result of the *in vivo* replacement of less toxic zinc from MT by cadmium. It has been shown (Webb, 1979) that acute effects of cadmium in testes can be prevented by pretreatment with zinc. The protection afforded was attributed to the induction of MT (Webb, 1972). Haffner and Rugstad (1976) isolated cadmium resistant human skin cell lines after growing in cadmium-containing medium. The resistant cell

lines showed protection against cadmium, were active in synthesizing MT, and showed cross tolerance to high concentrations of zinc. After injection of copper salts, Bremner and Davis (1976) isolated copper and zinc thioneins from rat liver which were similar to cadmium thionein in amino acid composition.

In this study, exposure to sublethal concentrations of zinc offered protection against copper and cadmium toxicity; a possible explanation stems from the fact that Cu, Cd, Zn, and Hg are major binders of MT (Ridlington, 1981). When fish are exposed to sublethal levels of Zn, induction and biosynthesis of MT occurs. It is possible that on exposure to a second metal (e.g. copper or cadmium) zinc would be displaced. Binding sites of MT bind other metals and protect the fish from their toxicity. It is known (Kägi and Nordberg 1979) that copper is much more tightly bound to MT than cadmium, and cadmium more tightly bound than zinc. In the presence of either copper and zinc, or cadmium and zinc, detoxification by MT would be in favor of copper or cadmium. Zinc is an essential trace element and Zn bound to zinc thionein can be reutilized in the body providing a source of zinc during deprivation. This could be a source of disparity in experiments where fish were acclimated to copper first before acute exposure to Zn was carried out as was done by Dixon and Sprague (1981).

Dixon (1980) suggested that a possible explanation to the phenomenon where fish acclimated to copper initially were more tolerant to zinc exposure, but after 2 days became less tolerant than the controls, could be with respect to MT. It was possible that the MT present during copper acclimation was initially able to bind zinc, resulting in early protection. As zinc exposure continued, it was possible that some zinc ions displaced copper ions from the MT, and that toxic copper ions caused the increased mortality. Although the affinity of copper for MT is considered to be greater than that of zinc, the 96-hr LC50 of zinc is very much higher than that of copper. The higher zinc concentration in the cytosol could favor the displacement of copper from MT by zinc. Acclimation of fish to copper could, as a result of zinc displacement from zinc thionein, make more zinc available. Further exposure to zinc in a subsequent acute test could

render zinc more toxic to the copper-acclimated fish than the controls because there are now fewer available metal binding sites. This would make the high molecular weight proteins vulnerable to zinc toxicity.

The probable explanation favouring cross tolerance as was observed in the present investigation is that sublethal zinc exposure results in the induction of thioneins capable of binding cadmium and copper which protect the animal from subsequent high doses. The fact that we found increased ^{203}Hg binding to MT following acclimation to zinc suggests the nonspecificity of the zinc-induced MT.

Applications and Conclusion:

The question of acclimation to toxicants in fish is undoubtedly important in evaluating water quality standards. Water quality standards are for protection of the aquatic ecosystems such that the designated beneficial uses are protected, but not necessarily over-protected. Over-protection, which places very stringent pollution control standards, penalizes both the industries and the consumers. If fish are capable of developing resistance to environmental toxicants, then some water quality standards could justifiably be relaxed and this would lead to some economic relief which would permit industries to operate in a less costly manner, while aquatic life would still be adequately protected. Most bioassays are performed with animals which have not been previously exposed to the toxicant under test. Acclimation is a factor that can modify the applicability of toxicity results. Acclimation makes uncertain the extent to which water quality criteria developed without taking it into account reflect the true measure of the hazard the toxicant presents to the fish. Water quality criteria set for a particular heavy metal, based on the laboratory data on the LC50s for fish with no previous exposure history will no longer suffice in all cases. The development of application factors, maximum allowable toxicant concentrations, 'safe' levels and incipient LC50s should take cognizance of the metal-acclimation history of the fish in question.

Laboratory acclimation experiments can be a further step in the recognition of interrelationships and interactions between organisms and their environment by quantifying the toxicological significance of

acclimation. Studies of acclimation are useful in understanding some discrepancies that arise between bioassay results which otherwise would be expected to be similar if other physical and chemical parameters were the same.

