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The Effect of Nickel Pre-exposure on the Lethal
Tolerance of the Zebrafish
(*Brachydanio rerio*)

Christine Elizabeth Searle

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Biology at
Concordia University
Montreal, Quebec, Canada

October, 1988

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ABSTRACT

The Effect of Nickel Pre-exposure
on the Lethal Tolerances of the Zebrafish
(Brachydanio rerio)

Christine Searle

The purpose of this study was to investigate the effect of nickel pre-exposure on the lethal tolerances of the zebrafish (Brachydanio rerio) to copper. Pre-exposure to waterborne nickel at a concentration of 1.40 mg/L for 7 d led to an apparent decrease in tolerance (i.e. sensitization) to lethal levels of copper. When test fish were pre-exposed to the same level of nickel for a 14 d period, a significant sensitization to copper resulted.

The increase in sensitization seen following pre-exposure could not be attributed to continuous accumulation of nickel within the body over the pre-exposure period, as steady state conditions were attained following 2 d of a 21 d exposure to three concentrations of nickel (0.35, 0.70, 1.40 mg Ni/L).

The enhanced sensitization was also not attributable to a retention of nickel within the body following pre-exposure, as nickel was found to be eliminated rapidly from the body. The depuration pattern of this contaminant appeared to be biphasic, with the bulk of nickel being eliminated during an initial fast phase lasting approximately 10 h. This rapid release of accumulated nickel leaves little time for interaction with copper presented sequentially at a lethal level. Pre-exposure to nickel

also did not enhance the uptake of copper during exposure to elevated levels of this contaminant.

The unique sensitization response seen in this study following pre-exposure to nickel is discussed with respect to tolerance modifications seen following pre-exposure to other metals.

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INTRODUCTION

The suggestion that fish have the ability to adapt to pollutants was first considered in the context of fish-stocking programs (King, 1937; Paul, 1952). For example, hatchery-raised trout introduced into the Sacramento River of California were found to suffer severe mortality due to the presence of elevated levels of copper within the river. However, a native population of the same species appeared to be unaffected, suggesting that these fish had adapted to the adverse conditions (Paul, 1952).

It was Schofield (1965) who determined that the high mortality rate of hatchery-raised fish could be reduced by pre-exposing these fish to the same contaminants found in the waters being stocked. His study showed that fingerling brook trout (Salvelinus fontinalis) raised in hatchery waters containing high levels of zinc could be successfully introduced into a zinc- and copper-contaminated lake, whereas viable populations of the same species could not be established from fish raised in zinc-free waters (Schofield, 1965). He attributed the observed metal tolerance to an ability of fish to physiologically adapt.

Adaptation may occur through the process of acclimation (physiological adaptation) or natural selection (genetic adaptation). Genetic adaptation to elevated metal levels has not been definitively found to occur in fish (Rahel, 1981; Klerks and Weis, 1987). In contrast, physiological adaptation of fish to heavy metals has been observed in situ (Benson and Birge, 1985) and in laboratory studies (e.g. Dixon and Sprague, 1981b). Acclimation refers to any compensatory physiological adjustments made by an organism in response to experimental manipulation of a specific environmental factor, which results in the reestablishment of homeostasis (Fry, 1971).

For any environmental factor, a range of intensity can be successfully tolerated by fish. This range is referred to by Fry (1971) as the 'zone of tolerance', the upper limit of which is termed the 'incipient lethal level' (ILL) (Fry, 1971). Beyond the ILL is the 'zone of resistance', and exposure of levels of a factor within this zone will be lethal if the duration of exposure is continued for a sufficient length of time. Research has demonstrated that the ILL can be shifted through acclimation to subthreshold levels of many stressors, thereby resulting in an alteration of the normal zone of tolerance. If homeostasis is reached through acclimation, the resultant tolerance modification will be constant over time (Fry, 1971).

Modifications in tolerance of fish were initially demonstrated for natural variables. For example, Shephard (1955) and Moss and Scott (1961) demonstrated that by gradual acclimation to low oxygen levels, fish could tolerate a lower level of oxygen than unacclimated fish. Similarly, the upper incipient lethal temperature for salmonids has been found to change 1°C for every 3°C change in acclimation temperature (Brett, 1952). Recently, the interest in this subject has focused on toxicant stressors, particularly heavy metals.

Acclimation of fish to metals has generally resulted in substantial increases in lethal tolerance. Early studies revealed that pre-treatment of rainbow trout (Salmo gairdneri) or flagfish (Jordanella floridae) eggs with sublethal levels of zinc or cadmium protected them against a subsequent toxicant challenge (Sirley et al., 1974; Spear, 1976). Other researchers have since conducted more detailed studies on tolerance modifications. For example, Dixon and Sprague (1981b) found that tolerance to copper could be augmented between 60% and 100% following pre-exposure to varying

concentrations of this metal. Increases in lethal tolerance ranging from 45% to 250% have been reported in fish following pre-exposure to aluminum (Orr et al., 1986), arsenic (Dixon and Sprague, 1981a), zinc (Bradley et al., 1985), and cadmium (Duncan and Klaverkamp, 1983). Acclimation to different metals therefore appears to lead to similar magnitudes of increased tolerance.

Toxicant acclimation has been reported, on occasion, to result in decreased tolerance or sensitization of fish to a subsequent metal challenge. Decreases in tolerance of between 8% and 60% have been reported following pre-exposure of fish to zinc (Hobson and Birge, 1986), copper (Dixon and Sprague, 1981b), nickel and silver (Reddy, 1987). The sensitization noted in Reddy's (1987) study following pre-exposure to nickel is particularly interesting in that nickel, unlike most other metals, has not yet been found to increase tolerance through pre-exposure. Reddy (1987) observed that pre-exposure to 0.5 and 1.0 mg Ni/L led to an apparent sensitization to lethal nickel levels, whereas 5.0 mg Ni/L resulted in significant sensitization. Fish pre-exposed to nickel at similar levels were even more sensitive to lethal levels of copper. These occurrences of sensitization are of considerable importance due to the greater risk associated with contaminants displaying this increased toxicity.

Sensitization resulting from metal pre-exposure is thought to be a consequence of either the pre-exposure concentration (review by Chapman, 1985; Roesijadi and Fellingham, 1987) or the length of the pre-exposure period (Hobson and Birge, 1986).

It has been suggested that levels of a toxicant that are too low to induce an enhancement of tolerance may lead to sensitization in some instances. Likewise, an exposure concentration that is excessive can evoke the same response due to the toxic

effects of the pre-exposure concentration itself (Roesijadi and Fellingham, 1987).

Sensitization is also thought to occur following a pre-exposure period that is insufficient for complete acclimation.

Acclimation to sublethal levels of metals is normally complete within a 5 to 7 d period (Dixon and Sprague, 1981b; Duncan and Klaverkamp, 1983; Bradley et al., 1985; Orr et al., 1986). Complete acclimation can be confirmed if there is no significant alteration in tolerance with a further extension of the pre-exposure period. In a recent study, a 7 d pre-exposure period to zinc resulted in sensitization to subsequent lethal metal challenge (Hobson and Birge, 1986). These authors found that by extending the pre-exposure period to 14 or 21 d, the magnitude of sensitization was decreased and control-level tolerance was eventually gained. Dixon and Sprague (1981a) noted the same pattern following pre-exposure to an organic contaminant, cyanide. These findings suggest that lengthening the pre-exposure period beyond 7 d can decrease the magnitude of sensitization by allowing fish more time to fully acclimate to the toxicant under consideration (Dixon and Sprague, 1981a).

The main objective of this thesis was to investigate how the length of the pre-exposure period may influence the ability of fish to acclimate to nickel. Previous studies on nickel pre-exposure had only used a 7 d acclimation period, which resulted in sensitization to lethal levels of nickel and copper (Reddy, 1987). I decided to explore the possibility that fish may require a longer period of exposure to acclimate to nickel. Support for this hypothesis is provided by the fact that no mortality occurred in any of Reddy's (1987) pre-exposure regimes, which would suggest that the sensitization

did not arise from toxic effects of the pre-exposure concentrations (Chapman, 1985; Roesijadi and Fellingham, 1987).

Experiments were therefore designed to test the hypothesis that an extension of the nickel pre-exposure period from 7 to 14 d would result in a reduction of sensitization to lethal levels of copper.

Copper was selected as the lethal metal for this study because copper and nickel are frequently found as co-contaminants (Moore and Ramamoorthy, 1984; NRCC, 1981).

Nickel and copper are heavy metal contaminants released through industrial activities such as fossil fuel combustion, mining and smelting (Moore and Ramamoorthy, 1984). Although nickel is ubiquitous in aquatic systems, its concentrations are often low enough to be of little consequence to resident populations (NAS, 1975). Streams and lakes across the United States contain an average level of 4.8 ug Ni/L, with many containing concentrations below the detection limit of 1 ug Ni/L (Snodgrass, 1980). Copper is found in natural waters at concentrations as low as 0.5 to 1.0 ug Cu/L (Spear and Pierce, 1979). Lakes in the vicinity of nickel mining operations, such as Sudbury, Ontario, where these two contaminants are released together into the environment, contain concentrations as high as 6.0 mg Ni/L and 200 ug Cu/L (Stokes et al., 1973).

There are substantial differences in the acute toxicity of these two metals to fish. Nickel is considered to be only moderately toxic when compared to copper, with their respective LC50 values being 4 to 45 mg Ni/L (Hale, 1977; Pickering and Henderson, 1966; Nebeker et al., 1985; Anderson et al., 1979) and 0.025 to 2.55 mg Cu/L (Spear

and Pierce, 1979). The toxicity of both metals varies with the species of fish under consideration, as well as the water quality conditions (Pickering and Henderson, 1966; Howarth and Sprague, 1979). When nickel and copper are presented in mixtures, their toxicity becomes synergistic at both lethal and sublethal levels (Weinstein, 1979). Reddy's (1987) work suggests that sequential exposures of these metals may also alter their toxicity.

To accompany the central objective of this study, a series of questions relating to the pharmacokinetic management of nickel within fish were also addressed.

Pharmacokinetic research involves a quantitative examination of the uptake and elimination patterns of compounds (Klaassen, 1980). Although accumulation patterns for nickel have been established for the mussel (Mytilus edulis) (Friedrich and Filice, 1976), sea urchin embryos (Lytechinus pictus) (Timourian and Watchmaker, 1972), diatom species (Phaeodactylum tricorutum) (Skaar et al., 1974) and a zooplankton species (Daphnia magna) (Hall, 1982), no information pertaining to whole body nickel uptake or elimination patterns in fish could be found in literature. A knowledge of these patterns could be useful in determining whether or not the sensitization seen following pre-exposure to nickel could be directly related to loading or to a lack of elimination of this toxicant.

Accordingly, additional experiments were designed to test the following hypotheses. The first hypothesis was that the steady state levels of nickel in whole fish would reflect the change in copper tolerance. To address this, an experiment examining accumulation patterns of nickel at three exposure concentrations over a 21 d period was conducted. The second hypothesis was that the sensitization seen with nickel pre-

exposure could be attributed to a persistence of nickel within the body following pre-exposure, thereby adding or interacting with lethal levels of copper. An experiment was conducted to investigate the depuration pattern of nickel from the body following a 7 d exposure to nickel. Also examined in part was the overall partitioning of nickel within two compartments of fish and the question as to whether or not nickel pre-exposure would lead to increased copper uptake.

MATERIALS AND METHODS

2.1. Test Organism and Holding Procedures

Zebrafish (*Brachydanio rerio*) were selected as the test organism for these studies as they are small, readily available throughout the year and relatively easy to maintain in laboratory aquaria (Fogel and Sprague, 1977).

Adult zebrafish were purchased from the Florida Fish Farm Co-op in Tampa, Florida, when required. Upon arrival, the fish were distributed into 50 L glass aquaria at a loading density of approximately 2 g/L. These holding tanks were supplied with a continuous flow of 25°C water at a rate of 1 L/min, which provided a 95% replacement rate of 2.6 h (Sprague, 1973). A three-week period of acclimation to laboratory conditions of overhead lighting (12 h dark - 12 h light) and to water quality was permitted. The tanks were cleaned regularly and the fish were fed once daily, ad libitum, with Aquafin tropical fish food. This feeding schedule was continued throughout the acclimation and experimental periods until 48 h prior to lethal bioassay testing (as suggested in Sprague, 1973), or 40 to 48 h prior to sampling for kinetic studies (to allow an adequate void time for ingested food).

2.2. Water Quality Analysis

City of Montreal source water supplied both the holding and test tanks. The water was degassed, dechlorinated, filtered and heated before entering the laboratory. Water quality was monitored at weekly intervals in the holding and acclimation tanks, and daily during the course of an experiment. The pH was measured with a digital pH meter, equipped with a Corning glass electrode and a Fisher reference electrode (model no. 503). Dissolved oxygen and temperature were measured using a YSI field meter

and a hand-held mercury thermometer, respectively. Alkalinity, Ca^{2+} and Mg^{2+} were measured only in experimental tanks. The former parameter was determined using the potentiometric titration technique (APHA, 1980). The latter two ions were measured directly from water samples on an atomic absorption spectrophotometer (Perkin Elmer 503). Hardness, as mg/L CaCO_3 , was then calculated following the recommended formulae in APHA (1980). Mean water quality characteristics for all experiments are given in Table 1.

