THE RELATIONSHIP BETWEEN WATER HARDNESS AND THE MODE OF MULTIPLE TOXICITY OF COPPER AND ZINC

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ABSTRACT

THE RELATIONSHIP BETWEEN WATER HARDNESS
AND THE MODE OF MULTIPLE TOXICITY
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The role of water hardness as a possible modifier of the acute lethal toxicity of copper, zinc, and their mixtures to the zebrafish (Brachydanio rerio) was investigated. Bioassays were performed at each of three water hardnesses: soft, moderately hard, and very hard water (means = 22.1, 127.9, and 306.2 mg/l as $CaCO_3$, respectively) under conditions of constant pH and alkalinity.

No chemical interactions between water hardness cations and copper and zinc, in aqueous solution, were observed as suggested by differential pulse polarographic analyses.

Empirical evidence in our studies suggested that physiological interactions involving hardness cations led to a lessening of the potency of each heavy metal and their mixtures but not to an alteration of respective modes of action.

Physiological responses, i.e. mucous production and hemorrhaging, to copper and/or zinc poisoning, through a hardness range did not appear to change in type but rather changed in severity.

Dose-response and time-to-mortality data supported the assumption that calcium and magnesium affect the potency of heavy

metals and their mixtures, and not their mode of action. The toxicity of copper in soft water (96-hr LC_{50} = 0.154 mg/1), moderately hard-"tap"(96-hr LC_{50} = 0.239 mg/1) and very hard water (96-hr LC_{50} = 0.672 mg/1) was greater than the toxicity of zinc (96-hr LC_{50}'s = 14.64, 17.68, and 25.30 mg/1, respectively) at each respective water hardness. The copper-zinc mixture exhibited a more-than-additive toxicity in the soft (96-hr LC_{50} = 0.133 mg/1), moderately hard (96-hr LC_{50} = 0.103 mg/1), and very hard water (96-hr LC_{50} = 0.366 mg/1) relative to the individual potencies of copper and zinc. The degree of enhanced toxicity, greater than that predicted according to the model of concentration-addition, increased with hardness. In absolute terms, the potency of copper-zinc mixtures decreases with hardness.

Results of a bioconcentration study (copper, zinc, calcium, magnesium, and sodium), in which fish were exposed for 48 hours to sublethal levels of copper and/or zinc, at each of the three water hardnesses, supported the rationale that water hardness, as a modifying factor, may decrease the potency of a toxicant. While calcium levels tended to increase in gill tissues, with hardness, copper and/or zinc levels were observed to decrease. The relative bioconcentration of copper and zinc was greater in both gill and body samples of fish exposed to their mixture than to pure solutions, respectively.

Possible mechanisms of calcium's and magnesium's interactions with heavy metal toxicity are proposed.

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INTRODUCTION

Water hardness[1] has been known for several decades to modify the toxicity of heavy metals[2] to freshwater teleosts (Doudoroff and Katz, 1953; McKee and Wolf, 1963; Skidmore, 1964). The main objective of this study is to examine the role of water hardness as a modifying factor of the toxicity of heavy metal mixtures. It would appear that no previous studies have examined this problem, possibly because experts have assumed that the hardness relationships established for single heavy metals apply directly to their mixtures.

Historical Summary

Much of the early work on the relationship between hardness and toxicity neither identified the separate elements comprising hardness nor distinguished between the effects of hardness and that of other closely related characteristics of water, such as alkalinity and pH (Inglis and Davis, 1972). These studies had little success in quantifying the relationship between hardness, alone and toxicity of heavy metals to fish. This failure has led to more sophisticated research projects which have attempted to identify

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[1]Water hardness is a characteristic of water represented by the total concentration of ions of the alkaline earths, primarily calcium and magnesium, expressed as ppm $CaCO_3$ (NAS, 1978).

[2]Heavy metals are defined as those metals with a density greater than 5 (Anderson and Weber, 1975a).

specifically the causal agents within hardness and their respective mechanisms of interactions with heavy metal toxicity.

Numerous studies of the alkaline earth metals[1], particularly calcium and magnesium, on cell and tissue function are found in the literature. Early classical experiments, such as those by Loeb and Osterhout (Osterhout, 1922) investigated the role of calcium, magnesium, and other alkaline earth metals on the permeability of tissues to sodium and potassium ions. In attempting to explain the observed decrease in membrane transport of alkali[2] ions, Loeb and Osterhout (Osterhout, 1922) formulated the "permeability theory". They proposed that an alkaline earth metal, such as calcium, antagonizes an alkali metal, such as sodium, by altering membrane porosity and, thereby reducing sodium influx into the tissues. This theory which has, for over 50 years, been widely accepted by the scientific community, was recently investigated by Meryman (1972). He carried out a comprehensive study of the potassium flux rates across artificial lecithin-cholesterol membranes in the presence of various cations of the alkaline earth and alkali metals. Results suggested that, of the cations added, calcium produced the greatest reduction in membrane permeability to potassium (in the order

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[1]The alkaline earth metals are elements of group 2a of the periodic classification. In order of increasing atomic number, they are beryllium, magnesium, calcium, strontium, barium, and radium (Van Nostrand, 1976).

[2]The alkali metals are elements of group 1a of the periodic classification. In order of increasing atomic number, they are hydrogen, lithium, sodium, potassium, rubidium, cesium, and francium. (Van Nostrand, 1976).

calcium > strontium > barium > magnesium > sodium, potassium, lithium > ammonium). Meryman (1972) inferred that divalent anionic sites which bind calcium, are located in or on these membranes. Calcium, when bound to these sites, reduces the hydration of exposed nonpolar groups and changes the configuration of adjacent molecules. These transformations ultimately lead to a "tightening" of membrane structure and an observed decrease in permeability.

Nielsen (1974) explored this proposed relationship further and showed that calcium and magnesium do bind to cell membranes. Furthermore, Nielsen noted that the passive binding of calcium or magnesium is influenced by the concentration of the other, suggesting competition between these two metals for the same binding sites.

Various alkaline earth metals are suspected of reducing the permeability of boundary membranes to heavy metals in a manner similar to their interaction with alkali ions. The resulting membrane transformation supposedly lessens the latter metals' uptake from the ambient environment. This inference has been popularized because of the repeated observation over the last several decades that the toxic potencies of certain heavy metals are reduced with an increase in the hardness of the aquatic medium. In fact, Jones (1938) suggested that calcium could be added to zinc solutions in amounts sufficient to eliminate zinc's toxicity to fish even when zinc's concentration was extremely high.
A later study by Jones (1939a) demonstrated that the alkaline, earth metals, in the order, strontium > calcium > magnesium > barium, reduce the toxic potency of copper nitrate to tadpoles of the toad,

Bufo bufo. Even exposure of fish to water of high calcium content, prior to transfer to a "softer" water, temporarily lessened the toxic effects of certain heavy metals (Lloyd, 1962). Jones (1938) and Herrera and Curran (1963) have suggested that this pattern is due to calcium decreasing the assimilation of heavy metals into tissues.

Most early studies related heavy metal toxicity to total hardness (Ellis, 1937), or merely to calcium content (Jones, 1938). However, the conclusion that calcium alone is the major deterrent of heavy metal uptake has been disputed for various reasons. Most significantly, magnesium, a common constituent of water hardness, may be an independently operating modifying factor of heavy metal toxicity. This possibility was not considered in the above-listed investigations.

The first recorded attempt to appreciate the role of each alkaline element of hardness as a modifier of heavy metal toxicity was taken by Lloyd (1960). He regulated the relative concentrations of ambient calcium and magnesium through a range of water hardness. Lloyd subjected rainbow trout fingerlings, Salmo gairdneri, to acutely lethal concentrations of zinc in both hard and soft water, which contained a constant ratio of 2.4 calcium to one magnesium. The decrease in toxicity was a linear function of the combined concentration of the two alkaline earth metals, in a ratio of 2.4 to 1. We can assume, therefore, that, if magnesium is a modifying agent, its effects are proportional to that of calcium, within the hardness range studied by Lloyd. Addressing the subject more astutely, Tabata (1969) examined the effects of various calcium to

magnesium ratios, on zinc toxicity. Daphnia sp. were exposed to acutely lethal levels of zinc at water hardnesses of 25 or 400 mg/l (as $CaCO_3$). Calcium:magnesium ratios varied from 10:0 to 0:10. Empirical results suggested that zinc toxicity tends to decrease with an increase in the proportion of calcium to magnesium in the test water from 0:10 to 8:2. This pattern was reversed in the proportionality range from 8:2 through to 10:0 (Tabata, 1969).

As much as these latter studies may suggest to some that magnesium, in addition to calcium, modifies heavy metal toxicity, problems in interpreting these data still exist. One reason for the controversy is that differences in alkalinity[1] usually accompany differences in hardness. These changes in alkalinity may account for the observed discrepancy in toxicity of a heavy metal between hard and soft water (Stiff, 1971). Pagenkopf et al (1974) reported that copper is highly complexed by carbonate ions in natural waters. Results of equilibrium calculations on copper data obtained from several sources in the literature (Lloyd 1961; Pickering and Henderson, 1966; Mount, 1968; Mount and Stephan, 1969) where alkalinity, total hardness, pH, and copper concentrations were all different, suggested that alkalinity is, itself, a significant modifying factor of copper toxicity (Pagenkopf et al, 1974). Compared to copper, relatively few studies have, apparently, been conducted on the interaction between alkalinity and zinc toxicity. For example, zinc has been shown to precipitate in alkaline waters, and as such, was relatively non-toxic (Anon, 1958).

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[1] Alkalinity is defined as a measure of the capacity of natural waters to neutralize acids (NAS, 1978).

It would appear that, besides distinguishing the respective roles of individual alkaline earth metals and alkalinity, any deliberation on water hardness' interaction with heavy metals should include the effects of free hydrogen ions. Howarth (1976) explored the effects of pH at different levels of hardness on the acute toxicity of copper to rainbow trout. He found that at a given degree of hardness, the threshold response to toxicity was altered with changes in pH. The following equation was derived to describe the variation in the 96-hr LC_{50} for rainbow trout (10.2 g) at various combinations of water hardness, ranging from 30 to 360 mg/l (as $CaCO_3$), and of pH, ranging from 5 to 9:

$$\text{96-hr } LC_{50} = \text{antilog}_{10} (2.00 + 0.58\ P_T + 0.54\ H_T + 0.16\ P_T^2 - 0.69\ P_T^3) \quad (1)$$

where $H_T = (\log_{10} \text{Hardness} - 2.01671)/0.62308$

and $P_T = (\text{pH} - 7.0)/2.0$ (Howarth, 1976)

High hardness decreased copper toxicity at any pH tested, while decreasing pH had a variable effect. For example, there was a decrease in toxicity from pH 9 to 8, but an increase from pH 8 to 6, compared to a further decrease from 6 to 5. These results were supported by Waiwood's (1977) separate investigations, in which both pH and hardness were examined as distinct modifying factors.

Hydrogen ion concentration may be directly related to the alkalinity of the aquatic media (Stumm and Morgan, 1970). A quantitative relationship between pH, hardness, and alkalinity,

as would occur in most natural waters, has been proposed by Waiwood (1977):

$$\log_{10} \text{alkalinity} = 49.18 \times 10^{-1} + 82.85 \times 10^{-2}\ \text{pH} + 5.16 \times 10^{-3}\ \text{Hardness} \quad (2)$$

This function indicates that an increase in alkalinity must be accompanied by an increase in pH and/or hardness (as calcium and magnesium ions).

Thus we conclude that all three components, water hardness, alkalinity, and pH can modify the toxicity of individual heavy metals. It may now be possible to explore the degree to which, and the manner by which calcium and/or magnesium modify heavy metal toxicity to the exclusion of pH and alkalinity, as variables. Tabata (1969), Zitko and Carson (1976) and Chakoumakos et al (1979) have offered some initial insight into this inquiry. Results of 24-hour lethal bioassays in which Daphnia sp. were exposed to various concentraions of zinc in solutions of constant alkalinity, but varying hardnesses, which ranged from 6 to 400 mg/1 (as $CaCO_3$), indicated that zinc's toxicity decreases about ten-fold from the soft water hardness to the very hard (Tabata, 1969). A study carried out by Zitko and Carson (1976), in which juvenile Atlantic salmon (Salmo salar) were exposed to acutely lethal levels of zinc in waters of varying calcium or magnesium content and constant bicarbonate conditions confirmed Tabata's (1969) findings. Zinc's potency was merely related to the concentration of water hardness

cations. With increasing calcium levels from 4 to 48 mg/l, zinc's toxicity decreases 10%, while with increasing magnesium levels, from only 1 to 7 mg/l, zinc's apparent toxicity completely decreases. This relationship between heavy metal toxicity and water hardness was further documented by copper toxicity studies carried out at a constant alkalinity with Daphnia sp. (Tabata, 1969) and cutthroat trout, Salmo clarki (Chakoumakos et al, 1979). With increasing water hardness from 6 to 400 mg/l (as $CaCO_3$), a six-fold decrease in copper's toxicity is observed with Daphnia sp. (Tabata, 1969); from 31.4 to 204 mg/l (as $CaCO_3$), a three-fold decrease in toxicity is observed with cutthroat trout (Chakoumakos et al, 1979).

A plausible explanation for the role of calcium and magnesium was advanced by O'Shea and Mancy (1978). They assumed that calcium and magnesium compete with heavy metals, such as cadmium, for organic binding sites in or on boundary membranes. They set up a model to explore this hypothesis and found that calcium inhibited trace metal compexation to organic ligands much more than did magnesium.

Numerous other researchers have investigated the relationship between heavy metal toxicity and water hardness, for example, Cairns and Scheier (1957), Lloyd (1961), Lloyd and Hebert (1962), Pickering and Henderson (1966), Anon (1967), Shaw and Brown (1974), Solbe (1974), and Davies et al (1976). None of these studies have examined the modification of heavy metal toxicity by individual physical-chemical factors, exclusive of other environmental var-

iables. Because of this inadequacy, these investigations do not contribute to any further understanding of how each modifying factor, inherent within water hardness, individually affects heavy metal toxicity.

In conclusion, the empirical evidence reported in the literature suggests that the three factors, pH, alkalinity, and hardness, each with different degrees of effectiveness, are significant determinants in the toxicity of heavy metals to aquatic organisms.

Toxicity of Copper and Zinc

Copper and zinc have been selected for the present study because the salts of these two heavy metals are predominant industrial and mining pollutants of natural waterways (McKee and Wolf, 1963; Sprague et al, 1964). Copper and zinc, amongst the more toxic heavy metals to aquatic organisms (Clarke, 1974), are the two most prevalent heavy metals mobilized by man from nonindustrialized sources, according to a 1970 MIT report. However, trace quantities of these heavy metals are essential to the growth and development of aquatic organisms.