Acclimation studies have application in fisheries management; for instance, the failure of salmonid stocking in waters with elevated metal levels when healthy salmonid populations occur in waters under otherwise similar conditions can be explained (Dixon, 1980). Acclimation of a fish stock to certain metals could make the stocking in the metal-polluted water feasible. The development of tolerance to some toxicants and not to others could be a useful tool in confirming and prioritizing the potential hazard of environmental toxicants to organisms.

Possible prevention of poisoning by a toxic metal by pretreatment with higher doses of a less lethal metal is a possibility which metal co-tolerance could offer, and has been demonstrated in the case of cadmium poisoning in mammals by pretreatment with less toxic zinc (Webb, 1979). When MT induction and biosyntehsis become sensitively and rapidly quantified in fish liver (as it is fast becoming), it could serve as a useful indicator of heavy metal pollution in the aquatic environment.

Future studies that can arise from the present study will include:

1) The existence of threshold concentrations for acclimation to Zn and other metals (as have been reported for copper), to aid in quick estimation of 'safe' levels; safe levels being the maximum exposure concentration which produces neither sensitization nor acclimation.
2) The possibility of cross tolerance among other metals other than Zn, Cd, Cu taking into account deacclimation time.
3) Further work on the hypothesis of sensitization by lower threshold concentrations is indicated.

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APPENDICES

APPENDIX 1. Water quality conditions measured during the bioassay experiments. The number, mean and standard deviations are given for each set.

Date of determination	Dilution water temperature (mean of 1 week)	pH		Alkalinity		Hardness		Dissolved Oxygen	
		Bioassay aquaria	Control	Bioassay aquaria	Control	Bioassay aquaria	Control	Bioassay aquaria	Control
9/25/81	13.0°C	6.92	7.06	31	30	30	30	9.3	9.4
10/16/81	13.0	7.04	7.11	22	23	31	31	9.8	9.1
9/25/81	13.0	6.71	6.78	20	18	31	31	9.8	9.7
10/08/81	13.0	7.06	6.90	26	26	31	31	9.0	9.4
10/15/81	13.0	6.60	6.80	27	26	32	31	10.1	10.0
10/22/81	12.5	6.52	6.45	26	26	26	27	10.4	10.3
10/30/81	12.5	6.60	6.50	25	26	29	30	10.3	10.0
11/24/81	12.5	6.21	6.60	25	27	30	31	10.1	10.3
5/14/82	12.3	7.12	7.38	38	38	48	48	10.1	10.2
6/08/82	12.5	6.50	6.64	30	28	40	40	10.0	10.0
6/11/82	12.5	6.40	6.86	26	30	44	44	10.1	10.3
6/18/82	12.5	6.86	6.76	25	24	40	40	10.0	9.9
6/29/82	12.0	6.48	6.54	21	22	32	32	10.0	9.9
7/08/82	12.0	6.84	6.93	27	27	34	33	10.4	10.1
9/14/82	13.0	6.00	6.10	23	23	33	33	10.5	10.2
9/29/82	13.0	5.80	6.10	21	22	30	30	10.1	10.0
10/14/82	12.5	6.14	6.02	22	22	31	31	10.2	10.1
10/22/82	12.5	6.26	6.20	20	21	31	31	10.1	9.8
10/25/82	12.5	6.35	6.36	20	21	29	29	10.0	9.8
11/11/82	12.5	6.65	6.35	202	22	31	31	10.2	10.3
N =	20	20	20	20	20	20	20	20	20
$\overline{x}$	12.600	6.553	6.620	25.000	25.200	33.000	33.175	10.025	9.940
SD_n	0.318	0.352	0.359	4.900	4.300	5.500	5.292	0.343	0.324

Appendix 2. Detailed explanation of the codes used in all bioassays, detailed results of regresion analysis of all bioassays and original data for each of the lethality bioassays undertaken during the research. The table presents the following: UNIT = the identification number of test tank (aquarium) used; DIED = the observed number of fish dead at the end of each experiment (96 or 120 hrs); PHAT (P) is the predicted mortality level for each concentration given by a linear regression of logit mortality on log concentration; LOG CONC = Log_{10} of the mean assayed toxicant concentration; Z = approximately the number of standard deviations by which the observed mortality differed from values from regression line (PHAT); n = the total number of fish used in tanks of equal toxicant concentration. Control mortalities are indicated where any occurred. The bioassays are coded as follows: Ac/UN-Me-*Ci-Dd-S-de. AC = Acclimated or pre-exposed; UN = Unacclimated or Control, Me = the toxicant to which the fish had been acclimated. Ci = the concentration in $\mu g \ell^{-1}$ of toxicant to which the fish had been acclimated. Dd = the number of days of acclimation at the indicated concentration. S = the toxicant with which the bioassays were subsequently carried out after accclimation (in this case Zn, Cd, or Cu). de. = deacclimation. *Acclimation concentrations above 150 $\mu g \ell^{-1}$ refer to the last acclimation concentration of the sequential series shown in Figure 2.