2.3. Toxicant Stock Solution Preparation

Toxicant concentrations for the copper bioassay were selected from preliminary static bioassay data, as well as information provided from other studies conducted at this facility using zebrafish (Reddy, 1987; Weinstein, 1979). The selection of the pre-exposure and accumulation concentrations of nickel was based on previous studies (Reddy, 1987), as well as a consideration of levels that would be relevant to environmental situations where nickel is a contaminant.

Toxicant solutions for all experiments were prepared in the same fashion. Industrial grade nickel sulphate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$) (Canadian Industries Ltd.) or reagent grade copper sulphate ($\text{CuSO}_4 \cdot 6\text{H}_2\text{O}$) (Fisher Scientific) was dissolved in deionized, glass-distilled water. Industrial grade nickel sulphate was considerably more economical than the reagent grade quality of this salt. The purities of the two grades were compared by Weinstein (1979) and the differences were found to be negligible. The solutions were subsequently acidified with trace metal grade nitric acid (Fisher Scientific) to a pH of approximately 2 to ensure that the toxicant would stay in solution.

Table 1. Chemical and physical parameters of laboratory water used during these studies. All units are in mg/L except where otherwise indicated.

Parameter	Mean	SD	N
Alkalinity (as CaCO ₃)	82.96	2.11	39
Calcium	32.34	0.61	30
Copper	n.d.*		30
Dissolved Oxygen (% saturation)	94.59	2.52	270
Hardness (as CaCO ₃)	110.06	0.89	30
Magnesium	7.12	0.33	30
Nickel	n.d.*		30
pH	7.84	0.15	367
Temperature (°C)	24.83	0.67	267

n.d. = non-detectable by carbon-rod atomic absorption spectrophotometry.

* = detection limits: copper: 0.02 ug/L
nickel: 0.10 ug/L

2.4. Toxicant Water Analysis

During experiments, duplicate water samples were taken daily from both control and toxicant dosed tanks. These samples were acidified to $\text{pH} < 2$ and stored at -4°C in polyethylene vials (Fisher Scientific) until analysis, to minimize precipitation and possible absorption of any metal to the walls of the container. Filtration of the water samples was not found to alter the toxicant concentration, and therefore unfiltered samples were taken, representing total nickel or copper concentrations.

All samples were analyzed for metal content within 2 d of the sampling date using a Perkin Elmer 503 atomic absorption spectrophotometer. Copper levels below 200 $\mu\text{g/L}$, and nickel levels below 0.35 mg/L were analyzed using the carbon-rod atomization (flameless) technique, whereas all other samples were analyzed using the volatilization (flame) method. Detection limits of these techniques for these two elements are provided in Table 2.

All concentrations given in this thesis are nominal levels. The means of the actual measured levels are presented in Table 2. These means were between 94% and 104% of the nominal levels and yielded an average coefficient of variation of 8.65%.

2.5. Acclimation to Experimental Conditions

Following the three week holding period, the test organisms were individually weighed using a water displacement technique. This method involves introducing each fish into a beaker of water of known weight. The beaker and fish are subsequently reweighed to determine the actual fish weight. Fish ranging from 0.3 - 0.8 g were selected for the experiments. Both males and females were used in these studies. After the weighing procedure, fish were randomly transferred to acclimation tanks and

Table 2. Concentrations of nickel and copper measured during the bioassay and accumulation studies (mean values of replicated exposure tanks).

Experiment	Nickel concentration (mg Ni/L)			Copper concentration (ug Cu/L)		
	mean	SD	N	mean	SD	N
bioassay studies	1.42	0.10	112	182	9.6	39
				206	17.9	40
				224	17.9	40
				244	20.3	39
				258	19.5	39
			282	26.9	39	
21 day accumulation study	0.33	0.05	88			
	0.69	0.09	88			
	1.42	0.18	88			
devisceration and depuration studies	1.45	0.18	32			
copper uptake study	1.35	0.08	28	218	14	12

detection limits: volatilization method: 10.0 ug Ni/L
 2.0 ug Cu/L
 carbon-rod method: 0.1 ug Ni/L
 0.02 ug Cu/L

allowed to adjust to the respective experimental conditions for an additional 7 to 14 d. Test lot sizes and specific acclimation conditions are explained in each experimental methodology section. During this period, the tanks were cleaned daily. Acclimation tanks were identical to experimental tanks in terms of size, water quality, water flow, and photoperiod. The experimental water flow rates, which ranged from 500 - 750 ml/min, provided a 95% replacement time of 3.4 to 4.8 h (Sprague, 1973). Toxicant flow was commenced in the experimental tanks 5 to 7 d prior to introducing the fish. This was done to allow for equilibration and stabilization of the concentrations.

2.6. Bioassay Studies

2.6.1. Pre-exposure and toxicant delivery system

The pre-exposure phase involved replicated exposure of fish to one concentration of nickel (1.40 mg Ni/L) or diluent water alone for 7 or 14 d.

The toxicant in this phase was supplied to the tanks using a Mariotte bottle system (Leduc, 1966), combined with a 2 L polyethylene mixing chamber. In accordance with Leduc's (1966) method, a calculated volume of the nickel stock solution (as prepared in Section 2.3.) was diluted with deionized glass-distilled water to obtain the concentration required for the 1.40 mg Ni/L exposure regime. Large volumes of this secondary solution were made to ensure consistent concentrations throughout the duration of the experiment. This solution was stored in 50 to 60 L acid-washed Rubber Maid garbage pails. Mariotte bottles (18 L) situated on shelves above the experimental aquaria were subsequently filled using a submersible pump (Little Giant Corp., model no. 2E-NT). It was necessary to refill the Mariotte bottles on occasion over the two week experimental period. During this time, flow to the

experimental tanks was temporarily redirected in order to maintain constant toxicant concentrations within each exposure tank.

A series of 8 exposure tanks were involved in the pre-exposure assembly. Toxicant was released from each Mariotte bottle via a feeder tube (Intramedic tubing, Fisher Scientific) into the mixing chamber, which was supplied with a constant flow of diluent water controlled by a flowmeter (Manostat Corp., New York). Two exit ports from this mixing chamber then supplied 2 adjacent tanks with equal quantities of the diluted nickel solution. Toxicant flow rates into each tank were controlled using flow meters (Manostat Corp., New York). The two adjacent tanks constituted one replicate, as they were supplied from the same Mariotte bottle. A second replicate was constructed in the same fashion. These two toxicant-supplied replicates were accompanied by two control replicates - each consisting of two tanks supplied by diluent water alone.

A group of 960 fish were acclimated to experimental conditions for a 14 d period. The acclimation procedure involved maintaining test lots of 15 fish in mesh cylindrical polyethylene cages suspended within the tanks (area = 0.18 m², mesh size 2.5 x 4mm). These cages allowed the test lots to be separated from each other, while being held under the same exposure conditions. Preliminary studies were conducted to ensure that toxicant levels and water quality were consistent between cages within a give exposure tank, and that water flow was not inhibited.

On day 0 of the experiment, all cages were randomly transferred from the acclimation tanks into the paired aquaria containing 1.40 mg Ni/L, or diluent water alone. Each paired aquaria unit contained a total of 14 experimental cages of fish (i.e.

7 per aquaria). An additional cage of fish was placed in each tank to compensate for density differences incurred during sampling on day 7 of the pre-exposure phase (total loading density/tank = 1.2 g/L). During pre-exposure, cages were rotated every 24 h within each tank to avoid the location within the tank from biasing the results.

On day 7 of the pre-exposure phase, 7 of the total 14 cages within each of the two paired aquaria were randomly transferred to the lethal bioassay tanks, as illustrated in Figure 1, to assess changes in tolerance. The remaining 7 cages were maintained under pre-exposure conditions for an additional 7 d.

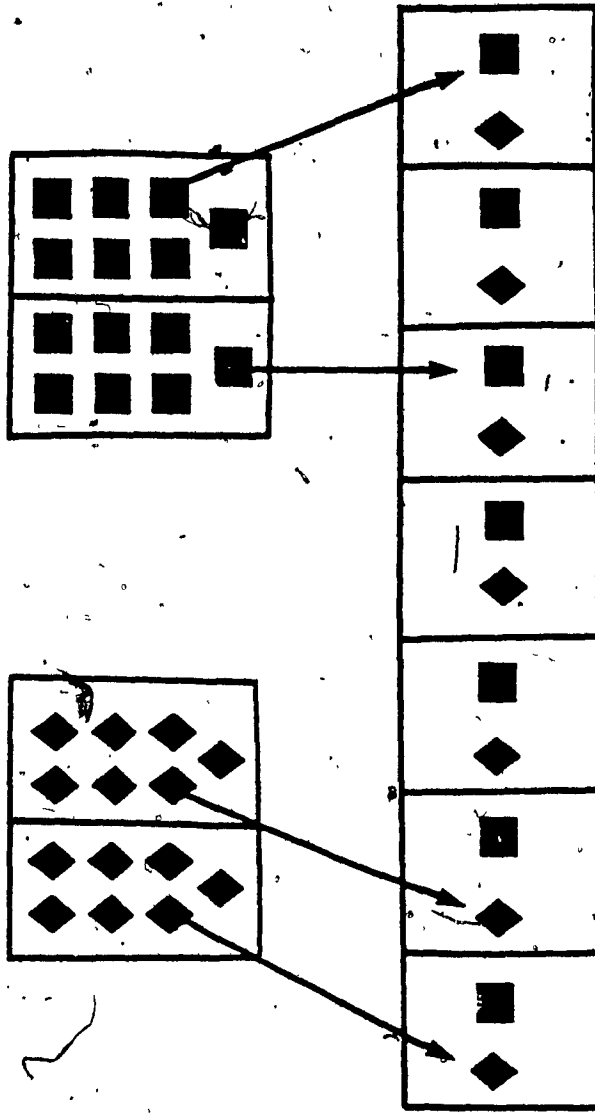
2.6.2. Lethality exposure and toxicant delivery system

The lethal bioassay involved exposure of the caged fish to six concentrations of copper (nominal range: 180 - 280 ug Cu/L) and one control over a 144 h test period. Following the transfer of the cages to the lethal bioassay tanks, each tank contained 4 cages (2 from replicated 1.40 mg Ni/L pre-exposure tanks, and 2 from replicated control tanks).

Copper was supplied to these exposure tanks using a serial diluter equipped with a Mariotte bottle. As in Section 2.6.1., a concentrated solution from the Mariotte bottle was dripped into a mixing chamber (see Figure 2) where it was combined with a continuous supply of diluent water. This solution flowed from the mixing chamber to level C on the front face of the diluter (see Figure 3). The solution was further diluted into the 6 exposure concentrations by adjusting the small glass faucets located on level C, such that different volumes per unit time were released into different chambers of the last diluting stage (level D) (see Figures 3 and 4). The diluting chambers of level D provided additional mixing of the toxic solution with diluent water in order to reach

Figure 1. The arrangement and transfer of groups of caged fish from paired pre-exposure tanks containing nickel or control water to the copper lethal exposure tanks.

PRE-EXPOSURE TANKS



LETHAL EXPOSURE TANKS

- control cage (15 fish)
- ◆ ni pre-exposure cage (15 fish)

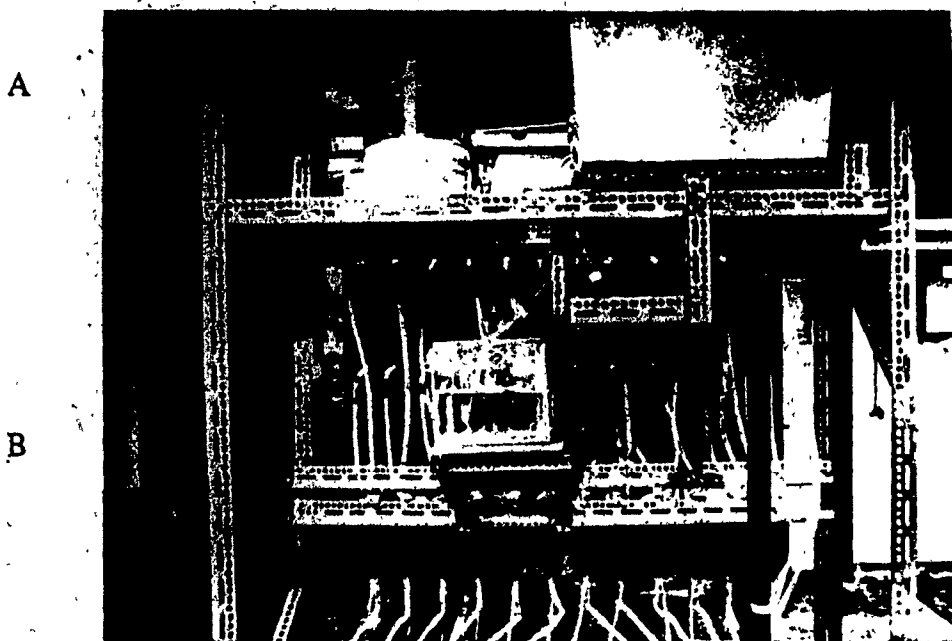


Figure 2. Photograph depicting the back of the serial diluter shown in Figure 3. The Mariotte bottle (point A) drips a concentrated solution of nickel into a mixing chamber (point B), which is supplied with clean water directly from the headbox. The mixing chamber then releases the diluted solution into level C of the diluter front (See Figure 3).