Copper sulphate, the most common salt of copper, is used widely in such industrial processes as tanning, dyeing, process engraving and pigment manufacture (McKee and Wolf, 1963). According one of the most significant sources of copper pollution is the use of copper sulphate as an algicide in reservoirs and streams.

Various physiological deformities have been observed in fish exposed to toxic levels of copper sulphate. Dilation of kidney tubules(Reichenbach-Klinke, 1975), renal necroses around the tubules, destruction of the hematopoetic tissues, as well as increased liver fat (Baker, 1969; Reichenbach-Klinke, 1975) have been documented for copper-exposed fish. In addition, structural gill damage, such as the swelling of gill epithelial cells, separation and sloughing off of epithelial cells from pilaster cells of the lamellae (Lloyd, 1962; Baker, 1969) as well as coagulation and precipitation of mucus on gill epithelium (DSIR, 1960; Lloyd, 1962) have been observed. Wide ranges of published 96-hr LC_{50} values, from .01 to 10 mg/l, are reported for fish exposed to copper (O'Hara, 1971). However, it may not be the amount of copper accumulated in gill tissue of exposed fish that correlates with lethality, but the rate at which it accumulates (Spear and Anderson, 1978).

Several significant sources of zinc pollution in aquatic systems are metal plating works, zinc mining processes, and small-arms ammunition plants. The chloride form of zinc, highly soluble in water, is often found in industrial wastes arising from the manufacture of paper, dyes, and glues (McKee and Wolf, 1963). Fish exposed to toxic levels of zinc show a cytological breakdown of gill epithelium (Lloyd, 1960; Skidmore, 1970; Skidmore and Tovell, 1972) similar to that found with copper (Skidmore, 1964), as well as mucus precipitation on gill epithelium (Carpenter, 1927; 1930; Jones, 1938; 1939b). Chronic exposure (55 to 95 days) to low lev-

els of zinc have been found to result in liver degeneration, under development of gonads and spleen, and expansion of kidney tubules (Crandall and Goodnight, 1963). A significant amount of research has been carried out on the toxicity of zinc to fish. Wide ranges of published 96-hr LC_{50} values for zinc, from .09 to 40 mg/l, are reported in the literature (Doudoroff and Katz, 1953; McKee and Wolf, 1963; Skidmore, 1964: NAS, 1978).

Variations in toxicity of copper or zinc may be particularly attributed to both the difference in sensitivities among the fish species and life stages and, as previously discussed, to environmental modifying factors (NAS, 1978).

The individual heavy metals, copper and zinc, are common pollutants which represent a hazard to aquatic organisms. These metals may contribute significantly to the inherent toxicity of many industrial effluents. However, a pollution problem of even greater dimension can be attributed to the toxic effects of mixtures of these heavy metals (Lloyd, 1961; Sprague, 1964; Eisler and Gardner, 1973; Anderson and Weber, 1975b; Lewis, 1978; Horovitch et al, in press). Sprague et al (1964) reports that pollution by both copper and zinc existing concurrently in an area such as the Miramichi River System in Northeastern New Brunswick is of such significant magnitude as to evoke environmental concern.

Little documentation of the physiological effects of toxic mixtures of copper and zinc on fish was found in the literature. Cusick (1967) examined the mucous cell response of the guppy,

Lebistes reticulatus, to mixtures of copper and zinc. Different sites of toxic activity were proposed for each of the heavy metals based on a reduction in numbers of mucous cells. More recently, Eisler and Gardner (1973) reported that exposure of the marine teleost, Fundulus heteroclitus, to mixtures of copper and zinc salts, resulted in damage of the kidney and the lateral line system similar to that reported for copper exposure alone. Empirical evidence suggests that the toxic response to each heavy metal at different anatomical sites may contribute to the increased mortality of the exposed fish (Eisler and Gardner, 1973).

Variations in the mode of multiple toxicity, claimed to occur with mixtures of copper and zinc, are reported in the literature (Bandt, 1946; Doudoroff, 1952; Lloyd, 1961; Sprague, 1964; Sprague and Ramsay, 1965; Brown and Dalton, 1970; Anderson and Weber, 1975; Lewis, 1978). Water hardness, as associated with alkalinity, has been suggested as the principle modifying factor of copper-zinc toxicity (Table 1). Bandt (1946) observed that the toxicity of copper and zinc mixtures, to the trout and roach in soft water, was five times greater than expected if the toxic effects of the metals were additive[1]. No experimental data, however, are available (Doudoroff and Katz, 1953). Doudoroff (1952) reported similar findings to Bandt (1946). While minnows (Pimephales sp.) were observed to tolerate for 8 hours, 8 mg/l zinc or .2 mg/l

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[1] Additivity may be defined as the form of multiple toxicity by which each constituent of a mixture contributes to a common response in proportion to its relative potency, eg. LC_{50} of EC_{50}.

Table 1. Relationship between water hardness and the mode of multiple toxicity of copper and zinc

Author	Bioassay	Hardness	Mode of Multiple Toxicity
Lloyd, 1961	LT 50	320 mg/l	additive
Lewis, 1978	96-hr LC 50	218 mg/l	more-than-additive[1]
Anderson, 1973	96-hr LC 50	124 mg/l	more-than-additive
Bandt, 1946	--	soft	more-than-additive
Doudoroff, 1952	LT 50	soft	more-than-additive
Sprague and Ramsay, 1965	LT 50	14 mg/l	additive
Lloyd, 1961	LT 50	15-20mg/l	more-than-additive
Sprague, 1964	LT 50	20 mg/l	more-than-additive

[1]The lethal response exhibited for the mixture was much greater than that expected relative to the individual potencies of the heavy metals, copper and zinc.

copper in soft water, significantly mortality occurred in the mixed solution of 1 mg/1 zinc and .25 mg/1 copper within this time period. Lloyd (1961) found that relatively low but lethal concentrations of copper and zinc were additive in their toxicity to rainbow trout in soft water. A more-than-additive lethal response was exhibited with higher concentrations (Lloyd, 1961). While Sprague (1964) suggests that in soft water (20 mg/1 as $CaCO_3$) relatively high concentrations of copper and zinc were more-than-additive in their lethal action, a later study by Sprague and Ramsay (1965), carried out in slightly softer water (14mg/1) and at a lower concentration of the heavy metals, suggests a simply additive response. Lloyd (1961) reported a strictly additive response for copper and zinc in hard water, while Anderson (1973) and Lewis (1978) found the mixtures to exhibit a more-than-additive action in moderately hard and hard water, respectively. These judgements are based on a fixed time period of 96 hours.

No copper-zinc multiple toxicity investigations have, to our knowledge, been undertaken with water hardness as a modifying factor, exclusive of pH and alkalinity as variables. Based on empirical results of copper-zinc lethal bioassays obtained from the literature (Table 1), the present study was designed to evaluate the exclusive role played by water hardness, as calcium and magnesium ions, in modifying the mode of toxic interaction of the two heavy metals, copper and zinc.

In the present study, lethal response data were collected

for zebrafish exposed to pure and mixed solutions of copper and zinc at each of three water hardnesses: 22, 128, and 306 mg/l total hardness (as $CaCO_3$), herein identified as soft, moderately hard or "tap", and very hard, respectively, according to the terminology recommended in a recent report of Water Quality Criteria (NAS,1978) (Table 2). To determine if hardness modification of copper, zinc toxicity was a tissue uptake phenomenon, a copper, zinc, calcium, and magnesium bioconcentration study was undertaken. A differential pulse polarographic analysis of heavy metal solutions was carried out in order to test the hypothesis that water hardness cations may alter the chemical speciation of copper and zinc in the test waters.

Zebrafish, Brachydanio rerio (Hamilton-Buchanan, 1822; 1823), tropical Cypriniforms representative of the family Cyprinidae, were selected for the present study as they are small, inexpensive, and relatively easy to maintain in laboratory aquaria (Sprague and Fogels, 1976; Laale, 1977); they have proven to be responsive to a wide range of mutagens, carcinogens, and teratogens, as well as directly lethal toxicants (Laale, 1977). In addition, Sprague and Fogels (1976) mention that the zebrafish may become a standard test species for the International Standards Organization. This species is not indigenous to Canada and may not adequately represent the response of a native species, such as the rainbow trout. However, the rainbow trout is not native to all parts of Canada and yet is frequently used as a standard test organism. Zebrafish have been found, on the average, to be 2.6 times more tolerant than

Table 2. Classification of water hardness according to the 1978 Water Quality Criteria

Total Hardness Range (ppm as $CaCO_3$)	Classification
0 - 75	soft
75 - 150	moderately hard
150 - 300	hard
300 and up	very hard

(NAS, 1978)

rainbow trout to certain toxicants. More variation in tolerance has been shown for the same species of rainbow trout to a toxicant between studies in the same lab or between labs (Brown, 1968; Black et al, 1976; Howarth, 1976, 1976; Fogels and Sprague, 1977) than is represented by the tolerance factor of 2.6 (Sprague and Fogels, 1976).

In summary, the two main objectives of the present study are as follows:

1. To investigate the effects of water hardness, as calcium and magnesium ions in fixed proportions, on the lethal toxicity of copper, zinc, and their mixtures in solutions of constant alkalinity and pH.

2. To examine the relationship between bioconcentration and lethal toxicity of zinc and copper through a range in water hardness.

The results are discussed in relation to possible mechanisms of calcium and magnesium interactions with heavy metal toxicity, and to the establishment of water quality criteria that effectively safeguard organisms against the toxic hazard of copper and zinc mixtures.

MATERIALS AND METHODS

Experimental Organism

Adult zebrafish, Brachydanio rerio, (Hamilton-Buchanan, 1822; 1823) were obtained from Riverview Water Gardens in Sebastian, Florida. The fish, upon arrival at the Water Pollution Laboratory of Concordia University, were quarantined for at least two weeks. During this period they were held in a 352-litre fibreglass tank supplied with a continuous flow of Montreal city tap water. The tap water was charcoal-filtered, dechlorinated, degassed and heated to 26° C before entering the laboratory. These stock fish were fed ad libitum twice daily with Tetramin Staple tropical fish food. Following the quarantine period, fish were transferred in lots of 25 to 21-litre glass tanks. These fish were allowed to acclimate for at least two weeks to one of three water hardness regimes: 22.1, 127.8, and 306.2 mg/l (total hardness as $CaCO_3$) herein identified as soft, moderately hard or "tap", and very hard water, respectively (Table 2). Prior to the acclimation period in the soft or very hard waters, test fish were gradually introduced to these hardness regimes (see Water Hardness Regulation). At least one week prior to experimentation, the fish were wet-weighed, separated into lots representing arbitrarily defined weight classes. Each lot was assigned to a specific acclimating tank.

Environmental Parameters

Certain physical-chemical characteristics of the water were measured twice weekly during the period for acclimation and at least once daily during experiments: alkalinity (as $CaCO_3$) by the mixed brom-cresol green, methyl red method (APHA et al, 1965); total hardness (as $CaCO_3$) by the titrimetric method (APHA et al, 1965) and the Hach method (Hach Catalogue); calcium, magnesium, and sodium by flame absorption spectrophometry (Perkin-Elmer 503); and pH with a Corning Model 10 pH meter. Temperature was monitored daily, using a hand-held glass thermometer, in both acclimating and experimental tanks. Dissolved oxygen and chloride were measured once during each experiment: dissolved oxygen by the azide modification of the idiometric method (APHA et al, 1965) and chloride by the ion diffusion technique using a chloride-specific electrode on a Metrohm pH meter.

Table 3A lists the mean values of the physical-chemical measurements obtained for water used in both experimental and acclimating tanks. Fish were subjected to a photoperiod of 12 hours light-12 hours dark. Mean values obtained for Montreal city water are presented in Table 3B.

Toxicants

The toxicities of two heavy metals, copper and zinc, were examined in this study. Copper stock solution was prepared by combining reagent grade copper sulphate, $CuSO_4 \cdot 5H_2O$ (Anachemia)

Table 3A. Water quality measurements** of laboratory water (mean values ± standard deviation)

Parameters	Water Types: Soft	"Tap"	Very Hard
Alkalinity (mg/l as $CaCO_3$)	81.3 ± 0.4	81.3 ± 0.3	81.3 ± 0.4
Total Hardness (mg/l as $CaCO_3$)	22.1 ± 2.8	127.8 ± 7.7	306.2 ± 9.9
Calcium (mg/l as $CaCO_3$)	16.1 ± 1.7	88.2 ± 7.6	220.0 ± 6.4
Magnesium (mg/l as $CaCO_3$)	6.0 ± 1.2	39.6 ± 3.3	91.8 ± 9.0
Sodium (mg/l)	64.0 ± 2.8	12.7 *	12.7 *
Chloride (mg/l)	30.0 *	20.0 *	100.0 *
pH	7.8 ± 0.1	7.7 ± 0.0	7.7 ± 0.2
Temperature °C	25.3 ± 0.3	25.6 ± 0.4	26.4 ± 0.2
Dissolved Oxygen (% saturation)	85.7 ± 0.7	89.5 ± 3.5	88.5 ± 0.7

** Flow-through systems

* Standard deviations not available

Table 3B. Water quality measurements of Montreal City water

Parameters	
Alkalinity (mg/l as $CaCO_3$)	84.0
Total Hardness (mg/l as $CaCO_3$)	128.0
Calcium (mg/l as $CaCO_3$)	92.8
Magnesium (mg/l as $CaCO_3$)	34.2
Sodium (mg/l)	12.3
pH	7.9
Sulfates (mg/l)	25.1
Iron (mg/l)	0.02

(Data supplied by the Municipal Water Works, City of Montreal)

with deionized, glass-distilled water. In order to increase the solubility of the metal salt, concentrated hydrochloric acid was added to lower the pH of the aqueous mixture to two. Zinc stock solution was prepared in a similar manner using anhydrous zinc chloride, $ZnCl_2$ (Anachemia). Samples from exposure and control tanks were collected daily during the experimental periods. The samples were acidified with a few drops of concentrated hydrochloric acid to reduce the tendency of copper and zinc to adsorb on the walls of the pyrex test tubes. Copper and zinc samples were analyzed by flameless (graphite furnace) and flame atomic absorption spectrophotometry (Perkin-Elmer 503) respectively, and mean concentrations computed for each test tank.