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-INIT-ZN	1	0.00	0.016	31	1.491	-0.56	20
	2	0.05	0.046	46	1.663	0.09	20
	3	0.20	0.232	88	1.744	-0.34	20
	4	0.65	0.578	150	2.176	0.65	20
	5	0.90	0.899	290	2.462	0.02	20
	6	0.95	0.980	530	2.724	-0.95	20

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	134	(109,169)
0.10	62	(39,80)
0.90	292	(222,472)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-50-7d-Zn	1	0.00	0.000	17	1.230	-0.08	20
	2	0.00	0.000	30	1.477	-0.08	20
	3	0.00	0.000	75	1.875	-0.08	20
	4	0.00	0.004	150	2.176	-0.28	20
	5	0.10	0.088	290	2.462	0.18	20
	6	0.70	0.707	560	2.748	-0.07	20

Mortality level	Concentration	95% Confidence Interval
0.50	468	(391,569)
0.10	298	(195,363)
0.90	733	(596,1172)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-50-7d-Zn	1	0.00	0.000	0	-2.000	-0.08	20
	2	0.00	0.000	0	-2.000	-0.08	20
	3	0.05	0.048	25	1.398	0.05	20
	4	0.95	0.975	515	2.712	-0.71	20
	5	1.00	0.977	535	2.728	0.69	20

Mortality level	Concentration	95% Confidence Interval
0.50	98	(48,193)
0.10	36	(13,70)
0.90	265	(138,683)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-50-14d-Zn	1	0.00	0.000	24	1.380	-0.08	20
	2	0.00	0.000	40	1.602	-0.08	20
	3	0.00	0.002	91	1.959	-0.19	20
	4	0.05	0.021	176	2.246	0.88	20
	5	0.15	0.196	332	2.521	-0.52	20
	6	0.80	0.780	671	2.827	0.22	20

Mortality level	Concentration	95% Confidence Interval
0.50	481	(939,610)
0.10	269	(170,339)
0.90	858	(662,1511)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-50-14d-Zn	1	0.00	0.005	10	1.000	-0.31	20
	2	0.00	0.006	11	1.041	-0.34	20
	3	0.05	0.035	29	1.462	0.37	20
	4	0.80	0.849	419	2.622	-0.62	20
	5	0.90	0.855	429	2.632	0.57	20

Mortality level	Concentration	95% Confidence Interval
0.50	168	(91,257)
0.10	52	(15,96)
0.90	537	(347,1078)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	Size
Ac-Zn-50-21d-Zn	1	0.00	0.000	24	1.380	-0.08	20
	2	0.00	0.000	43	1.633	-0.08	20
	3	0.00	0.000	74	1.869	-0.08	20
	4	0.00	0.001	142	2.152	-0.11	20
	5	0.05	0.045	279	2.446	0.10	20
	6	0.75	0.753	530	2.724	-0.03	20

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	446	(377,518)
0.10	318	(220,337)
0.90	626	(536,861)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-50-21d-Zn	1	0.00	0.029	18	1.255	-0.77	20
	2	0.25	0.094	34	1.531	2.40	20
	3	0.15	0.296	69	1.839	-1.43	20
	4	0.60	0.631	140	2.146	-0.28	20
	5	0.90	0.883	296	2.471	0.24	20
	6	1.00	0.968	601	2.779	0.81	20

Mortality level	Concentration	95% Confidence Interval
0.50	107	(81,142)
0.10	35	(19,50)
0.90	324	(225,615)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-50-7d-200-10d-Zn							
	1	0.00	0.000	25	1.398	-0.08	20
	2	0.00	0.000	80	1.903	-0.08	20
	3	0.00	0.002	160	2.204	-0.22	20
	4	0.10	0.048	320	7.505	1.07	20
	5	0.35	0.449	605	2.752	-0.89	20
	6	0.95	0.900	1050	3.021	0.75	20