Level A

Level B

Level C

Level D

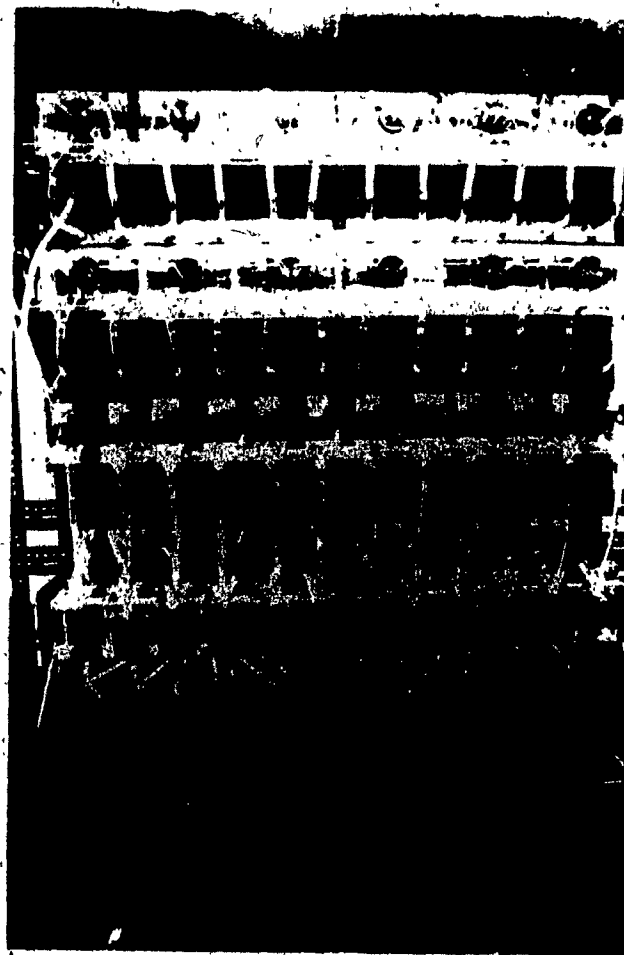


Figure 3. Photograph of the serial diluter employed during the lethal bioassay studies. Level A represents the stage containing clean water. Level B would be employed for a second toxicant in multiple toxicity studies. Level C represents the stage containing nickel which has been pre-diluted in the mixing chamber (Figure 2). Level D represents the final mixing chambers where the waters from levels A and C join prior to entering the individual test tanks.



Figure 4. Photograph depicting the rotating glass faucets located on the front stages of the serial diluter. Each faucet supplies water to a different exposure tank. By rotating the faucets upward, the volume of solution passing into the next stage is decreased. By moving the faucets downward, a greater volume of toxic solution passes into the final mixing chamber, resulting in a higher final exposure concentration. Flowrates are a function of hydraulic head and cross-sectional area of the glass faucet.

the desired concentrations, which were then delivered to the respective tanks. The method used to calculate the required flowrates from each level is explained in detail by Weinstein (1979).

Mortality was assessed by lack of response to gentle prodding. Dead fish were counted and removed from the cages at each observation time using a small modified net to minimize disturbance to the others. Observations on mortality were made every 2 to 3 h until the first death, and then at 1, 2 and 4 h. Observations were continued every 2 to 6 h until 96 h into the experiment. Between 96 and 144 h, the cages were observed every 6 and 12 h, as mortality had slowed considerably. The bioassays were run long enough to ensure that the incipient lethal level (ILL) had been attained. The ILL is an estimate of the concentration below which 50% of the test organisms may survive indefinitely (Sprague, 1973; Rand and Petrocelli, 1986). The estimated ILL is normally extrapolated from the asymptote of the toxicity curve defined by the LC50's for various time periods of exposure.

During the lethal tests, cages were rotated every 24 h within each tank. Water samples were taken on the same schedule as in the pre-exposure tanks. At the end of the 144 h test period, all remaining fish were removed and sacrificed. The 7 cages of fish still undergoing pre-exposure were subsequently transferred to the lethal bioassay tanks and assessed for copper tolerance. Mortality observations were made at the same time intervals as those used for the 7 d pre-exposure test lots.

2.7. Bioaccumulation Studies

2.7.1. Toxicant delivery

Toxicant solutions for each of these studies were introduced to the exposure tanks using the Mariotte bottle system (Leduc, 1966), as in Section 2.6.1. The mixing chamber design used in the pre-exposure apparatus was altered somewhat for these studies. Each exposure tank was serviced by an individual Mariotte bottle. The toxicant solution was delivered from these bottles via polyethylene tubing to a collecting funnel suspended over each tank, where it was then combined with a constant volume of diluent water. Control tanks were set up in the same fashion, but were supplied with diluent water alone.

2.7.2. 21 d accumulation study

Whole body accumulation of nickel was examined over a 21 d period. This experiment required 840 fish, which were acclimated for a 14 d period in test lots of 105. On day 0, each lot was sampled and randomly transferred from the acclimation tanks to one of 8 exposure aquaria, which contained a replicated series of 3 nickel concentrations (0.35, 0.70, 1.40 mg/L) and a control (loading density/tank = 1.05 g/L). In sampling, fish were removed from their respective tanks, allowed to free-swim in clean water for five minutes and then killed by a blow to the head. In order to obtain a quantity of tissue large enough to analyze, five fish were sacrificed for each sample. Triplicate samples were taken at each sampling interval. Tissues were collected in polyethylene vials (Fisher Scientific), immediately frozen to -20°C, and later freeze dried. Sampling of fish occurred on days 2, 4, 7, 11, 16 and 21 of the experiment in all tanks.

For nickel analysis, the freeze dried tissue was then ground to an even fine powder using an acid-washed mortar and pestle. A 200 mg aliquot was removed and analyzed using a slightly modified version of Aysola et al.'s (1987) tissue analysis method for heavy metals in biological samples. One ml each of trace metal grade nitric and sulphuric acids (Fisher Scientific) were added to the 200 mg tissue aliquot. The mixture was decanted into a teflon vessel and heated in a 700 watt microwave oven at high power for 2 to 3 minutes, to oxidize the organic matter present (Aysola et al., 1987). After cooling to room temperature, the mixture was transferred to a volumetric flask and diluted to 10 ml using double deionized water. Standards and blanks were prepared in the same manner. The accuracy of the technique was checked by spiking tissue samples with a known amount of nickel. Analysis of these samples yielded an average recovery of 99% (N = 40). Tissue analyses for nickel were performed by atomic absorption spectrophotometry. Toxicant-exposed test lots were analysed using the volatilization (flame) method, whereas control test lots required the lower detection limits of the carbon-rod (flameless) method.

2.7.3. Evisceration study

A preliminary study to investigate the distribution of nickel between the gill-organ compartment and an eviscerated body compartment was conducted. Further partitioning was not possible due to the size and number of zebrafish available.

This experiment was run in conjunction with the depuration study. These studies required a total of 540 fish, which were acclimated to experimental conditions for 7 d in test lots of 135. On day 0, each test lot was randomly transferred to one of 4 tanks containing either diluent water alone or 1.40 mg Ni/L (in replicate) and exposed for a 7

d period. At the end of this exposure period, 30 fish were removed from each test lot for the evisceration study, and individually sacrificed using the previously described methodology. The gills and the internal viscera (including the kidneys, liver, intestine, heart and the swim bladder) were quickly removed from the body. The viscera from ten fish were pooled together to form one sample. The corresponding eviscerated carcasses of these fish were pooled in separate polyethylene vials and analyzed separately. Analysis for metal levels was performed as previously described (Section 2.7.2). The remaining fish were required for the depuration study, and details pertaining to the experimental protocol are described in the following section.

2.7.4. Depuration study

Following the 7 d exposure to either 1.40 mg Ni/L or diluent water alone, each test lot of 105 fish was sampled according to the previously stated methodology (Section 2.7.1.) (time 0). Five fish were required for each sample and three samples were taken per tank, per sampling interval. Control and toxicant exposed test lots were then randomly transferred to separate aquaria supplied with diluent water alone. Depuration sampling occurred at 4, 7, 10, 24 and 48 h following this transfer. Tissue analysis was conducted using the previously described methodology (Section 2.7.1.). All samples were analyzed using the carbon-rod atomic absorption technique.

2.7.5. Copper uptake study

An examination of whether zebrafish pre-exposed to nickel would accumulate greater quantities of copper than non-pre-exposed fish was also undertaken. This experiment required 160 fish. Test lots of 20 fish were acclimated for 7 d in cylindrical polyethylene mesh cages (previously used in the pre-exposure study),

suspended in 4 exposure tanks. Each tank contained 2 cages (loading density/tank = 0.4 g/L). Following acclimation, the cages were transferred to replicated tanks containing 1.40 mg Ni/L or diluent water alone. After a 7 d exposure to these conditions, one cage of fish from each tank was sacrificed for whole body copper analysis. Each cage yielded three samples, with each sample containing 5 to 6 fish. These samples were taken to determine whole body copper concentrations in control and pre-exposed test lots of fish prior to copper exposure. A tissue analysis technique similar to that used for nickel determination was used. The sole alteration in the methodology occurred after the microwave digestion phase, wherein the tissue-acid mixture was transferred to a 25 ml volumetric flask and made to volume, rather than a 10 ml flask.

The remaining cages of fish were transferred to 2 tanks supplied with copper (nominal level: 220 ug Cu/L), such that each tank contained one cage of control exposed fish and one cage of nickel pre-exposed fish. At 48 h, the fish were sacrificed. Copper levels in these fish were compared to the levels found in fish prior to copper exposure.

2.8. Statistical Analysis

2.8.1. Bioassay data analyses

Dose-response data were analyzed using the Litchfield-Wilcoxon method (1949). This method was selected to permit comparison of the data with Reddy (1987), who utilized this same technique. This form of data analysis involved plotting the cumulative percent mortality for each lethal exposure concentration on log-probability paper at selected time intervals. An eye-fitted line was drawn, and the goodness-of-fit

was confirmed by a χ^2 test. Heterogeneous data sets resulted on occasion and were corrected for by replotting the lines and recalculating the χ^2 values. These plots yielded LC50 values for each time period, the slope of the dose-response line (or slope function, 'S'), and the 95% confidence limits of each of these. Significant differences between replicates or treatments were then determined by testing the slopes and relative potencies of the given dose-response lines at selected time intervals, as explained in Litchfield and Wilcoxon (1949). The LC50 values were subsequently plotted against time to give toxicity curves.

2.8.2. Kinetic data analyses

Accumulation data from the 21 d uptake and the depuration studies were analyzed using a two-way nested ANOVA by following the method outlined by Nie et al. (1975). If replicates were not significantly different they were pooled for subsequent a posteriori testing. The modified least significant difference test (LSD mod test) was used to examine significant differences between the control and toxicant-exposed samples at selected time intervals.

In addition, the depuration data were analyzed using a mathematical model in order to describe the distribution and elimination of nickel within zebrafish. A two compartment model was selected, based on the biexponential nature of the data when plotted on semi-log paper. Biexponential elimination events have been found to represent an initial 'fast' phase, when a toxicant is eliminated rapidly from a central compartment, and a 'slow' phase, when a toxicant is eliminated at a slower rate from a peripheral or deep compartment (Klaassen, 1980; Gillette, 1972). The two compartments examined in this model represent two different quantities of the toxicant

which have uniform kinetics, and do not constitute particular tissues or organs (Moriarty, 1978). A representation of the model is shown in Figure 5, where the fast compartment is denoted as C1, and the slow compartment as C2. The rates of transfer into (k_{01}), within (k_{12} and k_{21}), and out of (k_{10}) the compartments are also given. The subscripts of these k values correspond to the direction of transfer of the toxicant in question; i.e., k_{21} represents the rate at which nickel is transferred from compartment two (C2) to compartment one (C1). This model makes the assumption that uptake and elimination only occur through the fast compartment and that the rate at which a toxicant leaves a compartment is proportional to the amount of the pollutant in that compartment (Moriarty, 1978).

Data acquired from the evisceration and copper-uptake studies were also analyzed using two-way nested ANOVAs. No significant differences were found between replicates in either of these studies, and therefore the data were pooled and reanalyzed using a two-way ANOVA. The LSD mod test was again employed to test for differences between the treatment groups. All significant differences reported here are at the .05 level, unless otherwise indicated.

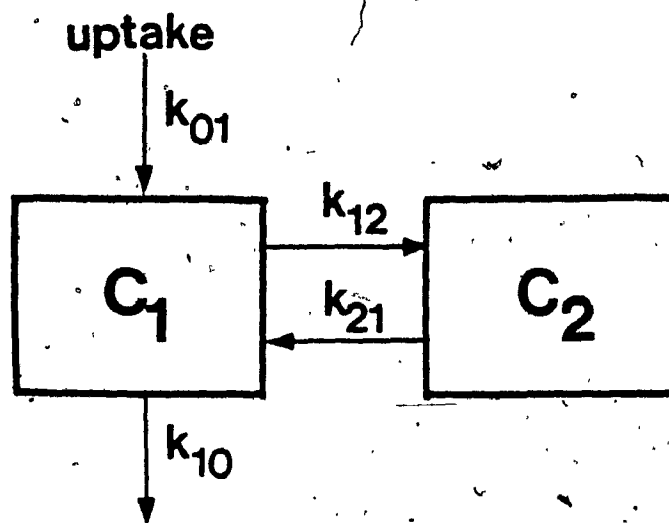


Figure 5. An illustration of the two compartment model (from Spacie and Hamelink, 1986) with the respective rate constants between each compartment.