Differential Pulse Polarographic Protocol

The solubility of copper or zinc at different water hardnesses was evaluated by differential pulse polarographic analyses (Metrohm Herisau E505, Polarecord E506). Pulse polarography measures the various dissolved fractions of a heavy metal in a aqueous medium, while atomic absorption spectrophotometry measures both the dissolved and bound species present. Each polarographic sample, consisting of 5 ml of heavy metal solution and .25 ml 1% gelatin solution, was diluted to 25 ml with a citric acid buffer. (Pulse polarographic analyses were performed on prepared samples of copper and zinc dissolved in soft, tap, or hard water.) The citric acid buffer served as the supporting electrolyte. Samples were degassed with nitrogen. Ag/AgCl (saturated KCl) was used as

a reference electrode. Zinc polarograms were run at a voltage range from -0.80 to -1.20, while those for copper from +0.14 to -0.38. In a given analysis, each peak height (μA) generated on the polarogram was assumed to represent a unique and dissolved heavy metal fraction. Reference solutions of copper or zinc salts were prepared in distilled water. The peak heights (μA) of these solutions were then compared to those fractions of the respective heavy metals dissolved in harder water. The relative difference in the peak heights of comparable fractions was an index of change in concentration. Relative differences in the spectrum of peak heights initiated a change in heavy metal species. No attempt was made to identify the chemical nature of the specific dissolved fractions portrayed by the polarography. Due to constraints in time and availability of facilities, few replicates of heavy metal polarograms could be run.

Bioassay Apparatus

The biassay apparatus consisted of the following components:

1. Diluter apparatus for water hardness regulation;
2. Diluter apparatus for toxicant regulation;
3. Acclimating and exposure tanks.

A schematic view of the biassay system is presented in Figure 1, and a photograph of the apparatus in Figure 2.

Water Hardness Regulation

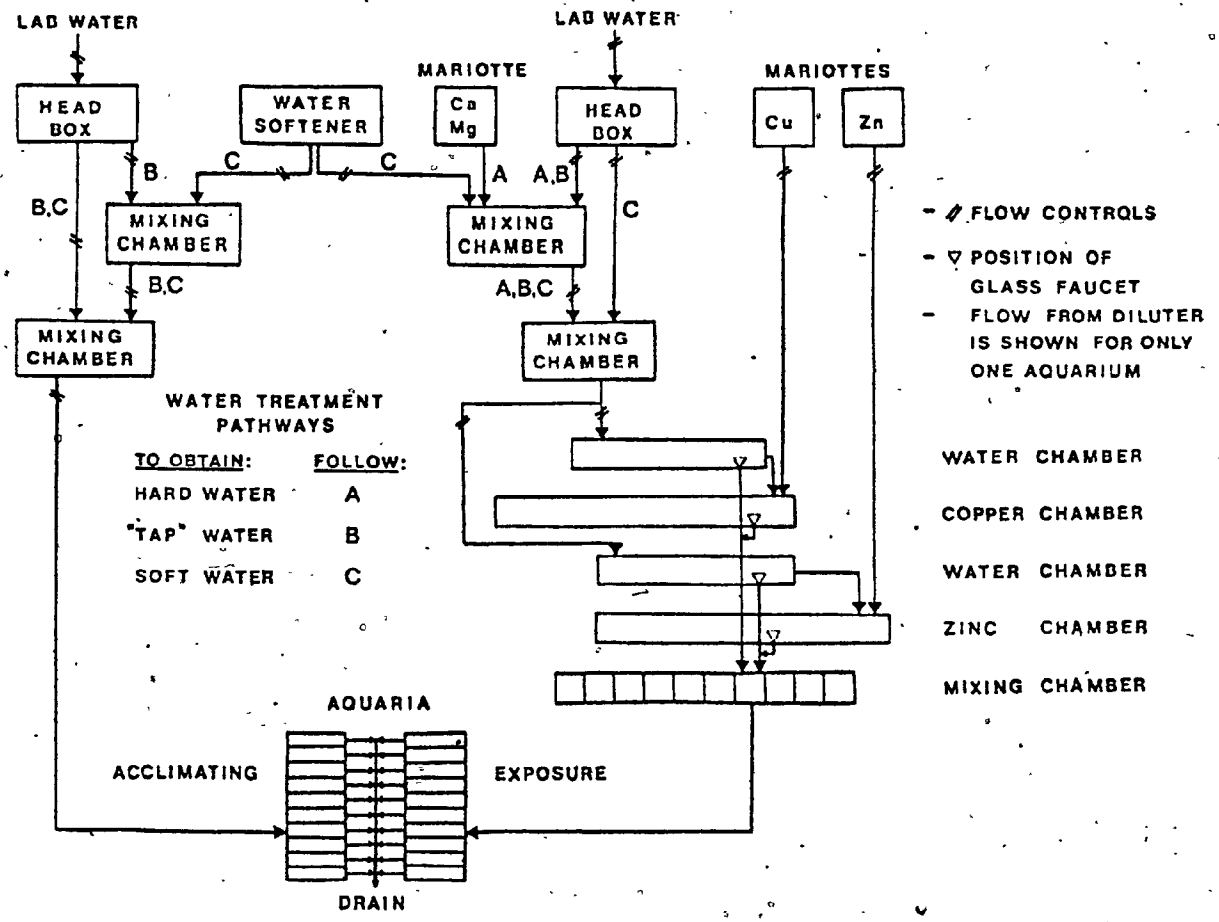

Figure 1. FLOW DIAGRAM OF BIOASSAY APPARATUS

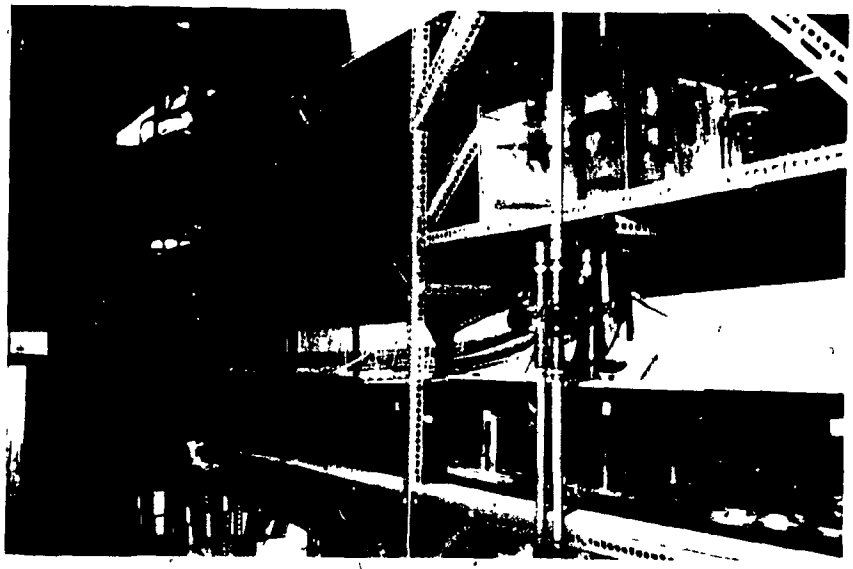

Figure 2. Photograph of bioassay apparatus. View of diluter and acclimating aquaria

The moderately hard water, which was provided by the laboratory tap water, closely approximated in hardness the water in which fish were reared and held in Florida (Aquaking Ltd., Montreal, personal communication). No special apparatus was necessary to maintain this regime.

A source of soft water was established by mixing tap water in specific proportions with water derived from two domestic water softeners, Aquafine No. ATE 201 (20,000 grains capacity) and No. ATE 401 (40,000 grains capacity) (Figure 3). (A grain is equivalent to 17.12 mg/l (as $CaCO_3$).) These water softeners operate on the ion-exchange principle by which calcium and magnesium are exchanged for sodium ions. These softeners were connected independently to the main diluter apparatus: an arrangement that allowed one unit to be shut down and thereupon regenerated with a brine solution, while the other continued to operate.

Fish to be used in tests with soft water were gradually acclimated to a soft water environment. They were kept in flow-through tanks in which the hardness of the water was gradually decreased over a one week period from tap to soft (22 mg/l as $CaCO_3$). As previosly mentioned, they were then then held in this soft water for an additional two weeks prior to tests (Figures 4 and 5).

A source of very hard water was obtained by adding a concentrated solution containing calcium and magnesium to moderately hard water. The calcium-magnesium stock solution was prepared by dissolving technical grade calcium chloride ($CaCl_2 \cdot 2H_2O$) and magnesium chloride ($MgCl_2 \cdot 6H_2O$) (Anachemia) in deionized glass-

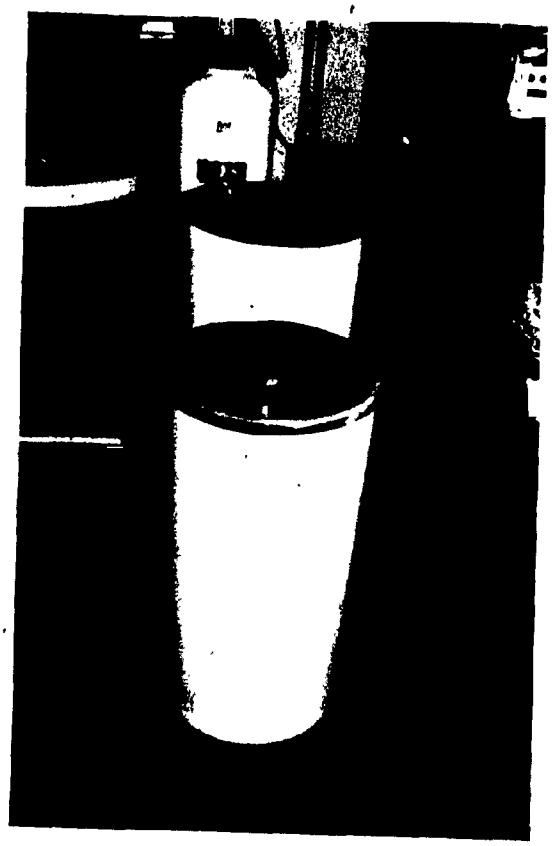

Figure 3. Photograph of domestic water softeners, Aquafine No. ATE 201 and No. ATE 401.

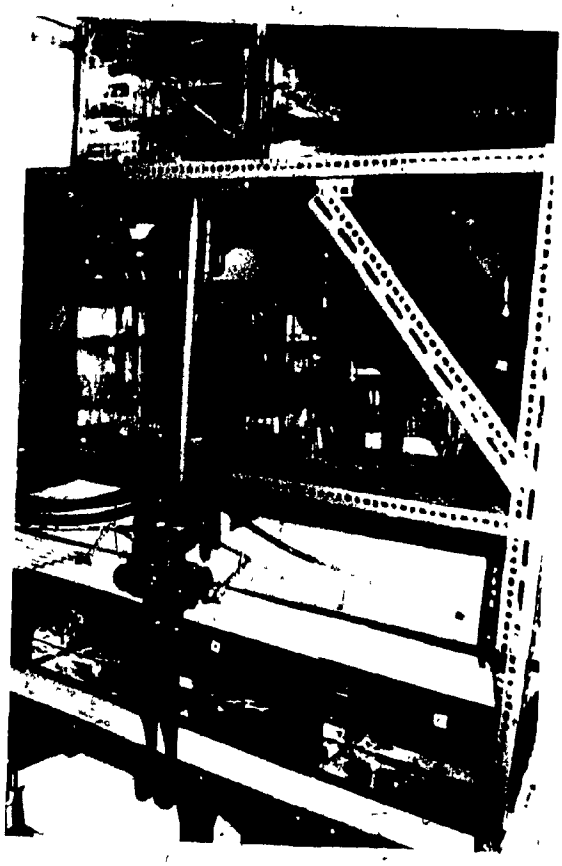

Figure 4. Photograph of diluter apparatus for hardness regulation for acclimating aquaria

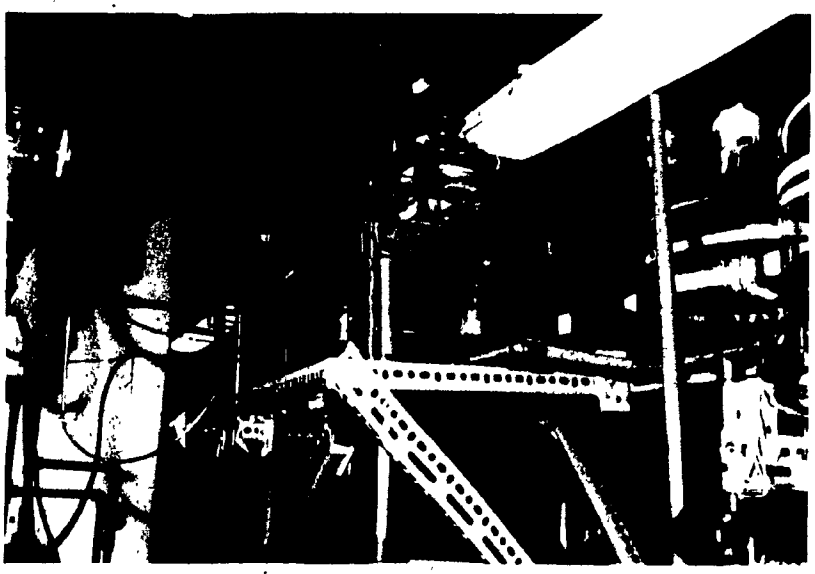

Figure 5. Photograph of diluter apparatus for hardness regulation for exposure aquaria

distilled water, in a ratio of 2.6 parts calcium to one part magnesium (both as $CaCO_3$). This ratio approximated the difference in the respective concentrations of calcium and magnesium in the tap and soft water regimes. The total hardness of the stock solution was,412,800 mg/l (as $CaCO_3$). Fish selected for tests with very hard water were allowed to acclimate in "static" tanks in which the hardness was gradually increased over a two-week period from tap to the assigned test level of 306 mg/l (as $CaCO_3$). The fish were then allowed to acclimate for an additional two weeks in waters at this hardness. The high costs that would have been incurred by using a flow-through system for this adjustment necessitated the adoption of a static system. The standing water in static tanks was renewed every two days by about 50%, after a gradual increase in hardness to 306 mg/l (as $CaCO_3$) was attained. Physical-chemical measurements of the standing water utilized for acclimation only were equivalent to those obtained in a flow-through system (Table 3A). To regulate the temperature of the holding water, the aerated static tanks were placed inside a 27^{o} C water bath.

A flow-through system replaced the static acclimation system during the 96-hour lethal bioassays. To achieve a continuous flow system, the calcium-magnesium stock solution was dripped at a controlled rate from a 45-litre pyrex Mariotte bottle into plexiglass mixing chamber. At this stage, the stock solution was proportionally diluted with tap water to give the acclimation level of 306 mg/l (as $CaCO_3$). The heavy metals were then added in a subsequent stage to the very hard water to provide a series of dilute solutions which were then delivered by tygon tubing to their respective test tanks.