Mortality level	Concentration	95% Confidence Interval
0.50	634	(531,760)
0.10	383	(249,470)
0.90	1051	(853,1634)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-50-7d,200-10d-Zn							
	1	0.00	0.002	10	1.000	0.18	20
	2	0.05	0.029	45	1.653	0.55	20
	3	0.15	0.133	105	2.021	0.23	20
	4	0.40	0.417	235	2.371	-0.16	20
	5	0.65	0.749	495	2.695	-1.02	20
	6	1.00	0.920	1000	3.000	1.32	20

Mortality level	Concentration	95% Confidence Interval
0.50	280	(207,380)
0.10	89	(43,131)
0.90	881	(594,1836)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-INIT-ZN	1	0.00	0.000	40	1.602	-0.08	20
	2	0.00	0.012	93	1.968	-0.48	20
	3	0.40	0.34	192	2.283	0.24	20
	4	0.95	0.964	388	2.589	-0.35	20
	5	1.00	0.999	770	2.886	0.13	20

Mortality level	Concentration	95% Confidence Interval
0.50	211	(179,256)
0.10	141	(90,169)
0.90	317	(260,530)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-100-1d-Zn	1	0.60	0.542	165	2.217	0.52	20
	2	0.70	0.785	280	2.447	-0.93	20
	3	0.95	0.936	535	2.728	0.26	20
	4	1.00	0.989	1230	3.090	0.47	20
	5	1.00	0.998	2600	3.415	0.22	20

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	152	(68,207)
0.10	54	(6,100)
0.90	427	(308,1097)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-1d-Zn	1	0.55	0.539	195	2.290	0.10	20
	2	0.80	0.795	645	20.538	0.05	20
	3	0.90	0.935	640	2.806	-0.63	20
	4	1.00	0.986	1350	3.130	0.54	20
	5	1.00	0.997	24	3.380	0.29	20

Mortality level	Concentration	95% Confidence Interval
0.50	181	(83,247)
0.10	64	(8,167)
0.90	514	(372,1201)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-100-2d-Zn	1	0.55	0.560	135	2.130	-0.09	20
	2	0.75	0.711	225	2.352	0.38	20
	3	0.85	0.891	570	2.756	-0.59	20
	4	0.95	0.955	1185	3.074	-0.15	20
	5	1.00	0.981	2400	3.38	0.62	20

Mortality level	Concentration	95% Confidence Interval
0.50	112	(32,181)
0.10	20	(0.7,54)
0.90	614	(377,2323)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-2d-Zn	1	0.55	0.539	195	2.290	0.10	20
	2	0.80	0.795	345	2.538	0.05	20
	3	0.90	0.935	640	2.806	-0.63	20
	4	1.00	0.986	1350	3.130	0.54	20
	5	1.00	0.996	2400	3.380	0.29	20

Mortality level	Concentration	95% Confidence Interval
0.50	181	(83,247)
0.10	64	(8,167)
0.90	514	(372,1201)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-80-3d-Zn	1	0.00	0.003	40	1.602	-0.25	20
	2	0.05	0.032	97	1.987	0.46	20
	3	0.15	0.188	200	2.301	-0.43	20
	4	0.65	0.615	410	2.613	0.32	20
	5	0.90	0.912	820	2.914	-0.19	20

Mortality level	Concentration	95% Confidence Interval
0.50	344	(270,445)
0.10	152	(86,205)
0.90	779	(571,1437)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-3d-Zn	1	0.55	0.539	195	2.290	0.10	20
	2	0.80	0.795	345	2.538	0.05	20
	3	0.90	0.935	640	2.806	-0.63	20
	4	1.00	0.986	1350	3.130	0.54	20
	5	1.00	0.996	2400	3.380	0.29	20

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	181	(83,247)
0.10	64	(8,167)
0.90	514	(372,1201)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-80-7d-Zn	1	0.00	0.000	117	2.068	-0.08	20
	2	0.00	0.003	247	2.393	-0.25	20
	3	0.05	0.057	490	2.690	-0.14	20
	4	0.60	0.575	1006	3.003	0.23	20
	5	0.95	0.965	2016	3.304	-0.35	20