RESULTS

3.1. Lethal Bioassays

Mortality did not occur in any of the test lots during either acclimation or nickel pre-exposure. Depressed appetite and sluggish behavior were noted during the latter days of the 14 d pre-exposure period in those fish being treated with nickel. Zebrafish are continuous breeders (Laale, 1977), and all caged test lots of fish continuously laid eggs during pre-exposure. When fish were transferred to the lethal bioassay tanks, only those cages suspended within the control tank were found to lay eggs. Copper exposed fish showed visible signs of distress during the first 24 h. Darker body colouration, surface swimming and rapid ventilation were frequently seen. There was no massive mucous production noted.

3.1.1. Copper lethal tolerance in controls

The LC50 data for the 7 and 14 d control test lots are shown in Figure 6 and Table 3, and the accompanying 95% confidence limits are given in Table 3. The replicates of each of these test lots were not significantly different from each other ($P > .05$) and were therefore pooled. Incipient lethal levels (ILL) were attained in both control groups by 120 h. The 144 h LC50's for the 7 and 14 d control groups were 260 and 265 ug Cu/L, respectively, which were not significantly different from one another ($P > .05$) (Table 3). The slope function values also did not differ significantly ($P > .05$), indicating that control test lots within a given exposure concentration responded in a similar fashion to copper (Table 3).

Figure 6. Toxicity curves illustrating the effect of pre-exposure to nickel (1.40 mg/L) for varying lengths of time (7 and 14 d) on the tolerance of zebrafish (Brachydanio rerio) exposed to lethal levels of copper.

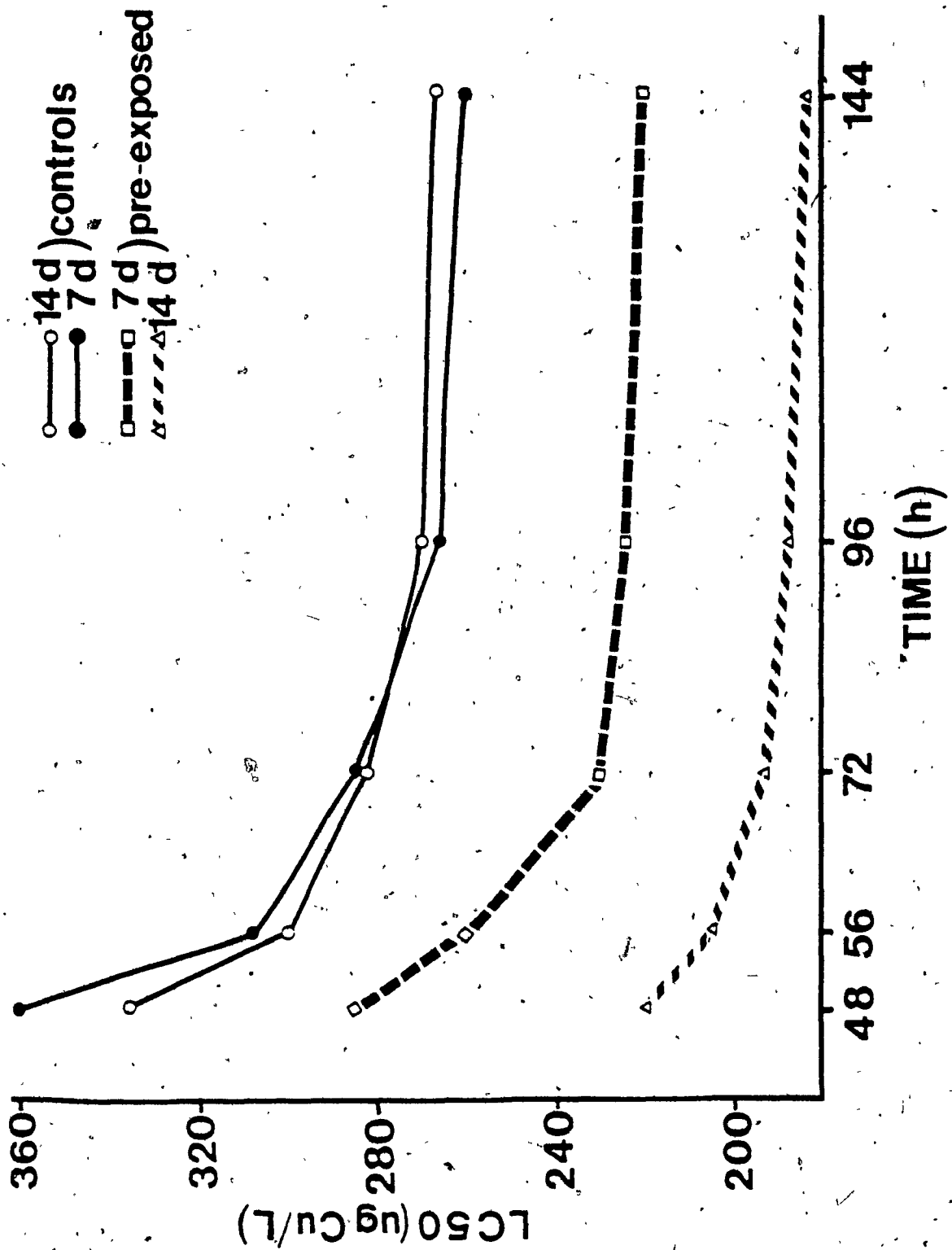


Table 3. LC50 and Slope function values ('S') of the dose-response lines with their respective confidence limits for zebrafish pre-exposed to diluent water alone or to 1.40 mg Ni/L for a 7 and 14 d period prior to lethal copper exposure (pooled data sets for replicate control and pre-exposed groups).

Time (h)	Group	LC50 (ug Cu/L)	Confidence Limits	'S'	Confidence Limits
48	7d C	360	(316-410)	1.4	(1.0 - 2.0)
	14d C	335	(299-275)	1.5	(1.1 - 2.0)
	7d P	285	(236-345)	1.4	(0.9 - 2.3)c
	14d P	220 a b	(195-249)	1.3	(1.0 - 1.6)c
56	7d C	308	(283-336)	1.3	(1.1 - 1.6)
	14d C	300	(275-327)	1.4	(1.1 - 1.7)
	7d P	260	(232-291)	1.7	(1.1 - 2.7)
	14d P	205 a b	(190-221)	1.2	(1.2 - 1.8)
72	7d C	285	(264-308)	1.3	(1.2 - 1.6)
	14d C	282	(233-341)	1.5	(0.8 - 2.6)c
	7d P	230 a	(209-253)	1.6	(1.2 - 2.1)
	14d P	193 a b	(179-208)	1.4	(1.2 - 1.7)
96	7d C	265	(225-318)	1.3	(0.9 - 1.9)
	14d C	269	(226-320)	1.4	(0.9 - 2.2)c
	7d P	225	(206-245)	1.5	(1.2 - 2.0)
	14d P	187 a b	(173-202)	1.4	(1.2 - 1.7)
144	7d C	260	(218-309)	1.3	(0.9 - 1.8)c
	14d C	265	(243-289)	1.5	(1.2 - 2.0)
	7d P	220	(202-240)	1.5	(1.2 - 1.9)
	14d P	183 a b	(169-198)	1.4	(1.2 - 1.7)

C = control exposure group

P = nickel pre-exposure group

7d = 7 d exposure period

14d = 14 d exposure period

a = significantly different from respective control (P<.05)

b = significantly different from 7 d pre-exposure group (P<.05)

c = corrected for heterogeneity

3.1.2. Copper tolerance in nickel pre-exposed fish

The LC50 data for fish pre-exposed to nickel for either 7 or 14 d are illustrated in Figure 6, and are also presented in Table 3 with the 95% confidence intervals. The replicate test lots of each of these exposure groups were not significantly different from one another ($P > .05$) and were therefore pooled. Incipient lethal levels were reached by 120 h in both the 7 and 14 d pre-exposure groups.

Fish acclimated to 1.40 mg Ni/L for a 7 d period were slightly less tolerant of copper than the controls (Figure 6). The 144 h LC50 of 220 ug Cu/L was not significantly different from that of the 7 d control group (260 ug Cu/L) ($P > .05$) (Table 3). Although the only statistically significant shift in tolerance between these two groups occurred at 72 h (Table 3), there was a constant 15% to 20% displacement of the nickel pre-exposed toxicity curve from that of the control group (Figure 6). The slope functions of the dose-response curves for the nickel-exposed group were not significantly different from those of the control (Table 3).

Increasing the period of pre-exposure to nickel from 7 to 14 d caused a significant decrease in zebrafish copper tolerance. The 14 d pre-exposure test lot yielded a 144 h LC50 of 183 ug Cu/L, which was significantly different from the accompanying control group (265 ug Cu/L), and the 7 d pre-exposure group (220 ug Cu/L) ($P < .05$). There was no apparent difference in the shapes of the three toxicity curves over the 144 h bioassay, which is clearly illustrated in Figure 6. The 14 d pre-exposure group exhibited a 30% decrease in tolerance relative to the controls. The slope function values (Table 3) of the individual dose-response data sets were again

found not significantly different from those of the control test lot or the 7 d pre-exposure test lot.

3.2. Accumulation and Elimination Studies

There was no mortality during any of the nickel accumulation or elimination experiments. However, mortality did occur during the copper uptake experiment (Section 2.3.4.) in the test lot pre-exposed to nickel and subsequently exposed to 220 ug/L copper. This mortality was not unexpected since the LC50 of copper at 48 h following a 7 d pre-exposure to nickel was 236 - 345 ug Cu/L. Dead fish in this particular experiment were not analyzed for heavy metal content.

3.2.1. 21 d accumulation study

Accumulation patterns of nickel at three waterborne levels are shown in Figure 7, and the accompanying data are given in Table 4. The replicate test lots were not significantly different and were therefore pooled ($P > .05$). Whole body levels of nickel in the zebrafish increased rapidly upon exposure to all three toxicant concentrations, and reached a steady state following two days of exposure. The stable levels attained were deemed steady state as there was no significant difference between these values and subsequent tissue levels for the remaining experimental period ($P > .05$) (Table 4). Control nickel levels were not significantly different from each other for the entire 21 d test period ($P > .05$). Nickel accumulation increased with increasing exposure concentration, and the mean values at steady state for test lots exposed to 0.00, 0.35, 0.70 and 1.40 mg Ni/L were 5.18, 10.61, 12.34 and 14.82 ug Ni/g tissue, respectively, as illustrated in Figure 8. When these values were tested against each other with a posteriori analyses (LSD mod test), the highest treatment group (1.40 mg

Figure 7. Whole body nickel concentrations in zebrafish during a 21 d exposure to 0.35, 0.70 and 1.40 mg Ni/L. Background levels in control fish over this period are also shown (mean \pm 2 S.E., N = 57).

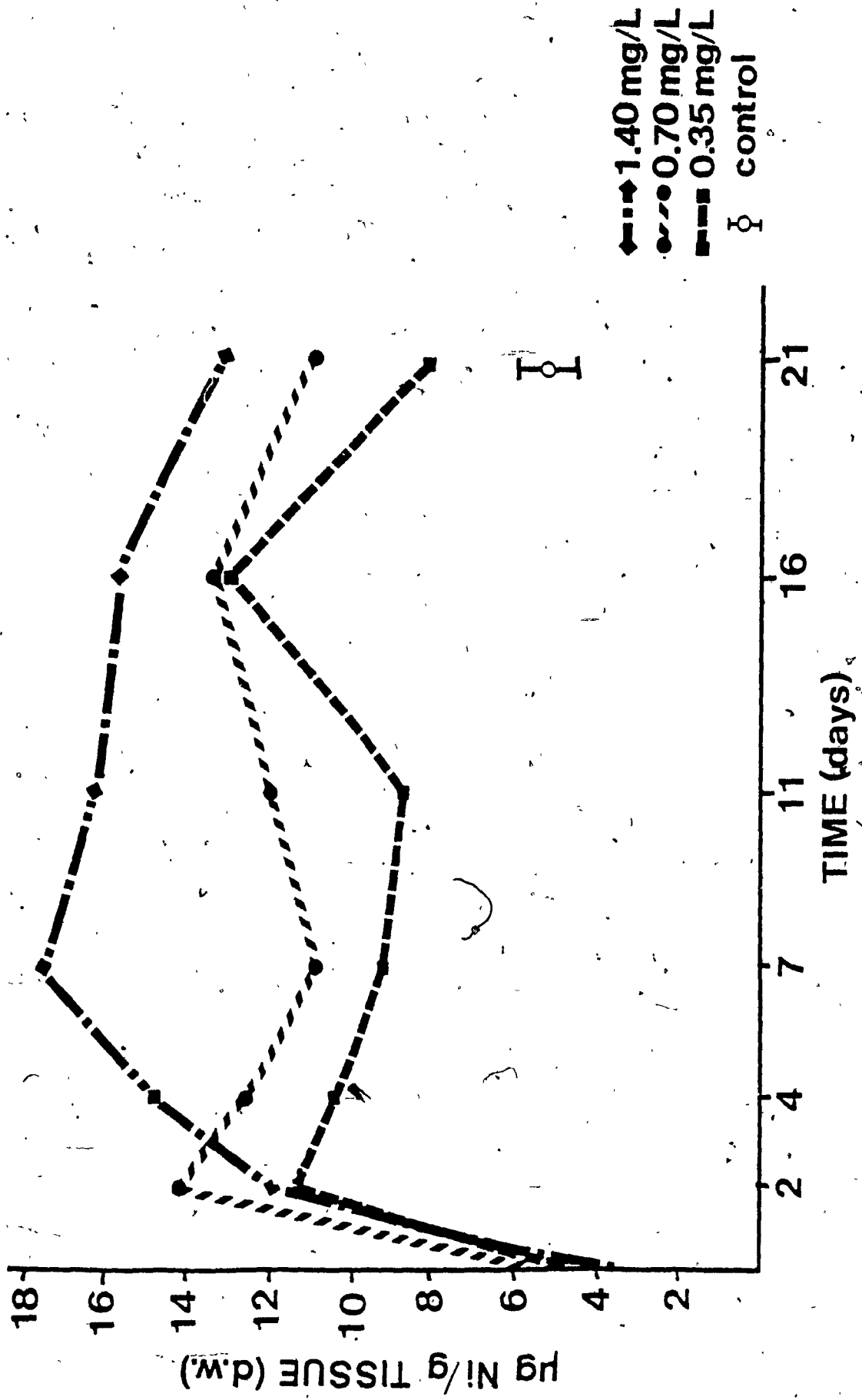


Table 4. Whole body burden of nickel in zebrafish over a 21 d exposure period to three concentrations of waterborne nickel.