Toxicant Regulation

Toxicant stock solutions, copper and zinc, were dripped at a constant rate, from polyethylene tubing fed from 45-litre Mariotte bottles, to a plexiglass diluter apparatus (Figure 6). Much of the diluter's design and its operational characteristics are as described by Weinstein (1978). A constant input of very hard, tap, or soft water was delivered through polyvinylchloride piping, with valve controls, to the toxicant diluter. Rotatory glass faucets, located in series at the front of each diluter chamber, controlled the gravity forced flow of the toxicant mixture and/or the diluting water into the final mixing chambers. These mixing chambers were connected in a 1:1 arrangent by glass and tygon tubing to a series of aquaria. A hydraulic head was established, with a glass standpipe, in each chamber of the bioassay apparatus. Short bursts of compressed air were forced through the glass faucets twice daily to prevent the accumulation of air bubbles which might air-lock the system. Flow rates were adjusted to deliver toxicant solutions continuously to exposure tanks at a rate of 300 ml/min. This flow rate was estimated to achieve a 99% replacement in five hours (Sprague, 1973).

The flow of toxic solutions was established at least 24 hours prior to introduction of the test fish. Preliminary tests by the author indicated that an apparent equilibrium between heavy metal ions adsorbed to the aquaria walls and ions in solution could be established within a 24-hour period. No toxicant was introduced into the control tanks. The concentrations of heavy metals in

Figure 6. Photograph of diluter apparatus for toxicant regulation

samples taken the control tanks were not above background levels of source water.

Exposure to Toxicants

Fish were deprived of food for a 24-hour period prior to being transferred from acclimation tanks to toxicant exposure tanks, as well as during the 96-hour exposure period. The procedure was introduced primarily to reduce the possibility of toxicant assimilation from injested food. Lots of 14 fish representing discrete weight classes were randomnly distributed to test tanks which contained a series of toxic solutions. Concentrations of toxicants were selected according to a method described by Weinstein (1978) to give equal intervals on a log scale (Appendix I). Observations were made at least once every four hours throughout the 96-hour exposure period. During this time period, test fish were examined for visible signs of toxic distress. Cessation of gill and heart movement, as observed by eye, was adopted as the criterion of death.

Data Analysis

Experiments to estimate the lethal toxicity of copper and zinc at each water hardness were conducted. Mortality times of test fish to either copper, zinc, or their mixtures were recorded at least every four hours during the 96-hour exposure period. Data representing cumulative probit mortality versus log cumulative time were plotted on probit-log paper. (Percent cumulative mortality was converted to probit mortality (Finney, 1971).) Log LT_{50}

(log time to 50% mortality) values were obtained from linear regressions computed for these data. LT_{50} is a measure of the median resistance time in a population of organisms exposed to a particular concentration of toxicant. Linear regressions were computed for the log-log distribution of LT_{50} versus toxicant concentration data, and correlation coefficients for these regressions obtained. Student's "t"-tests (Sokal and Rohlf, 1969) of slope values of these lines were carried out to determine whether the regression lines for each toxicant were not significantly parallel (at $p = 0.05$).

Dose-response data (mortality versus concentration) were obtained for each heavy metal at each level of hardness. Dose-response curves were then derived as follows:

Derivation of Dose-Response Curves - Individual Heavy Metals

When probit mortality is plotted against log concentration a dose-response curve is obtained. Dose-response data plotted in this way can usually be adequately described by a simple linear function (Equation 3). However , Anderson and Weber (1975b) have shown that the relationship between mortality and concentration may often be more precisely represented by a linear function which incorporates the weight of test organisms as a variable (Equation 4).

$$Y = a + b \log_{10} C \qquad (3)$$

$$Y = a + b \log_{10} (C/W^h) \qquad (4)$$

where Y = probit of % mortality

a = intercept

C = concentration of toxicant

W = mean wet weight of test organism exposed to C

h = proportionality constant

(Anderson and Weber, 1975b)

Both of these functions were computed for the data obtained in this study (Appendix IIa). The parameters of Equation 4 were determined using a computer program developed by Weinstein (1978). According to the Student's "t"-test, no significant improvement ($p = .05$) in the fit of the data to a linear regression results if weight is incorporated as a variable (Appendix IIc). Therefore Equation 3 was adopted in subsequent analyses.

Linear functions for each set of dose-response data were then determined by a modification of Finney's (1971) method of probit analysis. The analysis fits a regression to dose-response coordinates by estimating "maximum likelihood" through several successive iterations. The "maximum likelihood" is selected from a series of possible regressions through the use of a chi-square test (Appendix III). This test also indicates whether the assumption that dose-response data are linearly distributed is reasonable or not. The computer program for probit analysis was obtained from the Department of Mathematics and Statistics, University of Guelph, Guelph, Ontario and was not available for presentation in this manuscript. Besides identifying the parameters; y-inter-

cept,, and slope, of the "maximum likelihood" regression, the program determines the LC_{50} and its fiducial limits at 95% confidence.

A comparison of dose-response data for each heavy metal between each water hardness regime was performed. First, a chi-square test was employed to check the hypothesis that data collected could be reasonably assigned to three distinct dose-response sets corresponding to each hardness regime. Once this distinction was established, each set of data was fitted with a regression through the "maximum likelihood" method previously discussed. If these regressions were not significantly unparallel according to a chi-square test, a common slope was calculated in order to constrain them to parallelism as recommended by Bliss (1939) (Appendix IV). On the basis of the derived regressions, "relative potency factors" were determined (Appendix V). Besides comparing the relative potencies for single toxicants, copper or zinc, through a range of hardness, the relative potencies of copper and zinc, at each hardness regime, were also compared.

Empirical Models of Multiple Toxicity

In the aquatic environment, organisms are often exposed to mixtures of toxic pollutants. "Multiple toxicity" is the general term used to distinguish the toxic effects of chemical mixtures from those of single substances in pure solutions. Individual toxicants within mixtures may interact such that the overall toxicity of the mixture is greater or less than the toxicity of either constituent (Anderson and D'Apollonia, 1978).

Bliss (1939) conceived of two additive mechanisms of toxicant interaction which he called "similar" and "independent joint action". In theory, the similar joint action mechanism of multiple toxicity occurs when the constituents of a toxic mixture act at the same target sites in an exposed organism to produce similar effects. Bliss (1939) argued that in the case of similar joint action, the dose-response curves of the individual toxic constituents in pure solution are parallel. In comparison, the independent joint action mechanism occurs when constituents of a toxic mixture act at different target sites but produce effects which lead to a common response. According to Bliss (1939), such constituents do not necessarily generate parallel dose-response curves when they are applied in pure solution. Therefore parallelism between dose-response curves for individual constituents is not a criterion for independent joint action as it is for similar joint action.

Bioassays lasting 96 hours were conducted according to the standardized method of lethal toxicity testing (Doudoroff et al, 1951; Sprague, 1973). The bioassay procedure and analysis for multiple toxicity studies using aquatic pollutants have been documented in detail by Anderson and Weber (1975a, b). They proposed the terms "concentration-addition" and "response-addition" to delineate empirically Bliss' (1939) theoretical models of similar joint action and independent joint action, respectively. In the present study the additive models were delineated as follows:

Concentration-Addition

Linear regressions were derived through probit analysis for each set of lethal response data. These data represented the mortality at each water hardness in test populations after 96 hours of exposure to various concentrations of each heavy metal in pure solution. Linear regressions for copper and zinc at each water hardness were compared for parallelism. If these regression lines were not significantly unparallel, concentration-addition was hypothesized for the data. These lines were then constrained to parallelism (Appendix IV).

In order to express the concentrations of constituents within a given mixture as a single concentration for the mixture, the following procedure was taken. The measured level of zinc in a mixture was converted to an equipotent level of copper by multiplying the former by the relative potency factor (Appendix V). The relative potency factor was calculated on the basis of copper as the most toxic constituent. This converted value for zinc was then added to the active concentration of copper actually present in the mixture. The resultant sum was thus taken to represent the total concentration of the mixture as a single level of copper.

Linear regressions were derived for the coordinates of probit mortality versus log total concentration in accordance with Finney's (1971) method of probit analysis. The empirically derived line was then compared to the predicted regression for concentration-addition which was computed as follows:

$$Y_m = a_1 + b \log_{10}(C_1 + pC_2) = a_1 + b \log_{10}(C_1 + C_1') \quad (5)$$

where Y_m = probit of % mortality

a_1 = Y intercept of most potent toxicant

b = common regression coefficient

C_1 = concentration of most potent toxicant in mixture

C_2 = concentration of less potent toxicant in mixture

C_2' = concentration of less potent toxicant in mixture converted to an equipotent concentration of C_1

p = relative potency between first and second toxicant

A chi-square test was used to determine whether observed regressions were significantly different from those predicted for concentration-addition. If no significant differences could be found, then the concentration-addition hypothesis was not rejected. These tests were repeated at each water hardness.

Response-Addition

Probit response data obtained from multiple toxicity bioassays may not fit the model for concentration-addition when response curves of the constituent heavy metals in pure solution are unparallel, or if parallel, when the modes of toxic action of the constituents are different. If either of these conditions holds the empirical model of independent response-addition may be inferred. Lethal response data, represented as proportions of individuals responding to copper or zinc at each water hardness may be

substituted into the following equations in order to test whether observed responses for the mixture correlate with those predicted according to the alternative model.

If a negative correlation (r = -1) in tolerances to two response-additive toxicants is shown for organisms in a population, the proportion of individuals responding to the binary mixture can be represented by:

$$P_m = P_1 + P_2 \text{ if } P_1 + P_2 \leq 1 \qquad (6)$$

where P_m = proportion responding to the mixture

P_1 = proportion responding to toxicant "1" alone

P_2 = proportion responding to toxicant "2" alone

With no correlation in tolerances (r = 0), the proportion responding to the mixture is represented by:

$$P_m = P_1 + P_2 (1 - P_1) \qquad (7)$$

If the correlation is positive (r = +1), the relationship is expressed by:

$$P_m = P_1 \text{ if } P_1 \geq P_2 \qquad (8)$$

$$Pm = P_2 \text{ if } P_2 \geq P_1$$

The proportions (P_1 and P_2) responding to each toxicant alone were

calculated on the basis of the respective dose-response regressions previously derived. Mortalities predicted for the mixture were then computed using either equations 6, 7, or 8 (Plackett and Hewlett, 1952).

If no significant differences are found between observed and predicted responses, according to the chi-square test, then the response-addition hypothesis is not rejected. These tests were repeated at each water hardness.

Treatment of Non-Additive Response Data

If toxic response patterns do not fit models for either concentration or response-addition, we assume that interactions have occurred at either the biokinetic[1] or biodynamic[2] level, which in turn have led to either an enhanced or diminished response. At this point in time we are unable to identify mechanisms of interaction. Thus we cannot predict, from a knowledge of the toxicity of individual constituents, the degree or nature of non-additive multiple toxicity. We have chosen, therefore, to represent the non-additive data relative to that which one would expect for strict addition. When the response is greater than that predicted for strict addition, we call the pattern supra-additive, a form of synergism;

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[1]Biokinetic processes determine the actual quantity of toxicant available at receptors.

[2]Biodynamic processes mediate the observed reaction.

when the response is less than predicted for concentration-addition, the pattern is designated infra-additive, a form of antagonism.

Isobolograms[1] (Loewe, 1928; 1953; Ariens, 1972) are one method of illustrating an enhanced or diminished response relative to that expected for strict addition (Figure 7). Another method which quantitive is the relative enhancement factor estimation of Anderson and Weber (1975b) (Appendix VII).

Fish Tissue Analyses

Fish which had been allowed to acclimate to soft, tap, or very hard water regimes for at least two weeks were exposed, at their respective water hardnesses, to sublethal concentrations of copper, zinc, or mixtures of the two. Fish were transferred in lots of 21 individuals to aerated static tanks which were placed inside 27°C water baths. Physical-chemical measurements obtained were similar to those presented in Table 3A for the flow-through apparatus. All fish used in these experiments were from the same weight class. After the 48-hour toxicant exposure period, survivors were sacrificed, stored in glass test tubes, and immediately frozen. Wet weights of whole bodies as well as excised gill pairs from thawed fish were recorded. Tissue samples were dried overnight in a drying oven at 70°C. Dry weights were recorded. The fish samples

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[1]An isobologram is a two-dimensional diagram representing lines of equivalent response for given concentrations of a binary mixture of toxicants.

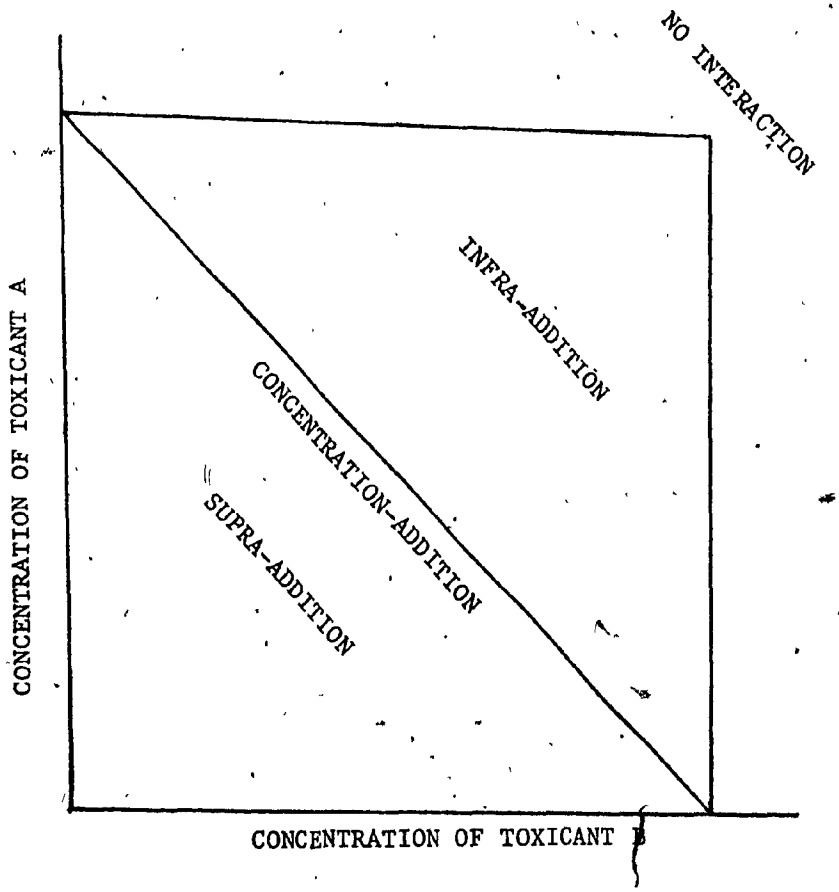

Figure 7. Isobole diagram for quantal responses of two toxicants A and B, each of which is active by itself (modified from Muska, 1977).

were then subjected to the wet acid digestion technique described in Leonard (1971). Digested tissue samples were analyzed by atomic absorption spectrophotometry. Determinations of mean dry tissue weight (µg/g) of calcium, magnesium, copper, zinc, and sodium in gills and whole bodies were made. Because of the limited sample size, the data did not lend themselves to rigorous statistical analyses. Data analyses were limited to testing of differences between mean concentration values (Schor, 1968).