Mortality level	Concentration	95% Confidence Interval
0.50	938	(769,1138)
0.10	564	(348,870)
0.90	1560	(1257,2493)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-80-7d-Zn	1	0.00	0.006	55	1.740	-0.36	20
	2	0.05	0.059	120	2.079	0.17	20
	3	0.25	0.268	220	2.342	-0.18	20
	4	0.85	0.758	460	2.663	0.96	20
	5	0.90	0.958	910	2.959	-1.29	20

Mortality level	Concentration	95% Confidence Interval
0.50	311	(78,648)
0.10	146	(89,192)
0.90	661	(497,1127)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-80-21d-Zn	1	0.00	0.000	160	2.204	-0.08	20
	2	0.00	0.002	330	2.519	-0.19	20
	3	0.05	0.015	487	2.688	1.26	20
	4	0.35	0.414	984	2.993	-0.58	20
	5	1.00	0.969	1980	3.297	0.80	20

Mortality level	Concentration	95% Confidence Interval
0.50	1050	(892,1267)
0.10	700	(455,836)
0.90	1574	(1294,2594)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-80-28d-Zn	1	0.00	0.000	186	2.270	-0.08	20
	2	0.00	0.000	390	2.591	-0.08	20
	3	0.10	0.100	763	2.883	0.00	20
	4	1.00	1.000	1600	3.205	0.08	20
	5	1.00	1.000	3153	3.499	0.08	20

Mortality level	Concentration	95% Confidence Interval
0.50	894	(801,1455)
0.10	762	(652,888)
0.90	1047	(895,2621)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-80-28d-Zn	1	0.00	0.600	138	2.140	-1.13	20
	2	0.30	0.237	277	2.442	0.66	20
	3	0.65	0.566	520	2.716	0.75	20
	4	0.75	0.866	1050	3.021	-1.53	20
	5	1.00	0.970	2133	3.329	0.78	20

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	462	(357,599)
0.10	176	(93,247)
0.90	1213	(870,2263)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-INIT-Zn	1	0.05	0.78	80	1.903	-0.47	20
	2	0.20	0.252	150	2.176	-0.54	20
	3	0.77	0.631	315	2.498	1.38	22
	4	0.85	0.898	665	2.823	-0.70	20
	5	0.95	0.978	1400	3.146	-0.86	20

Mortality level	Concentration	95% Confidence Interval
0.50	247	(187,322)
0.10	90	(47,128)
0.90	673	(478,1269)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-100-10d-Zn	1	0.50	0.004	224	2.352	3.34	20
	2	0.05	0.035	425	2.628	0.38	20
	3	0.15	0.294	855	2.932	-1.41	20
	4	0.90	0.838	1750	3.243	0.76	20
	5	1.00	0.980	3300	3.519	0.65	20

Mortality level	Concentration	95% Confidence Interval
0.50	1097	(887,1360)
0.10	586	(372,748)
0.90	2051	(1602,3263)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-10d-Zn	1	0.10	0.024	73	1.863	2.20	20
	2	0.05	0.188	150	2.176	-1.58	20
	3	0.65	0.603	275	2.439	0.43	20
	4	0.95	0.942	590	2.771	0.15	20
	5	1.00	0.993	1200	3.049	0.37	20

Mortality level	Concentration	95% Confidence Interval
0.50	240	(193,301)
0.10	118	(71,154)
0.90	489	(373,827)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-300-10d-Zn	1	0.00	0.000	175	2.243	-0.08	20
	2	0.00	0.001	336	2.525	-0.12	20
	3	0.50	0.037	690	2.839	0.31	20
	4	0.65	0.674	1405	3.148	-0.23	20
	5	1.00	0.988	2700	3.431	0.49	19

Mortality level	Concentration	95% Confidence Interval
0.50	1234	(1015,1334)
0.10	834	(511,1015)
0.90	1827	(1539,2706)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-300-10d-Zn	1	0.10	0.137	80	1.903	-0.48	20
	2	0.45	0.351	150	2.176	0.92	20
	3	0.60	0.655	285	2.455	-0.52	20
	4	0.85	0.884	580	2.763	-0.47	20
	5	1.00	0.973	1290	3.111	0.74	20