Sampling Time (d)	Exposure Concentration (mg Ni/L)	Body Burden (ug Ni/g, d.w)	Std Dev	N
0	0.00	3.50	1.09	4
	0.00	3.90	0.95	5
	0.00	5.60	1.68	6
	0.00	4.54	1.54	6
2	0.00	8.02	3.92	5
	0.35	11.20 a	4.62	5
	0.70	14.29 b	4.50	5
	1.40	12.00 c	5.02	6
4	0.00	7.52	2.75	5
	0.35	10.46 a	2.80	6
	0.70	12.50 b	2.03	5
	1.40	14.61 c	3.21	5
7	0.00	4.99	2.56	6
	0.35	9.21 a	1.48	5
	0.70	10.91 b	2.80	6
	1.40	17.50 c	2.10	6
11	0.00	3.28	1.19	6
	0.35	8.93 a	2.31	6
	0.70	12.05 b	3.71	6
	1.40	16.25 c	2.52	6
16	0.00	4.86	1.78	6
	0.35	13.09 a	2.37	5
	0.70	13.56 b	3.39	6
	1.40	15.74 c	2.87	6
21	0.00	5.54	1.56	8
	0.35	9.10 a	3.79	5
	0.70	11.09 b	2.74	6
	1.40	13.06 c	4.64	7

a,b,c = not significantly different from similarly marked samples within a given exposure concentration ($P > 0.05$).

Ni/L) was found to be significantly different from all other exposure groups ($P < .05$). Test lots exposed to the lower concentrations, 0.35 and 0.70 mg Ni/L, were not significantly different from each other, but were significantly different from the control ($P < .05$) (Figure 8).

Fish also showed an apparent ability to concentrate nickel to levels greater than those found in water. Fish exposed to nickel at 0.35 mg Ni/L acquired tissue levels 15 times the ambient water concentration, while at 0.70 and 1.40 mg Ni/L, tissue levels were 10 and 7 times greater, respectively.

3.2.2. Evisceration study

The levels of nickel which accumulated in the combined viscera and gill tissue were significantly higher than those found in the eviscerated carcass, as indicated in Table 5. Nickel levels in the eviscerated carcass did not differ significantly between fish exposed to diluent water alone or 1.40 mg Ni/L for a 7 d period of time (Table 5). Viscera-gill nickel levels in toxicant-exposed fish increased significantly by a factor of 3.9, or approximately 400% to 18.00 ug Ni/g when compared to control viscera-gill samples. In the 7 d toxicant-exposed test lot, nickel levels in the viscera-gill were 2.6 times higher than those in the eviscerated carcasses. In the control test lot, viscera-gill concentrations were not significantly different from the eviscerated carcass (Table 5). Thus the distribution of nickel under control conditions does not appear to favour either of the two compartments, whereas when fish are exposed to slightly elevated levels, the viscera-gill compartment loads significantly more of the toxicant than the remaining carcass.

Figure 8. Mean whole body burden of nickel in zebrafish established after 2 d and maintained over the following 19 d of exposure to different concentrations of waterborne nickel (Mean \pm 2 S.E., N = 30-34).

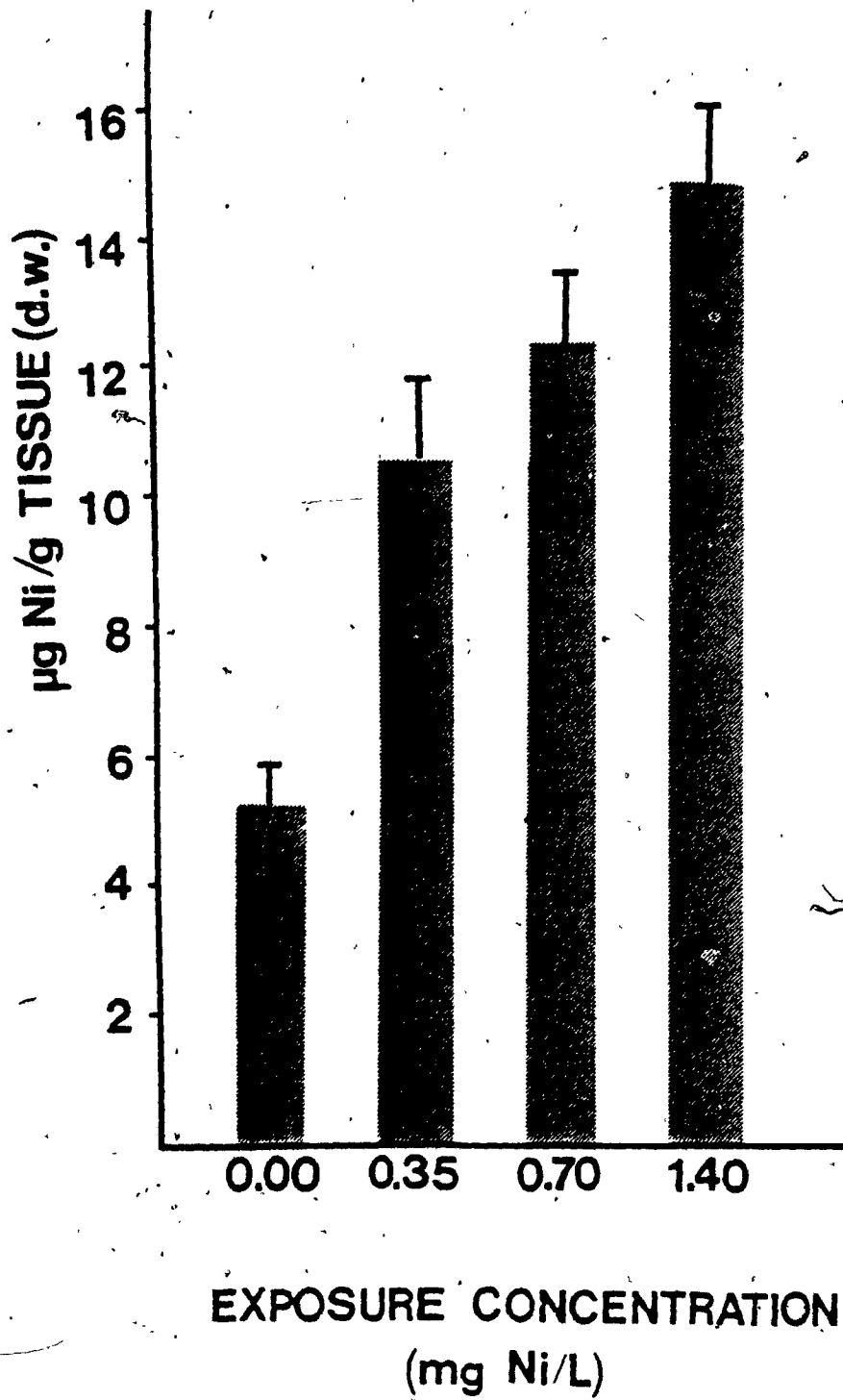


Table 5. Mean nickel content in viscera and gill versus the eviscerated carcass of zebrafish following a 7 d exposure to 1.40 mg Ni/L or diluent water ($\mu\text{g Ni/g}$ tissue, dry weight).

<u>Tissue</u>	<u>Treatment</u> (mg Ni/L)	<u>N</u>	<u>Tissue Concentration</u> ($\mu\text{g Ni/g}$)	<u>Standard Deviation</u>
Eviscerated Carcass	0.00	6	4.48	1.32
Eviscerated Carcass	1.40	4	6.85	1.90
Viscera-Gill	0.00	5	4.61	1.30
Viscera-Gill	1.40	4	18.00 a b	2.97

a = significantly different from control treatment ($P < 0.01$)

b = significantly different from carcass samples ($P < 0.01$)

3.2.3. Nickel depuration

The depuration pattern of nickel from zebrafish exposed to 1.40 mg/L for a 7 d period is illustrated in Figure 9 and the accompanying data are given in Table 6.

Nickel was rapidly eliminated from toxicant-exposed test lots and accumulated levels were not significantly different from control test lots following a 48 h period in clean water (Figure 9, Table 6) ($P > .05$). However, an ANOVA test revealed a significant difference between the replicate toxicant-exposed test lots ($P < .01$). This discrepancy appears to be a consequence of a difference in the initial body burdens, in that during the time period of depuration, mean tissue nickel levels between test lots remained proportional to each other and appear to decrease at a similar rate (Table 6). The pattern of displacement was consistent throughout the depuration period, except at the 10 h sampling interval, where the respective values appear to be reversed (Table 6, Figure 9). Due to these differences, nickel concentrations in the toxicant-exposed test lots are given separately in Table 6 and in Figure 9. The two test lots are labelled as A and B. Test lot A accumulated levels of nickel during the uptake phase equivalent to those reported in Section 3.2.1., whereas test lot B accumulated lower levels, and therefore appears to be an exception. The two control test lots were not significantly different over the course of the experiment ($P > .01$), and yielded a mean value over the 48 h experimental period of 3.62 ug Ni/g tissue (+/- 1.15).

3.2.4. Two compartment model

The depuration data were subsequently analyzed using a two compartment model. Although significantly different, the data of the two replicates were pooled in order to increase the sample size, in the hope that a population estimate of the rates of exchange

Figure 9. Elimination of nickel from zebrafish over a 48 h exposure period to clean water, following a 7 d exposure to 1.40 mg Ni/L. Background levels in control fish are shown (mean \pm 2 S.E., N = 33).

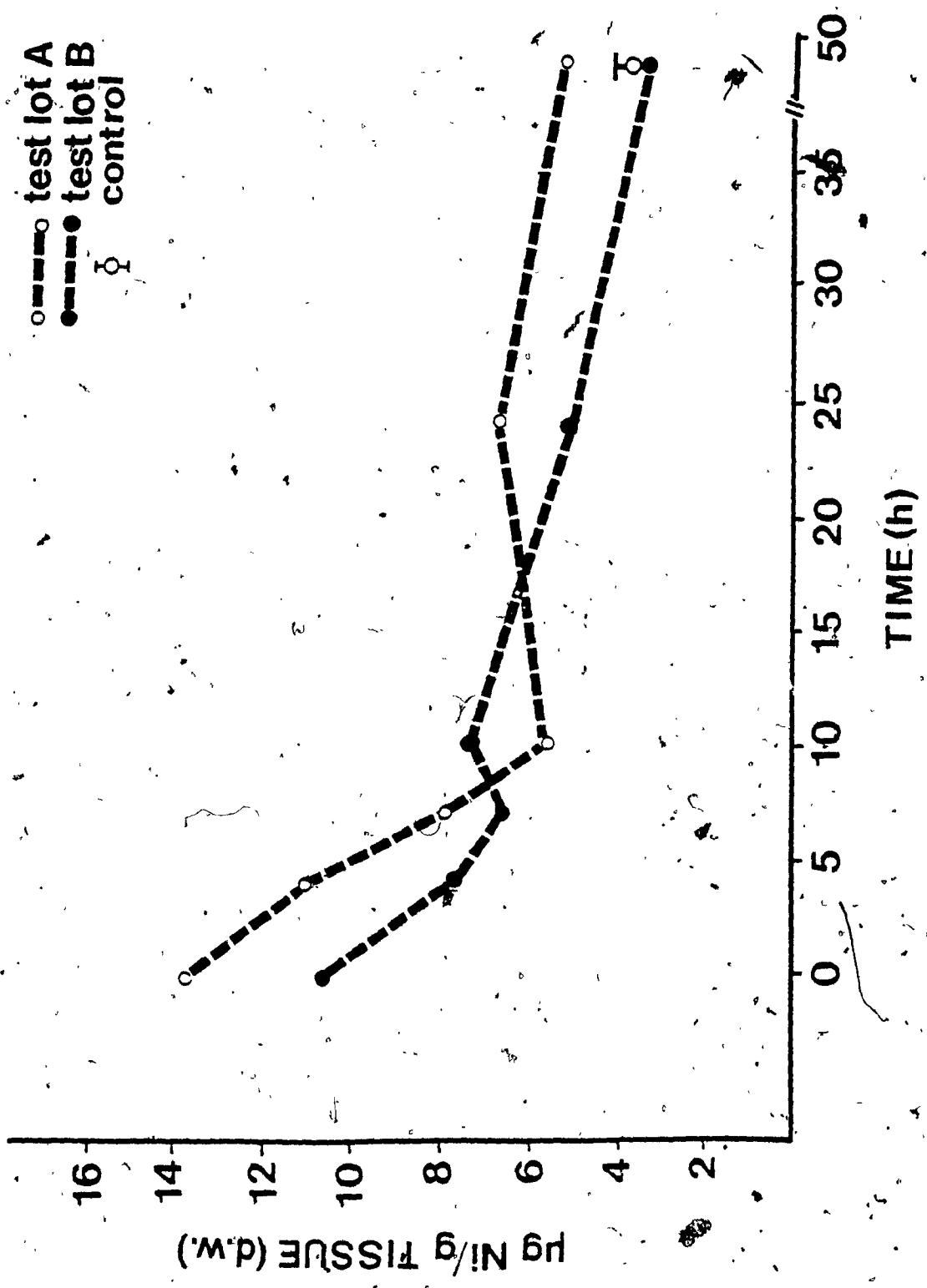


Table 6. Whole body nickel depuration in the zebrafish following a 7 d exposure to 1.40 mg Ni/L or diluent water alone (ug Ni/g tissue, dry weight).