RESULTS

Environmental Parameters

Certain chemical characteristics of the test waters are listed in Table 3A. These data represent the means of all measurements on water samples collected at each water hardness during the duration of the research program. The variations in alkalinity, pH, temperature, and dissolved oxygen levels between hardness regimes and throughout the period of experimentation were considered negligible.

The advantages of the cation exchange system in providing soft water at a pH and alkalinity similar to the source water was offset by high sodium levels. The sodium concentration in the softened water was approximately five-fold greater than in tap or very hard waters. The use of chloride salts of calcium and magnesium to create the hard water environment of 306 mg/l (as $CaCO_3$) resulted in test tank solutions containing significant amounts of chloride ions. Chloride levels in this latter system were approximately 100 mg/l, five-fold greater than the mean levels in either tap or soft water which were similar in chloride content. However, the desired fixed ratio of 4:1 between calcium and magnesium ions was achieved within each of the three water hardness regimes.

Differential Pulse Polarographic Determinations

Results of differential pulse polarographic determinations of

solutions of the chlorides of copper or zinc at various water hardnesses are presented in Table 4. Increasing the water hardness from 0 to 26 mg/l (as $CaCO_3$) resulted in an 11% decrease in the concentration of the apparent zinc dissolved fraction, and a 12% in that for copper. Concentrations of either dissolved fraction declined only by an additional 3% with zinc and 5% with copper as the water hardness was increased from 26 mg/l to 100 and 120 mg/l respectively. A final increase of water hardness from 100 to 200 mg/l did not result in a decrease of the concentration of the dissolved fraction of zinc. In fact, a 1% increase was observed. No analysis of copper dissolved in a 200 mg/l water hardness (as $CaCO_3$) was performed. Changes in the species composition of either heavy metal solution were not apparent since a change in the peak patterns was not observed through the hardness range studied.

External Morphological Damage

Exposure of zebrafish to high concentrations of copper and/or zinc salts resulted in certain toxicity signs that have been previously reported for these heavy metals. The degree of the two most apparent effects, mucous production and hemorrhaging, were assessed subjectively. The results are listed in Table 5. Fish exposed to lethal levels of copper appeared to produce excess mucus in soft and tap water and none in very hard water compared to controls. Hemorrhaging was extensive in the flank region of copper-treated fish in soft water, moderate in tap water, and minimal in very hard water.

Table 4. Differential pulse polarographic analysis of solutions of copper or zinc at various water hardnesses

Concentration heavy metal (mg/l)	Water hardness (mg/l)	Splitting of peak**	Peak height (uA)	% Peak height (uA) relative to standard	($E_{\frac{1}{2}}$) Peak position (v)
4 ppm Zinc (as Zn^{++})	0 (distilled)	None	0.077*	100	-0.980
	26 (soft)	None	0.068*	89	-0.980
	100 (moderately hard)	None	0.066*	86	-0.980
	200 (hard)	None	0.067*	87	-0.980
1 pm Copper (as Cu^{++})	0 (distilled)	None	0.0358 ± 0.002***	100	-0.058
	26 (soft)	None	0.0314 ± 0.001***	88	-0.058
	120 (moderately hard)	None	0.0302 ± 0.001***	84	-0.058

* no standard deviations available (one determination)

** in both expanded and unexpanded range

*** mean ± standard deviation

Table 5. Summary of relative physiological effects observed in zebrafish after exposure to copper and/or zinc in a series of pure and mixed solutions at various water hardnesses

Empirical concentration range (mg/l)		Water hardness	Magnitude of physiological response	
Copper	Zinc		Mucous production	Hemorrhaging
0.089 - 0.456	--	soft	*	***
0.130 - 0.350	--	tap	*	**
0.300 - 1.940	--	very hard	-	*
--	8.19 - 18.26	soft	***	***
--	6.69 - 37.66	tap	*	*
--	21.42 - 37.97	very hard	*	*
0.026 - 0.120	5.72 - 13.52	soft	***	***
0.019 - 0.094	2.79 - 9.76	tap	*	**
0.064 - 0.181	5.89 - 12.64	very hard	-	*

Key: Relative magnitude of physiological response observed in most exposed fish

*** Abundant
** Moderate
* Minimal
- None visible

Fish exposed to lethal levels of zinc were observed to produce an enormous amount of mucus in soft water, and minimal amounts in both tap and very hard water compared to controls. Long strands of mucus enveloped the flanks of fish in the soft water regime. Mucus from these fish also appeared to have been deposited on the walls of their taks. Hemorrhaging was considerable in the cardiac and abdominal regions of zinc-treated fish in soft water, and minimal in tap and very hard water.

In comparison with controls, fish exposed to mixtures of copper and zinc produced enormous amounts of mucus in soft water, minimal in tap, and none in very hard water. Hemorrhaging was extensive in soft water-exposed fish, moderate in tap, and minimal in very hard water.

Lethal Response Study - Time to Mortality

Cumulative probit mortality over time data for zebrafish exposed to copper, zinc, or their mixtures at three water hardnesses are presented in Tables 6a to 6i. (The fraction of fish which have died in each tank is expressed in terms of probit mortality (Finney, 1971).) Median mortality times (LT_{50}) were computed for each exposure regime (Tables 7a, 7b, and 7c). Linear regressions were computed for the log-log distribution of LT_{50} versus toxicant concentration data, and are listed in Table 8 (Figures 8 and 9). A key of graph symbols is listed in Table 9. Correlation coefficients were highly significant (at $p = 0.01$) for those linear regressions representing copper-tap and very hard, and copper-zinc-tap water

Table 6a. Observed cumulative mortality-time data for zebrafish exposed to pure solutions of copper in soft water.

Probit mortality	Fraction of fish in tank	[Cu++] mg/l						
		0.110	0.160	0.190	0.260	0.390	0.460	0.570
		Time (minutes)						
3.53	1/14	3,260	2,040	510	500	--	510	--
3.93	2/14	3,420	--	--	1,310	500	--	--
4.21	3/14	4,100	--	1,210	1,400	--	--	--
4.43	4/14	4,450	--	1,690	1,750	--	--	--
4.64	5/14	5,370	--	--	1,770	1,200	1,210	250
4.82	6/14	--	2,420	2,030	2,700	1,290	1,390	--
5.00	7/14	--	2,770	--	--	--	1,690	--
5.18	8/14	--	--	2,410	2,780	1,680	1,800	--
5.37	9/14	--	3,110	2,680	3,720	--	2,030	--
5.57	10/14	--	3,270	4,000	3,160	2,170	--	510
5.79	11/14	--	--	--	3,720	--	--	620
6.07	12/14	--	--	--	3,760	2,410	2,420	--
6.47	13/14	--	--	--	5,760	2,680	2,830	1,210
Slope			9.12	2.40	3.03	3.32	3.76	2.67
Intercept			-26.39	-3.20	-5.14	-5.32	-6.86	-1.73
LT_{50}			2,780	2,290	2,250	1,290	1,440	330

Table 6b. Observed cumulative mortality-time data for zebrafish exposed to pure solutions of copper in "tap"water.

Probit mortality	Fraction of fish in tank	[Cu++] mg/l 0.150	0.130	0.170	0.220	0.240	0.270	0.310	0.350
		Time (minutes)							
3.53	1/14	--	--	--	990	--	--	--	--
3.72	1/10	2,340	2,610	1,220	--	2,180	1,380	2,030	1,670
3.93	2/14	--	--	--	2,180	--	--	--	--
4.16	2/10	--	3,270	1,350	--	2,280	1,760	2,220	1,710
4.21	4/14	--	--	--	2,410	--	--	--	--
4.48	3/10	--	--	3,330	--	4,770	1,910	--	--
4.64	5/14	--	--	--	2,690	--	--	--	--
4.75	4/10	--	--	--	--	--	3,200	2,900	1,890
4.82	6/14	--	--	--	5,320	--	--	--	--
5.00	5/10	--	--	--	--	--	*	--	2,420
5.25	6/10	--	--	--	--	--	--	3,240	3,270
5.52	7/10	--	--	--	--	--	--	--	3,570
5.57	10/14	--	--	--	--	--	--	--	--
5.79	11/14	--	--	--	--	--	--	--	--
5.84	8/10	--	--	--	--	--	--	--	4,080
6.07	12/14	--	--	--	--	--	--	--	--
6.28	9/10	--	--	--	--	--	--	--	--
Slope							2.66	6.93	4.38
Intercept							-4.50	-19.14	-10.00
LT_{50}							3,670	3,040	2,650

* value missing

Table 6c. Observed cumulative mortality-time data for zebrafish exposed to pure solutions of copper in very hard water

Probit mortality	Fraction of fish in tank	[Cu++] mg/l				
		0.690	0.950	1.210	1.440	1.940
		Time (minutes)				
3.53	1/14	1,820	1,520	--	--	--
3.93	2/14	2,270	1,670	1,800	1,300	1,080
4.21	3/14	3,460	2,260	1,950	1,390	--
4.43	4/14	4,120	2,740	--	1,660	1,300
4.64	5/14	4,280	3,140	--	1,800	--
4.82	6/14	4,480	3,840	2,250	2,240	1,390
5.00	7/14	4,970	4,110	--	2,630	--
5.18	8/14	5,080	--	--	2,720	1,500
5.37	9/14	5,700	4,280	2,530	--	--
5.57	10/14	--	--	2,730	2,890	--
5.79	11/14	--	5,700	3,000	3,120	1,650
6.07	12/14	--	--	3,830	3,390	--
6.47	13/14	--	--	4,300	3,820	1,790
Slope		3.46	3.57	6.52	4.86	12.78
Intercept		-7.82	-7.75	-17.07	-11.25	-35.26
LT_{50}		5,060	3,780	2,430	2,210	1,410

Table 6d. Observed cumulative mortality-time data for zebrafish exposed to pure solutions of zinc in soft water

Probit mortality	Fraction of fish in tank	[Zn^{++}] mg/l 12.90	14.23	16.08	18.26
		Time (minutes)			
3.53	1/14	1,500	--	1,120	--
3.93	2/14	2,740	730	1,700	--
4.21	3/14	2,870	1,120	1,940	720
4.43	4/14	5,640	1,400	2,130	820
4.64	5/14	--	1,630	2,400	880
4.82	6/14	--	1,940	--	900
5.00	7/14	--	2,250	--	--
5.18	8/14	--	--	--	1,000
5.37	9/14	--	2,780	--	1,030
5.57	10/14	--	3,010	--	--
5.79	11/14	--	--	--	1,120
6.07	12/14	--	--	--	1,330
6.47	13/14	--	--	--	3,200
Slope			2.64		3.53
Intercept			-3.79		-5.51
LT_{50}			2,120		950

Table 6e. Observed cumulative mortality-time data for zebrafish exposed to pure solutions of zinc in "tap" water

Probit mortality	Fraction of fish in tank	[Zn^{++}] mg/l			
		17.65	22.09	29.76	37.60
		Time (minutes)			
3.53	1/14	340	--	--	--
3.93	2/14	570	1,350	330	490
4.21	3/14	630	1,460	--	--
4.43	4/14	--	--	480	580
4.64	5/14	1,120	--	570	630
4.82	6/14	1,720	1,710	590	710
5.00	7/14	2,340	--	620	--
5.18	8/14	3,550	1,870	860	--
5.37	9/14	4,110	2,040	1,010	760
5.57	10/14	--	--	--	--
5.79	11/14	5,020	2,680	1,110	870
6.07	12/14	--	5,460	1,460	960
6.47	13/14	--	--	2,040	1,020
Slope		1.69	3.35	3.23	7.76
Intercept		-0.66	-6.10	-4.18	-17.05
LT_{50}		2,200	2,040	700	690

Table 6f. Observed cumulative mortality-time data for zebrafish exposed to pure solutions of zinc in very hard water.

Probit mortality	Fraction of fish in tank	[Zn^{++}] mg/l			
		23.13	26.26	31.73	37.97
		Time (minutes)			
3.53	1/14	620	--	560	--
3.93	2/14	780	590	590	--
4.21	3/14	940	--	--	--
4.43	4/14	1,670	780	780	--
4.64	5/14	1,960	--	--	560
4.82	6/14	--	1,090	900	--
5.00	7/14	--	--	1,420	580
5.18	8/14	--	1,420	1,660	610
5.37	9/14	--	1,470	1,950	770
5.57	10/14	--	1,660	3,040	--
5.79	11/14	--	1,950	--	--
6.07	12/14	--	2,490	--	890
6.47	13/14	--	--	--	--
Slope			3.43	2.55	5.69
Intercept			-5.56	-3.08	-10.84
LT_{50}			1,190	1,480	610

Table 6g. Observed cumulative mortality-time data for zebrafish exposed to mixed solutions of copper and zinc in soft water

Probit mortality	Fraction of fish in tank	[Cu-Zn] [as Cu^{++}] mg/l			
		0.118	0.166	0.198	0.258
		Time (minutes)			
3.53	1/14	1,290	--	--	--
3.93	2/14	3,100	1,000	--	--
4.21	3/14	--	--	--	--
4.43	4/14	4,840	1,240	550	--
4.64	5/14	5,450	1,380	970	--
4.82	6/14	--	1,540	1,110	--
5.00	7/14	--	1,720	1,170	550
5.18	8/14	--	--	--	--
5.37	9/14	--	--	1,470	--
5.57	10/14	--	2,450	1,600	910
5.79	11/14	--	--	1,650	--
6.07	12/14	--	2,880	1,720	1,170
6.47	13/14	--	--	3,870	1,240
Slope			4.37	2.80	3.81
Intercept			-9.12	-3.43	-5.51
LT_{50}			1,710	1,030	580

Table 6h. Observed cumulative mortality-time data for zebrafish exposed to mixed solutions of copper and zinc in "tap" water

Probit mortality	Fraction of fish in tank	[Cu-Zn] [as Cu++] mg/l				
		0.088	0.147	0.132	0.201	0.225
		Time (minutes)				
3.53	1/14	1,730	580	720	500	--
3.93	2/14	2,190	690	880	--	--
4.21	3/14	--	--	1,030	--	--
4.43	4/14	2,700	740	1,070	730	360
4.64	5/14	3,420	850	--	780	--
4.82	6/14	3,880	940	--	--	--
5.00	7/14	4,120	1,000	1,370	890	--
5.18	8/14	--	--	1,390	--	--
5.37	9/14	--	1,040	1,480	910	--
5.57	10/14	--	1,340	1,730	1,040	500
5.79	11/14	--	1,360	--	1,140	--
6.07	12/14	--	3,390	1,890	1,380	590
6.47	13/14	--	5,730	3,420	1,490	650
Slope		3.73	2.66	4.75	6.16	7.69
Intercept		-8.52	-3.13	-9.92	-13.10	-15.17
LT_{50}		4,170	1,160	1,380	870	420