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	205	(152,272)
0.10	67	(31,100)
0.90	632	(435,1313)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-500-10d-Zn	1	0.10	0.014	343	2.535	3.21	20
	2	0.05	0.210	820	2.857	-1.76	20
	3	0.90	0.834	1525	3.183	0.79	20
	4	1.00	0.991	3320	3.521	0.43	20

Mortality level	Concentration	95% Confidence Interval
0.50	1009	(all values)
0.10	576	outside 1116,3101
0.90	1768	(all values)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-500-10d-Zn	1	0.00	0.017	55	1.740	-0.59	20
	2	0.20	0.223	135	2.130	-0.25	20
	3	0.80	0.701	264	2.422	0.97	20
	4	0.90	0.961	562	2.750	-1.43	20
	5	1.00	0.997	1325	3.122	0.23	19

Mortality level	Concentration	95% Confidence Interval
0.50	201	(159,252)
0.10	100	(57,176)
0.90	406	(310,694)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-500-9d.de3d-Zn							
	1	0.00	0.006	140	2.146	-0.25	10
	2	0.00	0.350	265	2.423	-0.60	10
	3	0.30	0.183	510	2.708	0.95	10
	4	0.60	0.732	1250	3.097	-0.94	10
	5	1.00	0.944	2400	3.380	0.77	10

Mortality level	Concentration	95% Confidence Interval
0.50	872	(593,1296)
0.10	396	(156,584)
0.90	1920	(1292,5009)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-500-9d,de-3d-Zn							
	1	0.20	0.212	135	2.130	-0.10	10
	2	0.70	0.664	265	2.423	0.24	10
	3	0.90	0.932	510	2.708	-0.40	20
	4	1.00	0.992	1100	3.041	0.28	20
	5	1.00	0.999	2400	3.380	0.09	20

Mortality level	Concentration	95% Confidence Interval
0.50	210	(131,296)
0.10	100	(23,151)
0.90	443	(224,852)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-500,de-7d-Zn							
	1	0.00	0.650	165	2.217	-0.83	10
	2	0.60	0.480	600	2.477	0.76	10
	3	0.90	0.957	625	2.796	-0.89	10

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
	4	1.00	0.998	1350	3.130	0.13	10
	5	1.00	1.000	2700	3.431	0.06	10

Mortality level	Concentration	95% Confidence Interval
0.50	306	(227,467)
0.10	184	(71,24)
0.90	507	(380,1437)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-500,de7d-Zn	1	0.20	0.249	165	2.217	-0.36	10
	2	0.80	0.702	310	2.491	0.68	10
	3	0.90	0.954	625	7.796	-0.82	10
	4	1.00	0.996	1400	3.146	0.20	10
	5	1.00	0.999	2650	3.423	0.07	10

Mortality level	Concentration	95% Confidence Interval
0.50	235	(145,331)
0.10	116	(23,173)
0.90	478	(338,1692)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-100-17d-Zn	1	0.00	0.000	190	2.279	-0.06	10
	2	0.00	0.001	330	2.519	-0.08	10
	3	0.60	0.600	640	2.806	0.00	10
	4	1.00	1.000	1500	3.176	0.06	10
	5	1.00	1.000	2400	3.380	0.06	10

Mortality level	Concentration	95% Confidence Interval
0.50	618	(all values)
0.10	516	outside (591,1706)
0.90	746	outside (324,663)

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-17d-Zn	1	0.70	0.687	210	2.322	0.09	10
	2	0.90	0.921	330	2.519	-0.24	10
	3	1.00	0.993	655	2.816	0.26	10
	4	1.00	1.000	1500	3.176	0.06	10
	5	1.00	1.000	2400	3.380	0.06	10

Mortality level	Concentration	95% Confidence Interval
0.50	170	outside (232,2031)
0.10	93	outside (163,766)
0.90	308	outside (65,227)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-100-17d-Cu	1	0.00	0.018	5	0.699	-0.43	10
	2	0.30	0.087	11	1.041	2.38	10
	3	0.10	0.213	18	1.255	-0.87	10
	4	0.30	0.476	32	1.505	-1.11	10
	5	1.00	0.925	110	2.041	0.90	10
	6	1.00	0.981	220	2.342	0.44	10