<u>Depuration Time</u> (h)	<u>Control</u> <u>Body Burden</u> ug Ni/g (S.D.)		<u>Experimental</u> <u>Body Burden</u> ug Ni/g (S.D.)	
			A	B
Replicate Test Lots				
0	2.65 b (0.16)	4.87 b (2.91)	13.96 a (4.45)	10.62 a (2.73)
4	3.59 (0.53)	3.69 (0.04)	11.17 a (3.52)	7.68 a (0.76)
7	3.72 (1.39)	4.65 (2.01)	7.89 a (0.17)	6.77 a (1.01)
10	2.28 (0.34)	2.97 (1.29)	5.70 a (0.66)	7.26 a (1.13)
24	3.70 (0.70)	3.71 (0.30)	6.63 a b (0.08)	5.00 a (1.19)
48	4.23 (0.17)	5.09 (0.34)	5.20 (1.53)	3.44 (0.33)

a = significantly different from controls (P < .05)

b = mean of 2 samples only, all other values are a mean of 3.

and elimination of nickel from fish could be obtained. This baseline set of values, although limited, could be elaborated on with future studies specifically designed to address the kinetic model.

To analyse the data for this purpose, the background nickel level of 3.62 $\mu\text{g/g}$ was initially subtracted from the raw data. A linear regression analysis was conducted on the adjusted data representing the 'slow' portion of the curve (time 10 - 48 h, Figure 10). The resultant equation was extrapolated to time 0 to obtain the Y-intercept (D), and subsequently used to calculate the corresponding 'fast' portion of the curve by employing the method of residuals or feathering (Klaassen, 1980). In feathering the extrapolated data points of the slow equation are subtracted from the actual collected data points (time 0, 4, and 7 h, Figure 10), and the difference is plotted to obtain the line representing the fast portion of the curve. A linear regression analysis is then performed on these data to yield an equation with slope c and Y-intercept C . The resultant linear regression equations, as well as the adjusted pooled data, can be found in Table 7.

The pooled data yielded linear regression equations which were statistically acceptable (Table 7). The Y-intercepts and slopes of each equation were subsequently used to calculate the rate of transfer of the toxicant from the fast compartment to the slow compartment, and vice versa, as well as the rate of elimination (K_{10}), which is assumed to occur only from the fast compartment (Figure 5) (Klaassen, 1980; Spacie and Hamelink, 1986).

Figure 10. The two compartment model representation of the depuration of nickel from zebrafish following a 7 d exposure to 1.40 mg Ni/L (pooled data from replicates A and B).

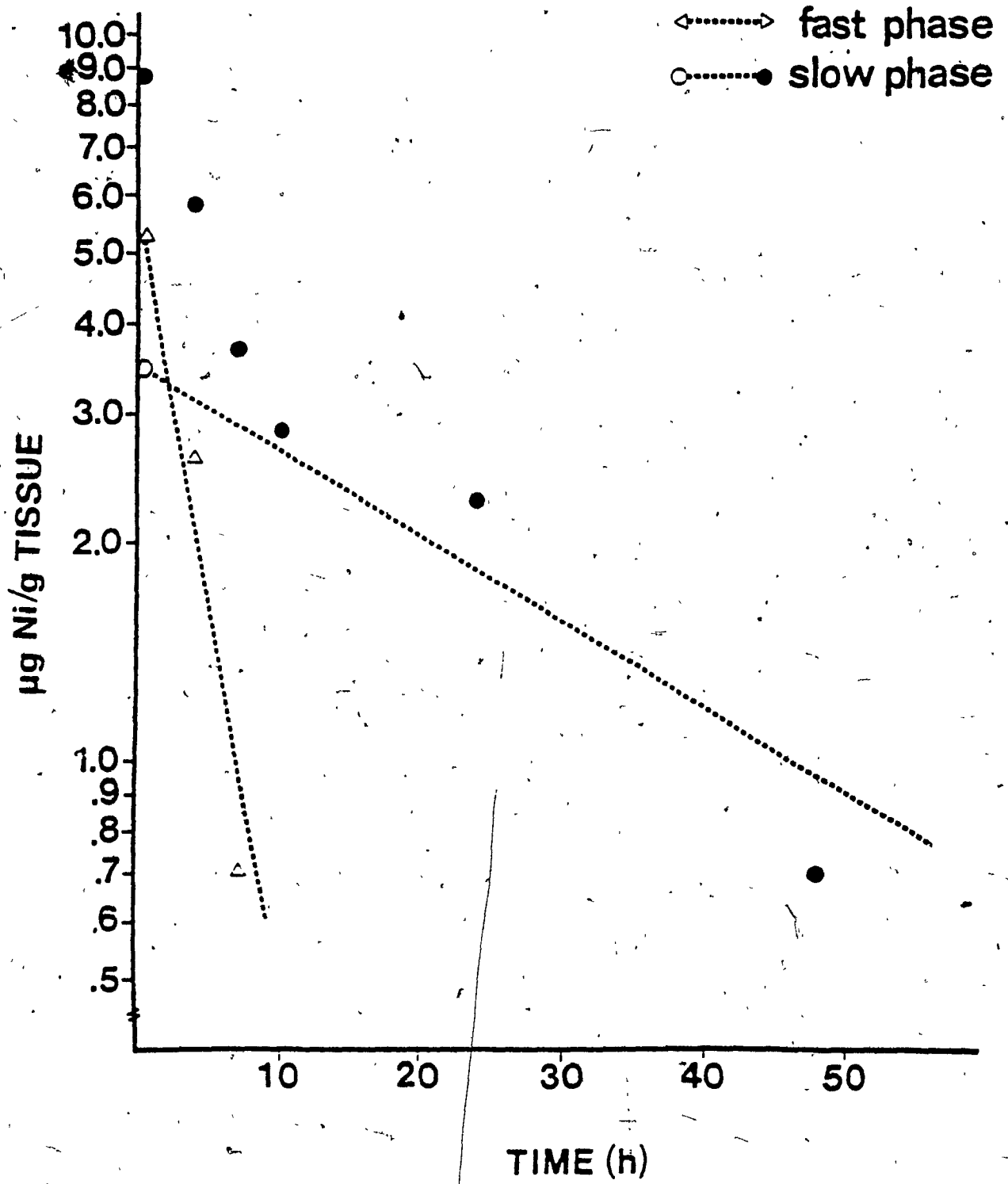


Table 7. Two compartment model equations and rate constants representing the depuration of nickel from the zebrafish.

Pooled data less the background level (3.62 ug Ni/g)

Time (h):	0	4	7	10	24	48
ug Ni/g tissue	8.67	5.75	3.71	2.85	2.08	0.70

Fast compartment (Compartment 1)

Linear regression equation	$Y = -.64 X + 5.17$ ($P > .05$)
Slope (c)	-.64
Y-intercept (C)	5.17

Slow compartment (Compartment 2)

Linear regression equation	$Y = -.06 X + 3.41$ ($P > .05$)
Slope (d)	-.06
Y-intercept (D)	3.41

Rate constants

k_{21}	0.30 ug Ni/g h
k_{10}	0.13 ug Ni/g h
k_{12}	0.27 ug Ni/g h

The rate of transfer from the slow compartment to the fast compartment was found to equate to 0.30 ug Ni/g h, as calculated by the equation:

$$k_{21} = Cd + Dc / C + D \quad (1)$$

where C = the Y-intercept of the fast compartment equation.

c = the slope of the fast compartment equation

D = the Y-intercept of the slow compartment equation

d = the slope of the slow compartment equation

The elimination rate constant, k_{10} , was subsequently calculated using the equation:

$$k_{10} = cd / k_{21} \quad (2)$$

This equation uses the slope values (c and d) as defined for equation 1, as well as the value of k_{21} calculated in equation 1. The elimination rate constant was found to equal 0.13 ug Ni/g h (Table 7).

The transfer of nickel from the fast compartment (1) to the slow compartment (2) was subsequently calculated by employing equation 3:

$$k_{12} = c + d - k_{21} - k_{10} \quad (3)$$

The k_{12} value was determined to be 0.27 ug Ni/g h (Table 7). Since this value is less than the calculated transfer value of k_{21} , the data is said to inadequately fit the model due to the lack of a significant slow or peripheral compartment.

3.2.5. Copper uptake study

Copper accumulation levels in zebrafish exposed to 1.40 mg Ni/L or diluent water for a 7 d period prior to lethal copper exposure are given in Table 8. Statistical analyses of the data revealed that there was no significant difference in any of the replicate tanks ($P > .01$), so the data were therefore pooled. Initial levels of copper in fish which had been exposed for a 7 d period to 1.40 mg Ni/L did not differ significantly from control fish held in diluent water (5.69 vs. 6.36 ug Cu/g; Table 8). Both nickel pre-exposed fish and diluent water-exposed fish accumulated copper following a 48 h exposure period to 220 ug/L Cu. These levels were not significantly different from one another but were significantly different from the initial copper levels (Table 8).

Table 8. Whole body burden of copper in zebrafish previously exposed to 1.40 or 0.00 mg Ni/L for a 7 d period, prior to and following a 48 h exposure to 220 ug Cu/L (ug Cu/g tissue, dry weight).

<u>Sampling Time</u> (h)	<u>Treatment</u> (mg Ni/L)	<u>N</u>	<u>Tissue Concentration</u> (ug Cu/g)	<u>Standard Deviation</u>
0	0.0	5	5.69	0.87
0	1.4	6	6.36	0.60
48	0.0	6	7.74 a	1.01
48	1.4	5	7.31 a	1.05

a \neq significantly different from time 0 samples ($P < 0.01$).

DISCUSSION

The results of the nickel pre-exposure study show that an extension of the pre-exposure period from 7 to 14 d led to a significant increase in sensitization of fish to lethal levels of copper (Figure 6, Table 3).

Since the present study was conducted under similar experimental conditions to those of Reddy's (1987), and the control responses to lethal levels of copper were virtually the same (Reddy: 261 ug Cu/L; current study: 260 - 265 ug Cu/L), direct comparisons of my 7 d pre-exposure results to those of Reddy (1987) can be made.

Reddy (1987) reported a significant decrease in tolerance to copper following a 7 d pre-exposure to nickel at 1.40 mg Ni/L. Since her work was not replicated, she could not definitively state whether this sensitization was due to nickel pre-exposure or to some chance event (Hurlbert, 1984). In the present study, this part of her work was repeated with replication. A sensitization to copper was found, but the shift in tolerance was only statistically significant at 72 h (Table 3). It is interesting to note that the relative increase in sensitization was greater in the current study (15% - 20%) than the 8% reported by Reddy (1987). The statistical discrepancies between these two studies may be attributable to wider confidence limits noted in the current study (Table 3). However, the trends resulting from the 7 d nickel pre-exposure within these two studies were consistent.

The significant increase in sensitization resulting from an extension of the pre-exposure period from 7 to 14 d confirms Reddy's (1987) initial observation that nickel pre-exposure at this level does lead to a decreased capacity of fish to tolerate copper (Figure 6, Table 3). This increase in sensitization also indicates that fish are not

acclimating to this level of nickel over the 14 d period tested, as there is no stabilization in the response to lethal levels of copper.

The mechanism of acute copper toxicity, which was recently attributed to the impairment of ion regulation at the gill (Lauren and McDonald, 1985), was not altered by pre-treatment with nickel. This is evident from the similar shape of the toxicity curves of the control and nickel pre-exposed test lots (Figure 6).

Apart from Reddy's (1987) preliminary work in this area, there are no other studies examining the effects of nickel pre-exposure on tolerance of fish to another metal (cross-tolerance). The sensitization results reported here stand apart from most of the cross-tolerance research currently published with reference to fish. For example, substantial increases in cadmium tolerance were reported in white suckers following pre-exposure to mercury (Duncan and Klaverkamp, 1983). Similarly, selenium pre-exposure has provided protection against subsequent mercury challenge in studies with goldfish (*Carassius auratus*) and northern creek chub (*Semotilus atromaculatus*) (Kim et al., 1979; Heisinger et al., 1979). Other authors have reported temporary increases in tolerance to the metals zinc (Dixon and Sprague, 1981b), cadmium and nickel (Reddy, 1987) following pre-treatment with copper, and cadmium following pre-exposure to selenium or zinc (Duncan and Klaverkamp, 1983). The contrast of my results to these and other studies will be discussed in the following sections, with reference to the factors influencing tolerance modifications and the possible mechanisms responsible for the sensitization seen following nickel pre-exposure.