Table 6i. Observed cumulative mortality-time data for zebrafish exposed to mixed solutions of copper and zinc in very hard water

Probit mortality	Fraction of fish in tank	[Cu-Zn] [as Cu^{++}] mg/l			
		0.356	0.364	0.487	0.520
		Time (minutes)			
3.53	1/14	500	580	--	230
3.93	2/14	2,570	1,430	--	280
4.21	3/14	2,770	--	--	350
4.43	4/14	3,170	--	--	--
4.64	5/14	3,480	1,670	860	--
4.82	6/14	--	2,650	--	--
5.00	7/14	--	3,160	--	870
5.18	8/14	--	--	1,110	--
5.37	9/14	--	--	1,250	--
5.57	10/14	--	--	1,530	1,120
5.79	11/14	--	--	1,860	1,340
6.07	12/14	--	--	2,280	1,540
6.47	13/14	--	--	2,560	1,980
Slope			2.04	3.48	2.93
Intercept			-2.18	-5.50	-3.35
LT_{50}			3,270	1,040	700

Table 7a. Median mortality times (LT_{50}) of zebrafish exposed to several concentrations of copper in soft, "tap" or very hard water

Water hardness	Heavy metal	Concentration (mg/l)	LT_{50} (min)
Soft	Copper	0.160	2,780
		0.190	2,290
		0.260	2,250
		0.390	1,290
		0.460	1,440
		0.570	330
"Tap"	Copper	0.270	3,670
		0.310	3,040
		0.350	2,650
Very hard	Copper	0.690	5,060
		0.950	3,780
		1.210	2,430
		1.440	2,210
		1.940	1,410

Table 7b. Median mortalit times (LT_{50}) of zebrafish exposed to several concentrations of zinc in soft, "tap", or very hard water

Water hardness	Heavy metal	Concentration (mg/l)	LT_{50} (min)
Soft	Zinc	14.23	2,120
		18.26	950
"Tap"	Zinc	17.65	2,200
		22.09	2,040
		29.76	700
		37.66	690
Very hard	Zinc	26.26	1,190
		31.73	1,480
		37.97	610

Table 7c. Median mortality times (LT_{50}) of zebrafish exposed to several concentrations of copper-zinc mixtures in soft, tap, or very hard water, expressed as equipotent concentrations of copper

Water hardness	Heavy metal	Concentration (mg/l)	LT_{50} (min)
Soft	Copper-zinc	0.166	1,710
		0.198	1,030
		0.258	580
"Tap"	Copper-zinc	0.088	4,170
		0.132	1,380
		0.147	1,160
		0.201	870
		0.225	420
Very hard	Copper-zinc	0.364	3,270
		0.487	1,040
		0.520	700

Table 8. Linear regressions representing Log LT_{50} vs Log concentration for zebrafish exposed to copper and/or zinc at each of three water hardnesses

			Correlation Coefficients	
Copper soft	Y =	2.49 - 1.30x	-0.843**	(9)
Copper "tap"	Y =	2.85 - 1.26x	-0.999*	(10)
Copper very hard	Y =	3.52 - 1.25x	-0.993*	(11)
Zinc soft	Y =	7.04 - 3.22x	-1.000*****	(12)
Zinc "tap"	Y =	5.63 - 1.81x	-0.932***	(13)
Zinc very hard	Y =	5.67 - 1.79x	-0.713****	(14)
Copper-zinc soft	Y =	1.30 - 2.46x	-0.997**	(15)
Copper-zinc "tap"	Y =	1.30 - 2.19x	-0.971**	(16)
Copper-zinc very hard	Y =	1.69 - 4.24x	-0.997**	(17)

* significant at 0.01 level ($p < .01$)

** significant at 0.05 level ($p < .05$)

*** significant at 0.10 level such that ($.10 > p > .05$)

**** not significant

***** too few data points

Table 9. Key for symbols representing the heavy metal-water hardness regimes depicted in Figures 8 to 18

KEY

	Soft	"Tap"	Very hard
Copper			
Zinc			
Copper-zinc			

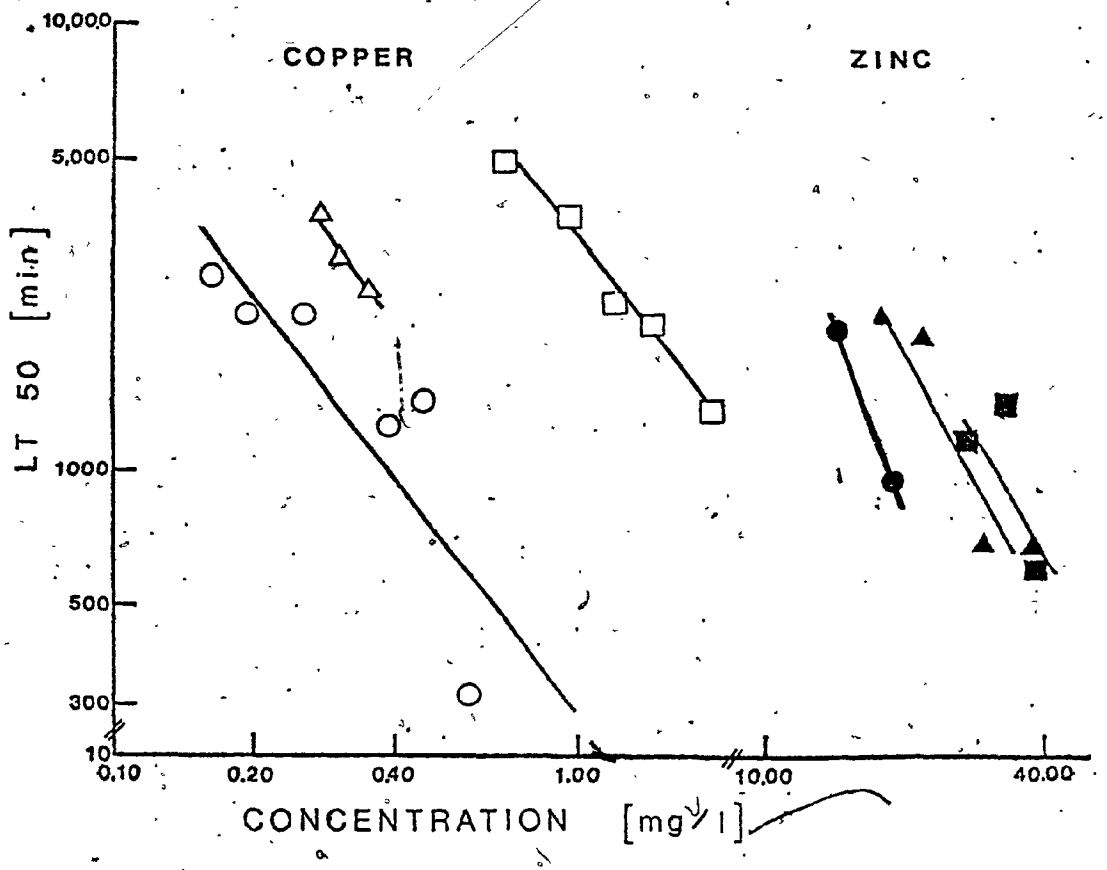

Figure 8. Median mortality-times (LT_{50}) of zebrafish exposed to several concentrations of copper or zinc in soft, moderately hard or "tap", or very hard water

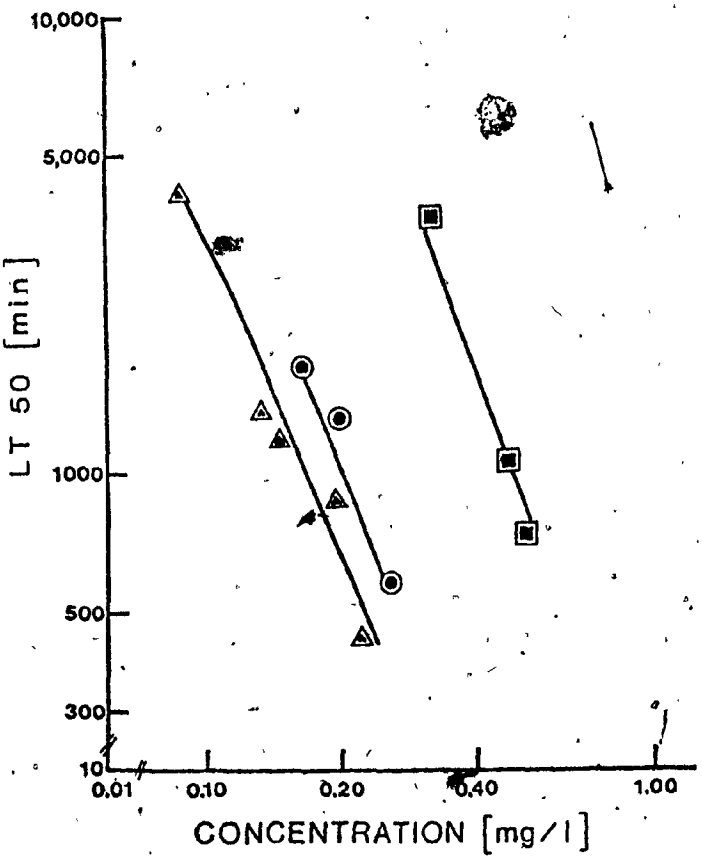

Figure 9. Median mortality-times (LT_{50}) of zebrafish exposed to mixtures of copper and zinc in soft, moderately hard or "tap", or very hard water

regimes. They were significant (at p = 0.05) for those fitted to data representing copper-soft, copper-zinc- soft and very hard water regimes. The lack of significant correlation of the linear regressions for zinc-soft, tap, and very hard water suggested that these regressions were poor fits to the data. Student's "t"-tests comparing regression lines which represented copper-soft, tap, and very hard water regimes, suggested that these lines were not significantly unparallel (at p = 0.05). Similar results were shown for the copper-zinc mixture. Lack of significant correlation of the log-log relationship of LT_{50} versus toxicant concentration for the zinc data prevented the application of these data to the statistical tests for parallelism. The toxicity curves for the copper-zinc mixtures were then compared with those predicted according to the model for concentration-addition (see test for concentration-addition). Results of an F-test (Schor, 1968) suggest that there was a significant difference (at p = 0.05) between observed and predicted median mortality times for soft, tap, and very hard water data (Figures 10, 11, and 12). These predicted lines are actually the individual regressions for copper at each of the respective water hardnesses.

Lethal Response Study - Dose-Response Data for Individual Heavy Metals

Dose-response data obtained from all 96-hour lethal bioassays, in which lots of 14 fish were exposed to copper or zinc at each water hardness, are compiled in Tables 10a and 10b. Chi-square

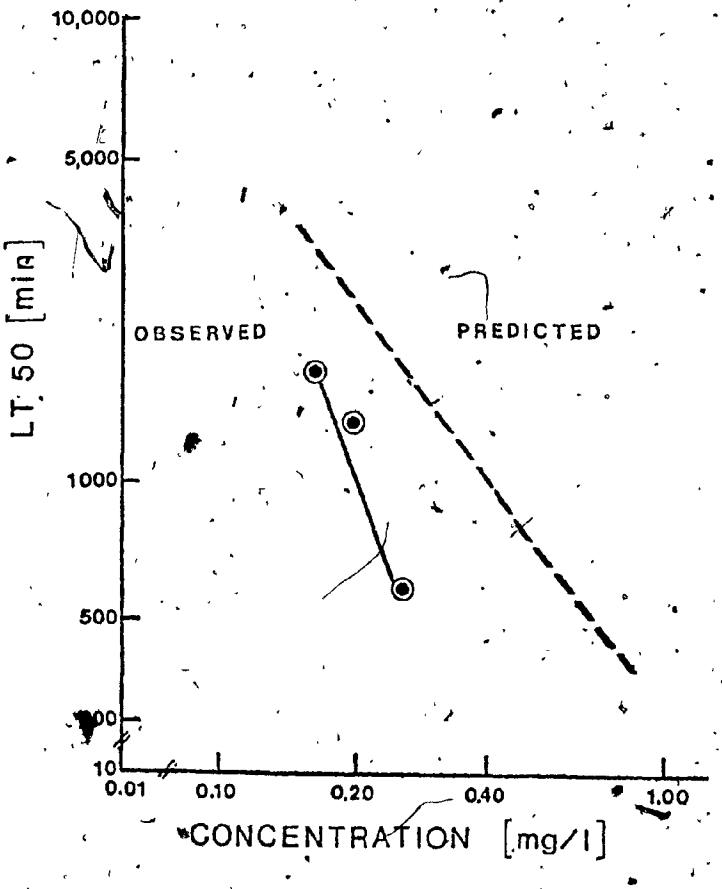

Figure 10. Observed median mortality-time curve for zebrafish exposed to mixtures of copper and zinc in soft water. The curve for copper (soft) as a discrete toxicant is depicted for reference. Concentration-time co-ordinates which are predicted for the mixture by the model of concentration-addition are found at the intersections of vertical lines raised from observed co-ordinates and the extrapolated lethal response curve for copper

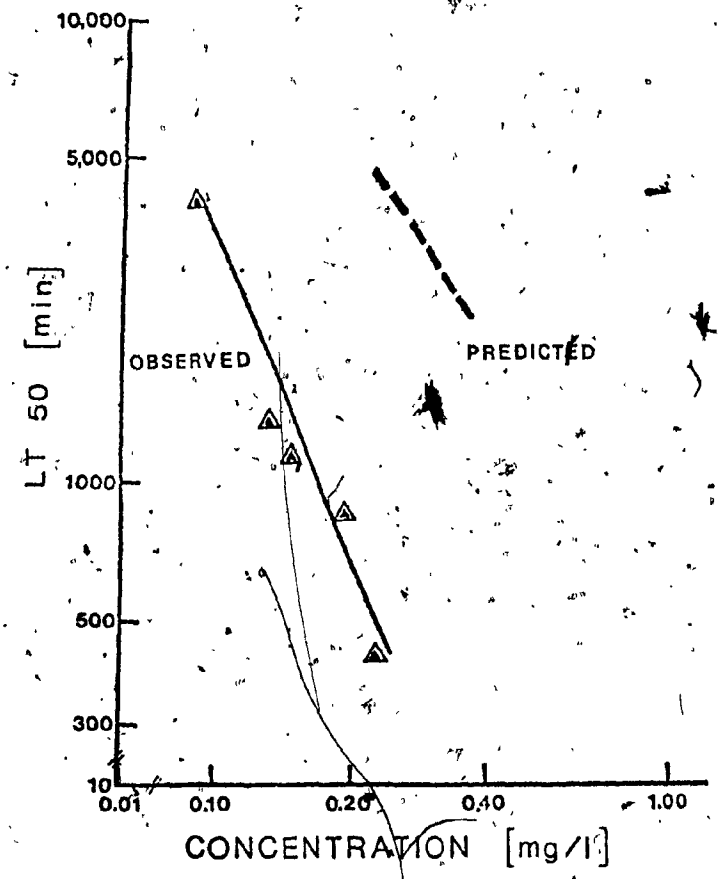

Figure 11. Observed median mortality-time curve for zebrafish exposed to mixtures of copper and zinc in moderately hard or "tap" water. The curve for copper ("tap") as a discrete toxicant is depicted for reference. Concentration-time co-ordinates which are predicted for the mixture by the model of concentration-addition are found at the intersections of vertical lines raised from observed co-ordinates and the extrapolated lethal response curve for copper