Mortality level	Concentration	95% Confidence Interval
0.50	33	(23,56)
0.10	12	(5,18)
0.90	95	(57,341)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-17d-Cu	1	0.40	0.398	5	0.699	0.02	10
	2	0.80	0.690	14	1.146	0.75	10
	3	0.70	0.782	21	1.322	-0.63	10
	4	0.80	0.885	40	1.602	-0.84	10

Appendix 2. Continued

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
	5	1.00	0.962	110	2.041	0.63	10
	6	1.00	0.983	220	2.342	0.42	10

Mortality level	Concentration	95% Confidence Interval
0.50	7	(1,12)
0.10	1	(0.01,3)
0.90	46	(24,413)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Ac-Zn-100-17d-Cd	1	0.00	0.001	0.5	-0.301	-0.12	10
	2	0.00	0.012	1	0.000	-0.35	10
	3	0.00	0.095	2	0.301	-1.03	10
	4	0.70	0.479	4	0.602	1.40	10
	5	0.80	0.921	90	0.954	-1.42	10
	6	1.00	0.995	19	1.279	0.29	10

Mortality level	Concentration	95% Confidence Interval
0.50	4.10	(3,6)
0.10	2.00	(0.86,3)
0.90	8.00	(6,21)

Bioassay description	Unit	Died	Phat	Conc	Log Conc	Z	n
Un-Zn-100-17d-Cd	1	0.00	0.001	0.6	-0.260	-0.07	10
	2	0.30	0.300	1	0.000	0.00	10
	3	1.00	0.999	2	0.301	0.10	10
	4	1.00	1.000	4	0.602	0.06	10
	5	1.00	1.000	9	0.954	0.06	10
	6	1.00	1.000	19	1.279	0.06	10

Appendix 2. Continued

Mortality level	Concentration	95% Confidence Interval
0.50	1.08	(0.95,1.40)
0.10	0.89	(0.6,1.00)
0.90	1.31	(1.14,3.00)

Appendix 3. Analysis of the difference between two LC50s by the method of the Standard Error of the difference Bioassays are coded as in Appendix 2.

Bioassay description	96-hr LC50	Upper fiducial limits	$f_{1\ 2}$	Ratio of median lethality	Significant difference ($P < 0.05$)
Steelhead:					
Ac-Zn-50-7d-Znf	468	569	2.030	4.775	*
Control	98	193			
Ac-Zn-50-14d-Zn	481	610	1.627	2.863	*
Control	168	257			
Ac-Zn-50-21d-Zn	446	518	1.161	4.168	*
Control	107	142			
Ac-Zn-50-7d,200-10d-Zn	634	760	1.056	2.264	*
Control	280	390			
Rainbow Trout:					
Ac-Zn-100-1d-Zn	152	207	1.554	1.198	
Control	181	248			
Ac-Zn-100-2d-Zn	112	181	1.77	1.616	
Control	181	247			
Ac-Zn-100-7d-Zn	938	1138	1.360	2.88	*
Control	311	395			
Ac-Zn-80-28d-Zn	894	1455	1.736	2.430	*
Control	462	599			
Ac-Zn-100-10	1097	1360	1.366	4.570	*
Control	240	301			
Ac-Zn-100,300-14d-Zn	1234	1448	1.384	6.02	*
Control	205	272			

Appendix 3. Continued

Bioassay description	96-hr LC50	Upper fiducial limits	$f_{1\ 2}$	Ratio of median lethality	Significant difference ($P < 0.05$)
Ac-Zn-100,300,500-9d-Zn					
	1009	1469	1.478	5.02	*
Control	201	224			
Ac-Zn-100,300,500-de3d-Zn					
	871	1296	1.691	4.148	*
Control	210	296			
Ac-Zn-100,300,500-de-7d-Zn					
	305	427	1.613	1.298	
Control	235	330			
Ac-Zn-100-17d-Zn	618(3)	655	1.211	3.211	
Control	170(3)	204			
Ac-Zn-100-17d-Cu(4)	33(3)	42	1.943	3.231	
Control	7(3)	13			
Ac-Zn-100-17d-Cd	4.1(3)	5.976	1.574	4.287	*
Control	1.1(3)	1.394			

* = Significant difference 'f' ($P < 0.05$).

† Bioassay coded as in Appendix 2.

$F_{1\ 2}$ = Explained in the methods.

(3) = 120-hr LC50 was determined for cross tolerance experiment

(4) = The toxicant used for bioassay after acclimation to zinc is indicated.