4.1. Factors Influencing Tolerance Modifications

4.1.1. Duration of the pre-exposure period

The tolerance modifications ensuing from pre-exposure to nickel were significantly affected by the length of the pre-exposure period (Figure 6, Table 3). This temporal relationship is somewhat unique in that previously reported studies have generally found that the maximum tolerance displacement occurs following a 7 d pre-exposure period. Further increases in the duration of pre-exposure to other metals have either had no effect on the maxima, or have reduced its value (Dixon and Sprague, 1981a, 1981b; Duncan and Klaverkamp, 1983; Bradley et al., 1985; Hobson and Birge, 1986; Orr et al., 1986). Nickel therefore differs in its tolerance-altering capacity from other metals, in that sensitization to copper continues to increase beyond a pre-exposure period of 7 d (Table 3, Figure 6).

4.1.2. Concentration of the toxicant employed in pre-exposure

The concentration of the toxicant employed during pre-exposure has been reported by several authors to be a critical variable modifying tolerance (Dixon and Sprague, 1981b, Reddy, 1987; Roesijadi and Fellingham, 1987). In a recent review of metal tolerance, Chapman (1985) suggested that the patterns of tolerance modification may vary in three distinct ways depending on the pre-exposure concentration. In demonstrating his theory, he normalized the pre-exposure concentrations of the toxicants to their respective incipient lethal level (i.e. in toxic units - T.U.). He then suggested that increased tolerance occurs through pre-exposure to metals between the lower and upper threshold levels of 0.2 and 0.7 T.U. At levels below 0.2 T.U., slight sensitization may occur, such as that reported by Dixon and Sprague (1981b) following

pre-exposure to 0.09 T.U. of copper. This is thought to be related to an inability of these levels to stimulate the mechanisms involved in tolerance induction. Lower threshold limits for tolerance induction were also reported by Reddy (1987) following pre-exposure to cadmium, copper or silver at T.U. concentrations ranging from 0.11 and 0.23. While no sensitization to these levels was noted, these concentrations were not able to induce significant increases in tolerance, unlike higher pre-exposure concentrations. The upper threshold limit of 0.7 T.U. reported by Chapman (1985) is thought to result from direct or latent toxic effects of the pre-exposure concentration, which are manifested by incidences of mortality. For example, symptoms of acute toxicity were noted in mussels (Mytilus edulis) during pre-exposure to 250 ug Zn/L (Roesijadi and Fellingham, 1987). This pre-exposure concentration sensitized the organisms to a subsequent mercury challenge, whereas mussels which were pre-exposed to only 50 ug Zn/L exhibited increased mercury tolerance.

It is difficult to compare the nickel-induced sensitization patterns reported here and by Reddy (1987) to tolerance modifications seen with other metals using Chapman's (1985) scheme, in that an ILL for nickel was not defined in either of these studies. Relative to the 144 h LC50, the pre-exposure concentration used in this study was 0.08, while those employed in Reddy's work were 0.03, 0.08 and 0.30. Although this latter exposure concentration is within the tolerance-inducing range suggested by Chapman (1985), Reddy (1987) reported greater sensitization with this pre-exposure concentration than that seen at the lower levels. These relative potency units for nickel may be significantly greater than currently suggested, should the true ILL of nickel prove to be below the 144 h LC50 of 18.2 mg Ni/L; as determined by Reddy (1987).

Nevertheless, it seems unlikely that the pre-exposure concentration used in the current study (0.08 T.U.) would be above Chapman's (1985) suggested upper threshold limit of 0.7 T.U., since there was no mortality during either pre-exposure or over the 21 d accumulation study run at the same exposure level.

In order to allow for comparisons, a new scheme of normalizing the data was developed. With this scheme, the respective water quality guideline levels (1987) for each metal were used as a reference point, rather than the ILL. Accordingly, the pre-exposure concentrations employed in any given study were normalized to the guideline limits for the given water quality conditions of each experiment. Comparisons of tolerance modification patterns relative to the normalized pre-exposure concentrations are given in Table 9.

This scheme separates nickel from other tolerance-modifying metals as a unique sensitizing agent, since the range of nickel pre-exposure concentrations explored by Reddy (1987) and myself is within that which causes an increase in tolerance with the metals aluminum, arsenic, copper and zinc (Table 9). The concentrations which represent the lower limit of tolerance induction appear to equate to 5 or 6 units, with sensitization (although not statistically significant) arising in one study at this level (Dixon and Sprague, 1981b). The upper tolerance limit is suggested by these data to be between 60 and 70 units in all metals except cadmium. Cadmium appears to induce tolerance at much higher concentrations relative to the guideline limit, but shows no evidence of sensitization.

Although zebrafish are the only species which have been examined with respect to nickel pre-exposure, the lack of a species effect following either cadmium or copper

Table 9: The effects of pre-exposure of fish to the metals aluminum, arsenic, copper, cadmium, nickel, and zinc after normalizing the pre-exposure concentration to the respective recommended guideline concentrations for each metal.

<u>Metal</u>	<u>Recommended Guideline Level (mg/L)</u>	<u>Fish Species</u>	<u>Range of Pre-exposure Levels (mg/L)</u>	<u>Normalized Values</u>	<u>Range of Effect of Exposure</u>
Al	0.005	rainbow	.085-.157	17 - 31	T 1/
As	0.05	rainbow	3.00	60	T 2/
Cd	0.0002	Wt. sucker	.22 -.71	1100 - 3550	NE to T 3/
Cu	0.0013	zebrafish	.20 - 2.0	154 - 1538	NE to T 4/
	0.003	zebrafish	.016-.076	5 - 25	NE to T 4/
	0.003	zebrafish	.029-.052	10 - 17	NE to T 4/
	0.006	rainbow	.030-.194	5 - 32	S to NE to T 5/
Ni	0.065	zebrafish	1.40	22	S (Cu) 6/
	0.110	zebrafish	.70 - 5.0	6 - 45	NE to S 4/
Zn	0.110	zebrafish	.50 - 6.0	5 - 55	NE to S (Cu) 4/
	0.03	rainbow	2.09	70	T 7/
	0.03	fatheads	.60 - 1.80	80 - 60	T to S 8/

NE= no effect compared to controls

T = increased tolerance to lethal metal presented

S = sensitization to lethal metal presented

1/ Orr et al., 1986; 2/ Dixon and Sprague, 1981a; 3/ Duncan and Klaverkamp, 1983;
4/ Reddy, 1987; 5/ Dixon and Sprague, 1981b; 6/ current study; 7/ Bradley et al., 1985
8/ Hobson and Birge, 1986.

pre-treatment suggests that the sensitization noted with nickel would hold true for other species (Table 9). This should be confirmed empirically with a native Canadian species, such as the rainbow trout.

It is therefore concluded that the sensitization resulting from nickel pre-exposure in the current study differs from the tolerance modifications seen with other metals examined within a similar relative concentration range and over similar lengths of time.

4.2. Possible Mechanisms of the Sensitization

4.2.1. A lack of metallothionein?

While the precise mechanisms involved in tolerance modifications are not currently known, investigators who have observed an increase in tolerance following pre-exposure have frequently attributed this to an induction of metallothionein (MTN) during the pre-exposure phase (Roch et al., 1982; Benson and Birge, 1985, 1987; Bradley et al., 1985). MTN's are metal-binding proteins, rich in cysteine and sulphhydryl groups, induced through previous exposure to the metals copper, cadmium, zinc and mercury (Cherian and Nordberg, 1983). These proteins function in maintaining homeostasis of essential metals (such as copper and zinc), and are thought to play a role in detoxifying certain non-essential metals (such as mercury and cadmium) by binding to them. When bound to MTN, metals are no longer available to bind high molecular weight proteins or sensitive tissue sites, thereby causing toxicity (Kagi et al., 1981; Webb and Cain, 1982). The binding of metals to MTN has been found to be somewhat non-specific. For example, Liber and Miya (1976) reported that pre-treatment of mice with zinc resulted in the induction of MTN, which subsequently bound significant quantities of cadmium, thereby increasing cadmium tolerance. This

non-specificity is attributed to the ability of metals to compete for binding sites on the apoprotein or thionein, and, in some cases, displace ions that are already bound (Cherian and Nordberg, 1983; Bremner and Campbell, 1978; Liber and Miya, 1976).

This phenomenon has been documented in other mammalian studies (Bremner and Campbell, 1978; Webb, 1972), and may account for some of the instances of cross-tolerance noted in the work of Duncan and Klaverkamp (1983) and Reddy (1987).

Since increased copper tolerance was not found in this study, it may be possible to assume either that metallothionein is not induced through pre-exposure to nickel, or that it does not function efficiently enough to provide protection against subsequent metal exposure. No evidence of fish inducing these proteins through nickel pre-exposure could be found in the literature. Mammalian studies have revealed that MTN induction can occur through pretreatment with this metal (Sunderman and Fraser, 1983), but to a far lesser degree than that seen with other metals (Mathur and Tandon, 1979). Investigators have also determined that nickel binds very weakly to thionein, and other sulphhydryl-rich compounds in mammals (Suzuki and Yoshikawa, 1976; Sunderman and Fraser, 1983; Mathur and Tandon, 1976). With this weak binding capability and low induction potential, the ability of nickel-induced MTN to protect against subsequent metal exposure would be greatly reduced when compared to other inducers of this protein. This observation is supported not only by the results of the current study and Reddy's (1987) work, but also by Yoshikawa (1970), who found that mice pretreated with nickel did not exhibit increased tolerance to a subsequent nickel challenge.

4.2.2. Metal accumulation and its possible role in nickel sensitization

4.2.2.1. Nickel accumulation

The results of the nickel accumulation study indicate that whole body levels of this metal do not reflect changes in copper tolerance. This study clearly demonstrated that steady state conditions are rapidly attained in fish exposed to low levels of nickel (Table 4, Figure 7). Although accumulation of this metal in whole fish and individual organs has been examined extensively in both the field and laboratory (Hutchinson et al., 1975; McConnell and Stokes, 1985; Van Hoof and Nauwelaers, 1984; Bradley and Morris, 1986), these studies have involved only static tissue measurements taken at one point in time, and therefore do not reveal the rapidity with which a plateau is attained. While no report of whole body accumulation patterns of this metal in fish could be found in the literature, apparent steady state conditions within 2 d were also reported by Bureau (1988) in rainbow trout gill, liver and kidney exposed to nickel at similar concentrations. The rapidity with which steady state was attained in the current study is similar to that found with copper (Dixon, 1980) within whole fish, but differs from patterns noted with selenium (Bertram and Brooks, 1986). These discrepancies may be a function of the bioavailabilities of the metal ions, or of the process by which they are accumulated (Spacie and Hamelink, 1986).

The lack of a significant dose-response relationship between the 0.35 mg Ni/L and the 0.70 mg Ni/L exposure test lots (Figure 8) suggests that increasing the ambient concentration did not elicit a significant increase in accumulation within this concentration range.

This observation may be a consequence of the use of whole fish analysis, as opposed to individual organ analysis in these kinetic studies. Nickel was found to accumulate in the soft organs and gill of zebrafish at levels 2.6 times greater than those found in the eviscerated carcass (Table 5). These results are supported by previous studies indicating that muscle tissue, which is the main component of the eviscerated carcass, is a minor contributor to nickel whole body burdens in exposed fish (Van Hoof and Nauwelaers, 1984; Dallinger and Kautzey, 1985). The slow-metal-accumulating tissues, such as the skin and muscle, comprise 57% of the zebrafish organo-somatic index, while the accumulating tissues (gill, kidney, liver, gonads and alimentary canal) make up only 18% (Karlsson-Norrgren and Runn, 1985). By incorporating the whole fish in my analyses, the concentrations of nickel in soft tissues would have been diluted by the presence of the musculature, thereby reducing the chance of detecting subtle differences in accumulation between exposure groups.

While this suggestion is possible, it is more likely that the lack of a significant dose-response relationship between these two exposure lots is due to a decreased uptake or increased elimination of nickel over the concentration range examined. The histogram in Figure 8 supports this suggestion, in that the steady state accumulation levels do not lend themselves to first order kinetic principles (Spacie and Hamelink, 1986). A lack of a significant increase in tissue metal levels with increased exposure concentration has been seen with other metals. For example, Spehar (1976) found that flagfish (*Jordanella floridae*) exposed to a range of either cadmium or zinc concentrations exhibited an increase in tissue concentrations which was not always dose-related. Cadmium tissue levels eventually reached a plateau, whereas zinc tissue

concentrations gradually increased with increased ambient zinc levels, such as that found in the current study.

The lack of a significant dose-response relationship between the 0.35 and 0.70 mg Ni/L exposed test lots could also be a consequence of increased nickel elimination with increased exposure concentration. Gill chloride cells are thought to aid in elimination of heavy metals, as well as their assumed role in ion regulation (Evans, 1987). In recent studies, chloride cell density and activity was positively correlated to exposure concentration in sticklebacks (Gasterosteus aculeatus) exposed to a range of either zinc (Matthiesson and Brafield, 1973) or cadmium (Oronsaye and Brafield, 1984) concentrations. Oronsaye and Brafield (1984) reported that while low level cadmium exposure caused a net increase in chloride cell numbers over time, higher exposure concentrations resulted in a peak which eventually declined. Although somewhat speculative, this could play a role in the lack of significant difference between the 0.35 and 0.70 mg Ni/L test lots, and the significant accumulation at 1.40 mg Ni/L.