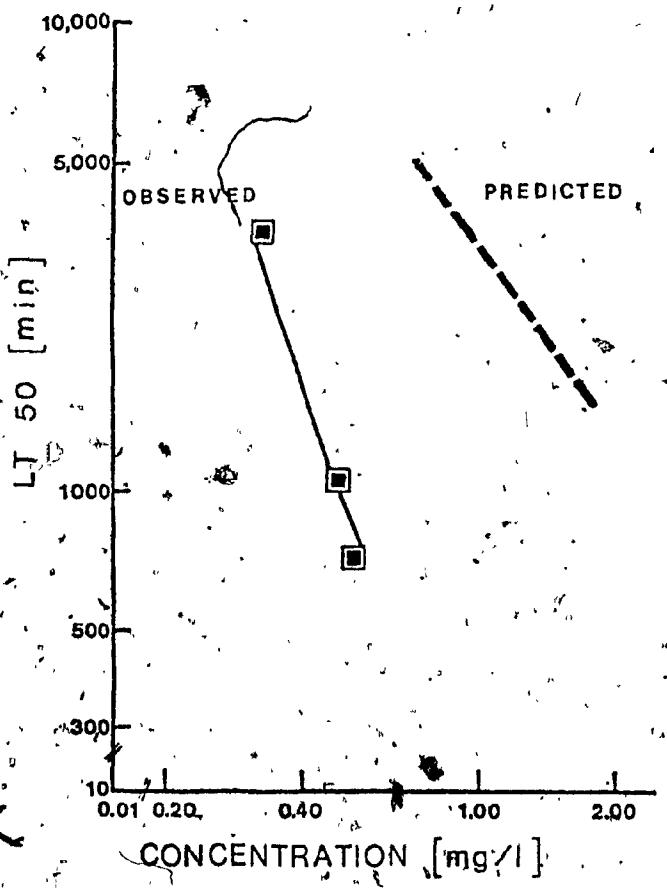

Figure 12. Observed median mortality time curve for zebrafish exposed to mixtures of copper and zinc in very Hard water. The curve for copper (very hard) as a discrete toxicant is depicted for reference. Concentration-time co-ordinates which are predicted for the mixture by the model of concentration-addition are found at the intersections of vertical lines raised from observed co-ordinates and the extrapolated lethal response curve for copper

Table 10a. Lethal response data for zebrafish exposed to several copper concentrations at each of three water hardness regimes

Water hardness regimes	Mean assayed level of copper (mg/l ± SD)	# of fish	Mean wet weight of fish (g ± SD)	Observed % mortality in 96 hrs
Soft	0.089 ± 0.01	14	0.54 ± 0.02	0
	0.092 ± 0.01	14	0.74 ± 0.03	0
	0.105 ± 0.01	14	0.75 ± 0.03	14
	0.115 ± 0.01	14	0.46 ± 0.02	36
	0.157 ± 0.02	14	0.63 ± 0.03	71
	0.193 ± 0.01	14	0.63 ± 0.03	71
	0.256 ± 0.01	14	1.01 ± 0.06	86
	0.386 ± 0.03	14	0.75 ± 0.03	100
	0.456 ± 0.03	14	0.84 ± 0.03	100
"Tap"	0.050 ± 0.02	10	0.29 ± 0.03	0
	0.090 ± 0.02	10	0.42 ± 0.04	0
	0.130 ± 0.03	10	0.42 ± 0.04	10
	0.150 ± 0.04	10	0.29 ± 0.03	20
	0.170 ± 0.05	10	0.29 ± 0.03	30
	0.220 ± 0.08	10	0.42 ± 0.04	30
	0.220 ± 0.05	14	0.32 ± 0.03	43
	0.240 ± 0.07	10	0.42 ± 0.04	30
	0.270 ± 0.04	10	0.42 ± 0.04	50
	0.310 ± 0.03	14	0.43 ± 0.03	100
	0.310 ± 0.06	10	0.42 ± 0.04	60
	0.350 ± 0.05	10	0.42 ± 0.04	80
Very hard	0.300 ± 0.01	14	0.34 ± 0.03	0
	0.420 ± 0.03	14	0.26 ± 0.03	7
	0.690 ± 0.03	14	0.26 ± 0.03	64
	0.952 ± 0.01	14	0.34 ± 0.03	79
	1.211 ± 0.04	14	0.26 ± 0.03	93
	1.442 ± 0.04	14	0.33 ± 0.03	100
	1.941 ± 0.05	14	0.33 ± 0.03	100

Table 10b. Lethal response data for zebrafish exposed to several zinc concentrations at each of three water hardness regimes

Water hardness regime	Mean assayed level of zinc (mg/l ± SD)	# of fish	Mean wet weight of fish (g ± SD)	Observed % mortality in 96 hrs
Soft	8.19 ± 1.23	14	0.63 ± 0.03	0
	8.51 ± 0.03	14	0.75 ± 0.03	0
	9.80 ± 0.03	14	0.63 ± 0.03	14
	9.83 ± 1.50	14	0.84 ± 0.02	7
	10.96 ± 1.50	14	0.74 ± 0.03	14
	11.86 ± 0.30	14	0.75 ± 0.03	14
	12.34 ± 1.65	14	0.55 ± 0.03	14
	12.95 ± 0.50	13	0.54 ± 0.02	31
	14.23 ± 1.97	14	0.55 ± 0.03	71
	16.08 ± 2.39	14	0.74 ± 0.03	36
	18.26 ± 2.65	14	0.55 ± 0.03	93
"Tap"	6.69 ± 0.37	14	0.63 ± 0.03	0
	7.84 ± 0.49	14	0.75 ± 0.03	0
	10.17 ± 1.16	14	0.84 ± 0.03	7
	14.33 ± 0.19	14	0.65 ± 0.03	14
	15.35 ± 0.74	14	0.65 ± 0.03	14
	17.65 ± 0.68	14	0.64 ± 0.04	79
	18.23 ± 1.23	14	0.56 ± 0.02	43
	22.09 ± 0.91	14	1.09 ± 0.06	86
	29.76 ± 1.16	14	0.73 ± 0.05	93
	37.66 ± 2.29	14	0.83 ± 0.02	100
Very hard	21.42 ± 2.22	14	0.34 ± 0.03	14
	23.13 ± 2.41	14	0.43 ± 0.03	36
	26.26 ± 2.75	14	0.47 ± 0.06	86
	31.73 ± 3.33	14	0.34 ± 0.03	71
	37.97 ± 3.00	14	0.47 ± 0.06	86

tests indicated that the dose-response coordinates listed in these tables[1] could be treated as discrete sets of data. Linear regressions were thus fitted to the lethal response data of copper or zinc at each hardness regime (Table 11; Figure 13). However significant heterogeneity between observed and predicted responses were found for the zinc-hard water regression at the 0.05 probability level. Results of chi-square for parallelism tests suggests that linear regressions representing relationships between and in copper and zinc toxicity at different water hardnesses are parallel. The slopes of these regressions were not significantly different at the 0.05 probability level.

Comparison of the 96-hr LC_{50}'s for copper and zinc at each water hardness regime (Table 12) indicate that at any of the water hardnesses tested copper is much more toxic than zinc. Relative potency factors between copper and zinc, determined at each level of hardness, are listed with 95% fiducial limits in Table 13. In addition, comparison of the 96-hr LC_{50}'s for copper or zinc at different water hardnesses (Table 12) indicates that for each heavy metal there is a progressive decrease in potency with increase in water hardness (Table 14). Linear regressions were therefore fitted to the coordinates which represent the relationship for copper and

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[1]Most of the copper-tap water bioassay tanks contained only ten fish each. These data were obtained from Arthur Hewitt, Concordia University, Montreal, Quebec.

Table 11. Linear regressions fitted by probit analysis to dose-response data recorded for copper or zinc bioassays at each of three water hardness regimes

Metal	Hardness	Regressions	LC_{50}	95% (fiducial limits)	
Copper	soft	Y = 10.601 + 6.881x	0.154	(0.138 - 0.173)	(18)
	"tap"	Y = 8.312 + 5.333x	0.239	(0.213 - 0.271)	(19)
	very hard	Y = 6.151 + 6.673x	0.672	(0.572 - 0.771)	(20)
Zinc	soft	Y = -5.512 + 9.023x	14.62	(13.69 - 16.05)	(21)
	"tap"	Y = -5.133 + 8.125x	17.67	(16.21 - 19.33)	(22)
	very hard	Y = -5.604 + 7.558x	25.30	(22.10 - 28.01)	(23)

where Y = probit of % mortality

x = log of concentration

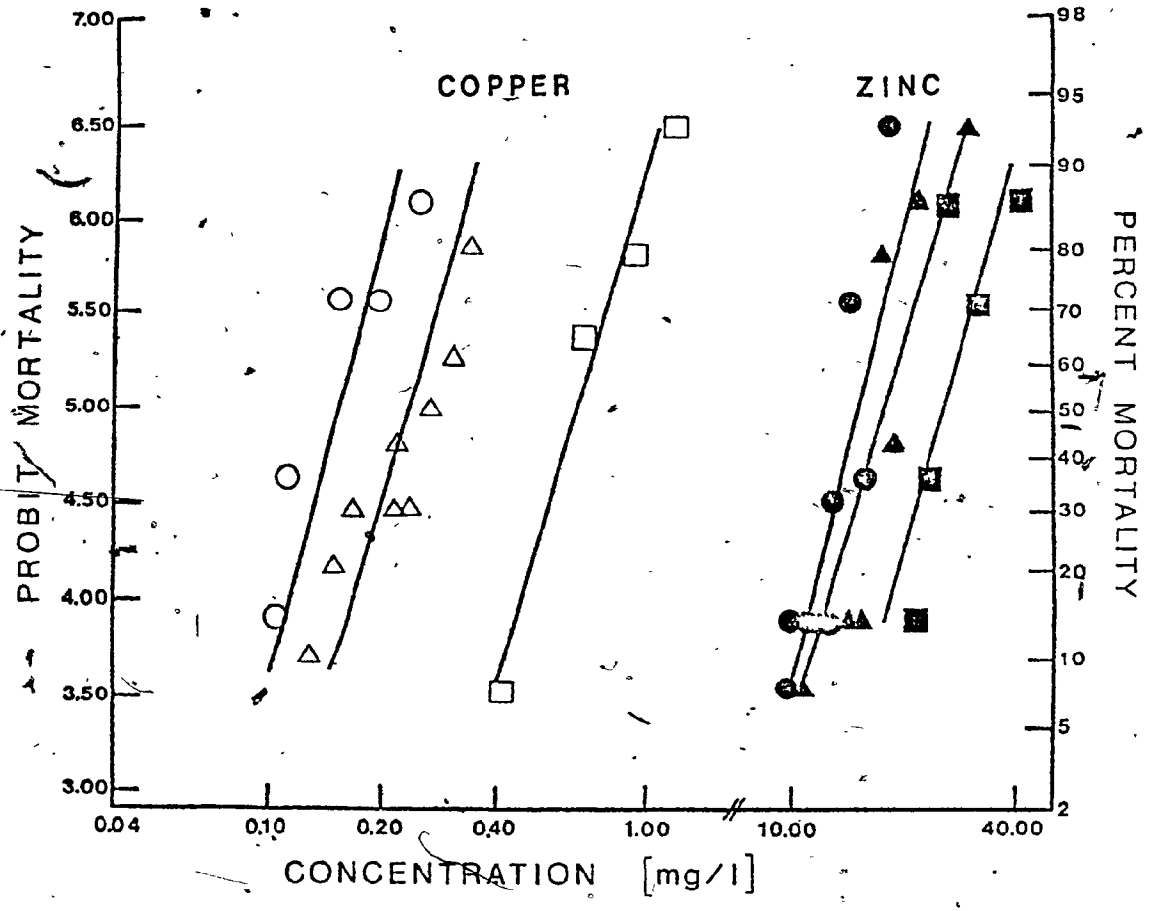

Figure 13. Lethal response curves for zebrafish exposed for 96 hours to several concentrations of copper or zinc in soft, moderately hard or "tap", or very hard water

Table 12. 96-Hour LC_{50}'s obtained from bioassays of copper, zinc, or their mixtures at each of three water hardnesses

Heavy metal	Water hardness	96 hr LC_{50} (fiducial limits)
Copper	soft	0.154 (0.138 - 0.173)
	"tap"	0.239 (0.213 - 0.271)
	very hard	0.672 (0.572 - 0.771)
Zinc	soft	14.62 (13.60 - 16.05)
	"tap"	17.67 (16.21 - 19.33)
	very hard	25.30 (22.10 - 28.01)
Copper-zinc (expressed as "copper")	soft	0.133 (0.123 - 0.145)
	"tap"	0.103 (0.095 - 0.113)
	very hard	0.366 *

* not available

Table 13. Relative potency factors between single toxicants, copper and zinc, at each of three water hardnesses (regressions constrained to parallelism)

Hardness	Relative potency factor	95% Fiducial limits
Soft	0.010	(0.009 - 0.012)
"Tap"	0.013	(0.012 - 0.015)
Very hard	0.027	(0.022 - 0.032)

eg. Zn. is only 0.010 times as potent as Cu in soft water, or conversely, Cu is 97.97 times more potent than Zn. in soft water.

Where relative potency factor $= \frac{LC_{50}\ \text{Copper}}{LC_{50}\ \text{Zinc}}$

Table 14. Relative potency factors for single toxicants, copper and zinc, between different water hardnesses (see Table 12)

Toxicants	M	N	Relative potency factor	95% Fiducial limits
Copper	Soft	"Tap"	0.693	(0.597 - 0.807)
	"Tap"	Very hard	0.359	(0.301 - 0.434)
	Soft	Very hard	0.245	(0.207 - 0.293)
Zinc	Soft	"Tap"	0.834	(.0752 - 0.938)
	"Tap"	Very hard	0.697	(0.614 - 0.799)
	Soft	Very hard	0.579	(0.518 - 0.664)

where relative potency factor $= \frac{LC_{50}M}{LC_{50}N}$

and M and N represent the hardness regimes under comparison

zinc respectively (Table 12; Figure 14). However, these regressions only poorly represented the distribution of coordinates for copper and zinc potency at each level of hardness in this study (at p = 0.02) (Table 23). A comparison of the slopes of these regressions indicates that water hardness cations, both calcium and magnesium, had a possibly greater influence in increasing the tolerance of fish to copper than to zinc.