Although the processes by which nickel is accumulated in fish have not yet been clarified, the lack of a significant dose-response relationship noted in Figure 8 could be due to the presence of an active transport system. Evidence supporting this theory is provided by the fact that fish in the current study were found to accumulate nickel against a concentration gradient, thereby leading to tissue levels between 7 and 15 times the ambient concentration. Hutchinson et al. (1975) also reported low nickel bioconcentration factors in fish, relative to those found in plant and invertebrate species in the nickel-contaminated Sudbury, Ontario region. These authors suggested that fish may have a selective uptake and elimination mechanism for this metal.

The steady state levels of nickel (Figure 7) therefore fail to explain the increased sensitization found to occur between the exposure periods of 7 and 14 d (Figure 6). Although steady state conditions have been found to reflect changes in tolerance with other metals, such as copper (Dixon, 1980) and zinc (Bradley et al., 1985), nickel does not appear to behave in the same fashion.

This accumulation study did not address the possibility that a change in nickel compartmentalization could have occurred during pre-exposure. Bureau (1988) did examine nickel's compartmentalization in several organs and found steady state concentrations were attained in all tissues by 2 d. Unfortunately, this study was limited to 60 h, and therefore does not indicate whether the relative distribution of nickel within the organs changed over a longer period of exposure, such as that employed in the current study.

4.2.2.2. Copper accumulation

Alterations in metal accumulation as a result of pre-exposure have been found to occur in several studies. For example, Bradley et al. (1985) and Kasprzak et al. (1986) found that pre-treatment with zinc or magnesium acetate mitigated the toxic effects of subsequent challenges with zinc or nickel by decreasing their uptake at selected organ sites. In the current study, nickel pre-exposure for a 7 d period was found to have no effect on subsequent copper accumulation (Table 8). Although these findings statistically support the lack of significant difference in copper tolerance noted between the non-pre-exposed control group and the 7 d pre-exposed group (Table 3), a slight increase in copper levels was anticipated due to the apparent sensitization noted in Figure 6. Nickel pre-exposure could have resulted in a surge of copper uptake early

on, which would not have been detected. Initial enhancements in metal uptake have been noted previously in the gills of copper-exposed rainbow trout (Matos, 1987), and in sticklebacks exposed to zinc (Matthiessen and Brafield, 1977). The copper concentrations reported here (Table 8) could also reflect levels in the resistant members of the test population, as mortality was incurred prior to sampling. Although the sensitization seen with pre-exposure to nickel cannot be attributed at this time to an increased uptake of copper, a change in the compartmentalization of copper during pre-exposure may have played a role. Rainbow trout exposed to nickel have been reported to experience a decrease in gill copper levels and an increase in liver copper levels, even though the fish were not exposed to copper (McConnell and Stokes, 1985). Similar findings have been reported in rats pre-treated with nickel, in that pre-treatment led to an increase in spleen copper levels (Chmielnicka et al., 1982). A shift in copper compartmentalization during pre-exposure could result in an increase in copper concentrations at critical target sites. This could augment the effective dose of copper, thereby leading to an enhancement of potency such as that noted in Figure 6. In light of the findings of McConnell and Stokes (1985), an investigation of the compartmentalization of these two contaminants relative to the issue of sensitization following pre-exposure would be interesting to examine in future studies.

4.2.3. Metal elimination and its possible role in nickel sensitization

In contrast to my hypothesis, nickel was not found to persist within the body of fish for an extended period of time, as control nickel levels were attained within a 48 h depuration period (Figure 9, Table 6). The elimination pattern suggests a fast phase dominating for the first 10 h, followed by slow phase lasting 38 h (Figure 9). A

biphasic elimination pattern of nickel has been noted in other organisms, such as rats (Kalliomaki et al., 1984) and zooplankton (Hall, 1982). The fitting of the data to a two compartment model was found to be unsuccessful (Table 7; Figure 5), which may be a result of the paucity of data points collected, compared to the volume normally required to accurately assess the slopes of each of the two phases (Spacie and Hamelink, 1986). The exponential constants (or slopes) derived from the model can be estimated to be close to 0.65 h^{-1} (fast compartment) and 0.06 h^{-1} (slow compartment) (Table 7). Although these constants cannot be used in direct comparisons, it is interesting to note that they are similar to those reported for nickel in mammalian studies (Kalliomaki et al., 1984).

The bulk of accumulated nickel within the body of the zebrafish was found to be eliminated within the first 10 h following exposure (Figure 9). Although specific elimination routes were not examined in the present study, this rapid release of nickel probably originates from the highly vascularized areas of the gill and kidney (Spacie and Hamelink, 1986; Klaassen, 1980). As mentioned previously, the gills are thought to play a role in metal elimination through the activity of the chloride cells (Matthiessen and Brafield, 1973; Oronsaye and Brafield, 1984), whereas the kidney is the primary organ by which nickel is eliminated in mammals (Sakar, 1984).

The nickel being eliminated during the ~~the~~ sampling intervals may be more tightly bound to either plasma components or critical tissue sites (Gillette, 1972). In mammals, nickel is known to bind effectively to albumin (Sakar, 1984), and has been found to compete with copper for albumin binding sites (Sunderman, 1977). Although this same phenomenon has yet to be confirmed in fish, the availability of these binding

sites would be decreased through metal pre-exposure. Copper ions would therefore be left to bind critical tissue sites, resulting in an increased mortality of pre-exposed fish. The effect would be dependent on the rapidity of the release of nickel from the albumin sites following an arrest of nickel exposure.

The results of the depuration study lead to the conclusion that, following a 7 d exposure to nickel, the bulk of this contaminant is quickly eliminated from the body when fish are allowed to depurate in clean water (Figure 9, Table 6). The possibility of these two toxicants interacting kinetically still exists, as some nickel was found to linger within the body from 10 to 48 h post-exposure. A pilot study conducted to examine the influence of copper on the depuration pattern of nickel suggested that the pattern may be altered in the presence of copper. Unfortunately, the results of this study were inconclusive, and showed a wide variability in response (see Appendix 1). It would be interesting to examine this further, as there is a suggestion in these data of a retention of nickel within the body in the presence of high levels of copper. If the depuration pattern of nickel were altered, the combined presence of the two toxicants could contribute to the sensitization pattern seen with nickel pre-exposure, in that the toxicity of nickel and copper, when presented in mixtures, has been found to be synergistic (Weinstein, 1979).

4.3. Alternative Hypotheses for Sensitization

4.3.1. Sublethal toxicity

Since the enhanced sensitization seen with increased pre-exposure to nickel is apparently unexplained by nickel's accumulation and elimination patterns, sublethal damage may be resulting from pre-exposure to this metal.

Sublethal effects of nickel pre-exposure could involve kinetic changes in the compartmentalization of nickel or copper, as mentioned previously, or could be related to a reduced ability of key target organs, such as the gills, to function due to structural damage arising from nickel pre-exposure. The gills are the main site of toxicant accumulation in fish (Evans, 1987). Nickel has been found to affect the ability of rainbow trout gills to transfer oxygen from the water to the blood at concentrations as low as 2.0 mg Ni/L, due to a thickening of the secondary lamellae (Hughes et al., 1979). Full recovery of the oxygen diffusing capacity took 19 d following a 3.5 d exposure to this concentration of nickel. One could speculate that gill damage incurred from nickel pre-exposure, although not resulting in lethality, could significantly augment the effects of lethal copper exposure, in that copper's acute mode of action has been attributed to impairment of gill ion regulation (Lauren and McDonald, 1985).

Since some behavioral aberrations were noted during the last days of the nickel exposure periods in the current study, the possibility of sublethal damage can be confirmed. It is interesting to note that the concentration selected for this study is below the level at which Weinstein (1979) reported seeing decreased egg viability and other reproductive effects in zebrafish (1.80 mg Ni/L). While adverse effects on reproduction are usually a sensitive endpoint for assessing sublethal toxicity, nickel may be affecting other systems at the exposure concentration used in this study.

4.3.2. Latent toxicity

The sensitization arising from nickel pre-exposure may be related to the longer time period nickel requires to express its acute toxicity, relative to other metals. Latent toxic effects of pre-exposure have been suggested by Chapman (1985) as a potential

reason for sensitization. As previously mentioned, Reddy (1987) did not obtain an ILL for nickel during her 144 h acute bioassay tests. The lack of an ILL for nickel seen in her work is not an isolated occurrence. Several publications examining acute toxicity of this metal to fish, from exposure periods of 96 h to 10 d, did not obtain an incipient level (Hale, 1977; Nebeker et al., 1985; Anderson et al., 1979; Pickering, 1974; Pickering and Henderson, 1966; Blaylock and Frank, 1979). Two metals which are considered highly toxic, mercury (Hewitt, 1980) and silver (Reddy, 1987), have also been found to lack an ILL within the standard 4 - 6 d bioassay period. An ILL for nickel was reported in rainbow trout at 20 d of a 35 d exposure period (British Department of the Environment, 1971). These findings, combined with the steady state conditions found following the 21 d accumulation study (Table 4, Figure 7) indicate that nickel will reach an ILL, but that it takes a longer period of time than most metals to do so. If the enhanced sensitization seen with pre-exposure to nickel is related to latent toxicity, then nickel's status as a moderate toxicant (NRCC, 1981) would merit further examination.

4.4. Environmental Implications of Nickel Sensitization

The increased sensitization resulting from previous exposure to low levels of nickel seen in both the current study and Reddy's (1987) work indicates that nickel may be a greater environmental risk than currently believed. The temporal relationship of increased sensitization with increased pre-exposure time (Figure 6) should be viewed as a considerable hazard. If this pattern, upon further examination, is found to continue over longer periods of time and occur at lower nickel concentrations, then the currently enforced 'safe' levels of this metal will probably be put in question.

The synergistic toxicity associated with nickel and copper when they are presented in mixtures has been established (Weinstein, 1979). This study has confirmed that sequential exposures of nickel followed by copper can also greatly enhance toxicity, which could have devastating effects on fish populations inhabiting areas receiving fluctuating concentrations of these two contaminants.

The 'biological costs' to fish of developing and maintaining a tolerance modification mechanism have yet to be fully evaluated, but are known to include decreased growth and poor recruitment (Dixon and Sprague, 1981b; Duncan and Klaverkamp, 1983). The cost of being unable to induce mechanisms such as these is obviously much greater, in that it leads to immediate decreased survival of organisms, which could greatly reduce species diversity in natural ecosystems and decimate susceptible populations encountering toxic effluents or contaminated spring run-off.

SUMMARY AND RECOMMENDATIONS FOR FUTURE STUDIES

The findings of this study have shown that nickel pre-exposure at 1.40 mg/L for 7 d results in an apparent sensitization of fish to lethal levels of copper, which is significantly increased by extending the pre-exposure period to 14 d. This sensitization could not be attributed to either a continual accumulation of nickel within the whole body, or increased copper titres due to pre-exposure to this compound. Although nickel is rapidly eliminated from fish in an uncontaminated environment, there is some suggestion that the pattern may differ in the presence of copper, which could result in interaction between these two metals. Nickel appears to differ from certain other metals by not having a capacity to induce increased tolerance over a similar range of pre-exposure concentrations, and by exhibiting an increase in sensitization following an elongation of the pre-exposure period. Although enhanced tolerance to nickel has been reported in bacteria (Kasan and Stegmann, 1987) and algae (Stokes et al., 1973), to my knowledge it has not yet been found to occur in vertebrates.

Future studies examining the temporal relationship found to exist between the pre-exposure period and increased sensitization should be conducted to see if this pattern continues to increase over extended periods of time. An examination of the incipient lethal level of nickel, as well as the effect of lower pre-exposure concentrations on the lethal tolerances of fish to metals, would be beneficial in elaborating on the unique patterns found in this study and the work of Reddy (1987).

A compartmentalization study of nickel and copper could provide valuable information on the possible mechanisms involved in nickel-induced metal sensitization.

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APPENDIX

Raw data set for a pilot study conducted to examine nickel depuration in the presence of copper. Test lots of fish were held for a 7 d period in either 1.40 mg Ni/L or diluent water alone. Fish were then transferred to exposure tanks containing 220 ug Cu/L and held under these conditions for a 48 h period. During this time, samples were taken to examine the pattern of nickel depuration. Sampling occurred just prior to transferring the fish to copper (time 0), and 4, 7, 10, 24, and 48 h during copper exposure. Control nickel levels were only measured at the 48 h sampling interval. All values are ug Ni/g tissue, on a dry weight basis.

sampling time (h)	control replicate 1	control replicate 2	depuration replicate 1	depuration replicate 2
t = 0	--	1.97	10.70	10.55
	2.54	6.08	13.30	18.99
	2.76	--	7.85	12.33
t = 4			14.02	8.54
			11.99	15.03
			14.51	15.92
t = 7			2.53	9.34
			5.77	10.51
			7.77	9.43
t = 10			6.88	20.98
			6.70	19.22
			12.53	12.34
t = 24			15.50	11.58
			20.92	13.16
			12.65	13.54
t = 48	4.21	5.00	6.92	5.77
	4.25	4.73	--	8.94
	1.13	6.15	9.05	4.23

-- =lost sample