Test for Concentration-Addition

The slopes of the regressions for the individual heavy metals, copper and zinc, at each water hardness were not found to be significantly different (at p = 0.05) according to the chi-square test. To critically test the hypothesis of concentration-addition, these regression lines were constrained to parallelism. The "constrained" regression equations are listed in Table 15. These new functions were examined for their effectiveness to represent the respective dose-response data. All lines fitted were representative of the original data (at p = 0.05), excluding zinc-tap and zinc-very hard regressions which were acceptable at only the 0.02 level ($0.02 < p < 0.05$).

Lethal response data for mixtures of copper and zinc at each water hardness are presented in Table 16 (Figure 15). As previously mentioned, copper is the more toxic of the two heavy metals at each hardness regime and therefore, was selected as the reference toxicant. Mean assayed concentrations of zinc in each mixture of the

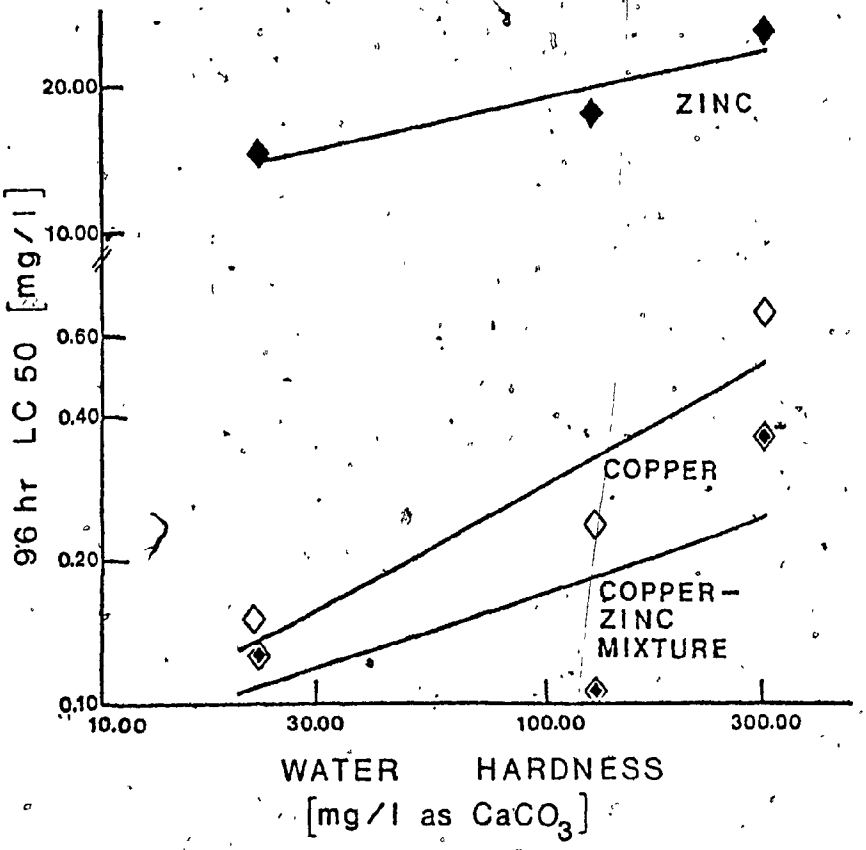

Figure 14. Linear regressions representing the relationship between water hardness and the 96 hour LC_{50} of copper and/or zinc for the zebrafish. The curve for copper as a discrete toxicant, is depicted for reference to the copper-zinc regression

Table 15. Computed probit analysis regressions (constrained to parallelism) of copper or zinc data at each of three water hardnesses.

Copper soft	$Y = 11.308 + 7.724\ x$	(24)
Zinc soft	$Y = -4.070 + 7.724\ x$	(25)
Copper "Tap"	$Y = 9.080 + 6.549\ x$	(26)
Zinc "Tap"	$Y = -3.178 + 6.549\ x$	(27)
Copper very hard	$Y = 6.182 + 6.914\ x$	(28)
Zinc very hard	$Y = -4.685 + 6.914\ x$	(29)

Table 16a. Lethal response data for zebrafish exposed to mixtures of copper and zinc at each of three water hardnesses. Responses predicted in accordance with the model of concentration-addition

Mean assayed level of copper (mg/l ± SD)	Mean assayed level of zinc (mg/l ± SD)	Zinc as equipotent level of copper (mg/l)	Total level of mixture as copper (mg/l)	Copper as proportion of total level of mixture	Observed mort. in 96 hrs (%)	(probit)	Predicted mort. in 96 hrs (%)	(probit)
Soft								
0.020 ± 0.01	5.05 ± 0.18	0.052	0.072	0.28	7.14	3.53	0.60	2.48
0.023 ± 0.01	5.29 ± 0.23	0.054	0.077	0.30	7.14	3.53	1.10	2.71
0.026 ± 0.01	5.72 ± 0.16	0.058	0.084	0.31	0.00	–	2.30	3.00
0.030 ± 0.01	6.27 ± 0.23	0.064	0.094	0.32	14.28	3.93	5.20	3.38
0.034 ± 0.01	6.68 ± 0.16	0.068	0.102	0.33	0.00	–	8.90	3.65
0.041 ± 0.01	7.52 ± 0.36	0.077	0.118	0.35	35.70	4.64	19.50	4.14
0.046 ± 0.02	8.18 ± 0.33	0.083	0.129	0.36	21.42	4.21	28.70	4.44
0.070 ± 0.01	9.45 ± 0.56	0.096	0.166	0.42	100.00	–	61.20	5.28
0.090 ± 0.02	10.58 ± 0.25	0.108	0.198	0.45	92.82	6.47	81.00	5.88
0.120 ± 0.02	13.52 ± 0.76	0.138	0.258	0.47	100.00	–	96.10	6.76
Tap								
0.019 ± 0.00	3.47 ± 0.31	0.047	0.066	0.29	0		0	1.35
0.019 ± 0.00	2.79 ± 0.29	0.037	0.056	0.34	7.14	3.53	0	0.88
0.025 ± 0.01	3.90 ± 0.21	0.052	0.077	0.32	0	–	0	1.79
0.036 ± 0.01	3.84 ± 0.30	0.052	0.088	0.41	50.00	5.00	0.20	2.17
0.045 ± 0.02	4.68 ± 0.18	0.063	0.108	0.42	28.56	4.43	1.20	2.75
0.055 ± 0.01	6.88 ± 0.26	0.092	0.147	0.37	92.82	6.47	8.50	3.63
0.059 ± 0.04	5.42 ± 0.32	0.073	0.132	0.45	92.82	6.47	4.70	3.32
0.090 ± 0.02	8.27 ± 0.21	0.111	0.201	0.45	100.00	–	31.40	4.52
0.094 ± 0.00	9.76 ± 1.36	0.131	0.225	0.42	100.00	–	43.50	4.84
Very Hard								
0.064 ± 0.01	5.89 ± 0.51	0.158	0.222	0.29	0		0	1.66
0.074 ± 0.01	7.28 ± 1.11	0.195	0.269	0.28	0	3.00*	0.30	2.24
0.104 ± 0.00	9.70 ± 0.99	0.260	0.364	0.29	50.00	5.00	3.20	3.15
0.108 ± 0.01	9.26 ± 0.68	0.248	0.356	0.30	35.10	4.64	2.70	3.08
0.181 ± 0.01	12.64 ± 0.98	0.339	0.520	0.35	100		21.70	4.22
0.184 ± 0.01	11.29 ± 0.98	0.303	0.487	0.38	100.00	7.00*	16.40	4.02

* Corrected mortalities determined according to Litchfield & Wilcoxin's (1949) Best-Fit Method.

Table 16b. Linear regressions, fitted to concentration response data, recorded for multiple copper-zinc bioassays at each of three water hardness regimes. Mixture levels are expressed additively in terms of copper

Hardness	Regression	
Soft	$Y = 10.521 + 6.326x$	(30)
"Tap"	$Y = 12.184 + 7.100x$	(31)
Very hard	$Y = 11.794 + 15.559x$	(32)

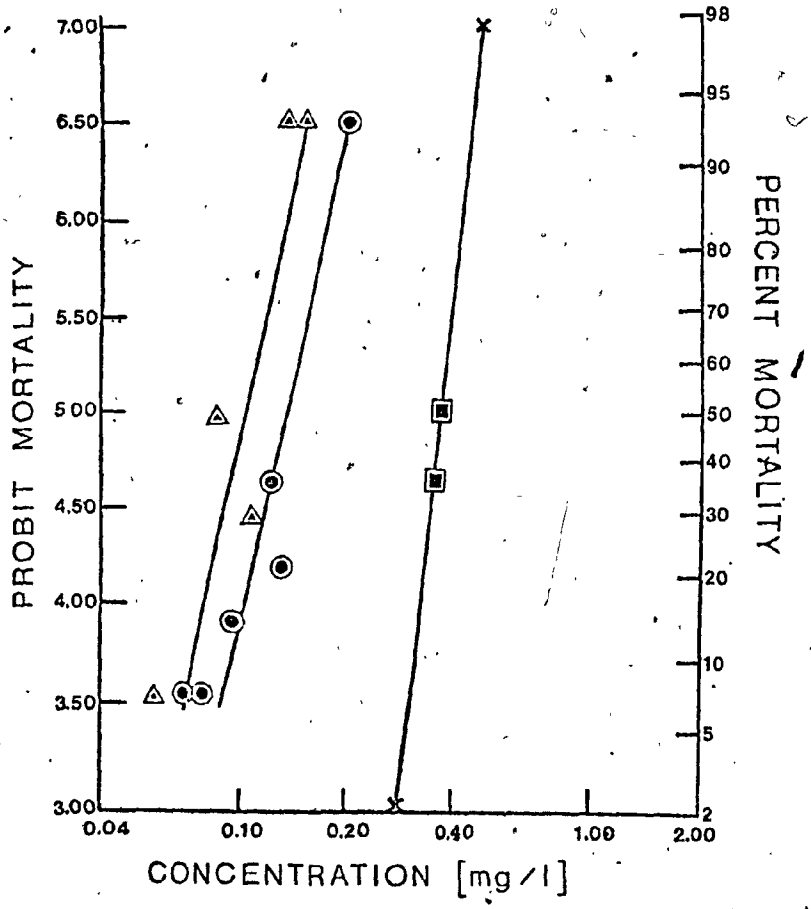

Figure 15. Lethal response curves for zebrafish exposed for 96 hours to mixtures of copper and zinc in soft, moderately hard or "tap", or very hard water

test series was converted to copper equivalents (Materials and Methods). These values, as well as the total effective concentration of each mixture, as copper equivalents, are listed in Table 16. Results of chi-square tests comparing observed responses with those predicted, according to the model of concentration-addition, for mixtures of copper and zinc, suggest that there was a significant difference (at $p = 0.05$) between observed and predicted responses for soft, tap, and very hard water data (Figure 16, 17, and 18). The concentration-addition hypothesis was thus rejected for the mixtures.

Test for Response-Addition

After rejection of the concentration-addition hypothesis, the assumption that the dose-response data fitted the response-addition model was tested (Materials and Method). Mean assayed concentrations of individual heavy metals, copper and zinc, present in the mixtures used, are listed in Table 17. The concentration of each constituent was substituted into the respective linear regression for that heavy metal, and the predicted response in percent mortality was computed. Responses, converted from percentages to proportions, were then substituted into the equations 6, 7, and 8 (Materials and Methods) for models of response-addition representing correlations of tolerances of zebrafish to copper and zinc. The responses predicted in accordance with the model of response-addition are listed in Table 17. Predicted responses were lower than observed. The response-addition hypothesis was thus rejected.

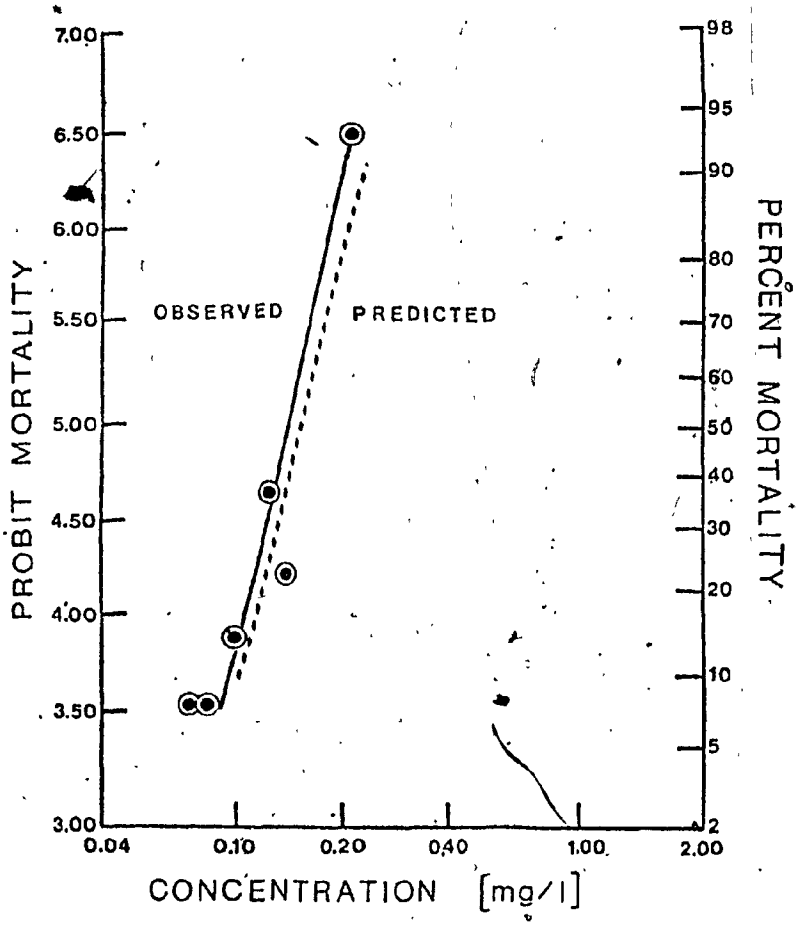

Figure 16. Observed lethal response curve for zebrafish exposed for 96 hours to mixtures of copper and zinc in soft water. The curve for copper (soft) as a discrete toxicant is depicted for reference. Dose response co-ordinates which are predicted for the mixture by the model of concentration-addition are found at the intersections of vertical lines dropped from observed co-ordinates and the extrapolated lethal response curve for copper

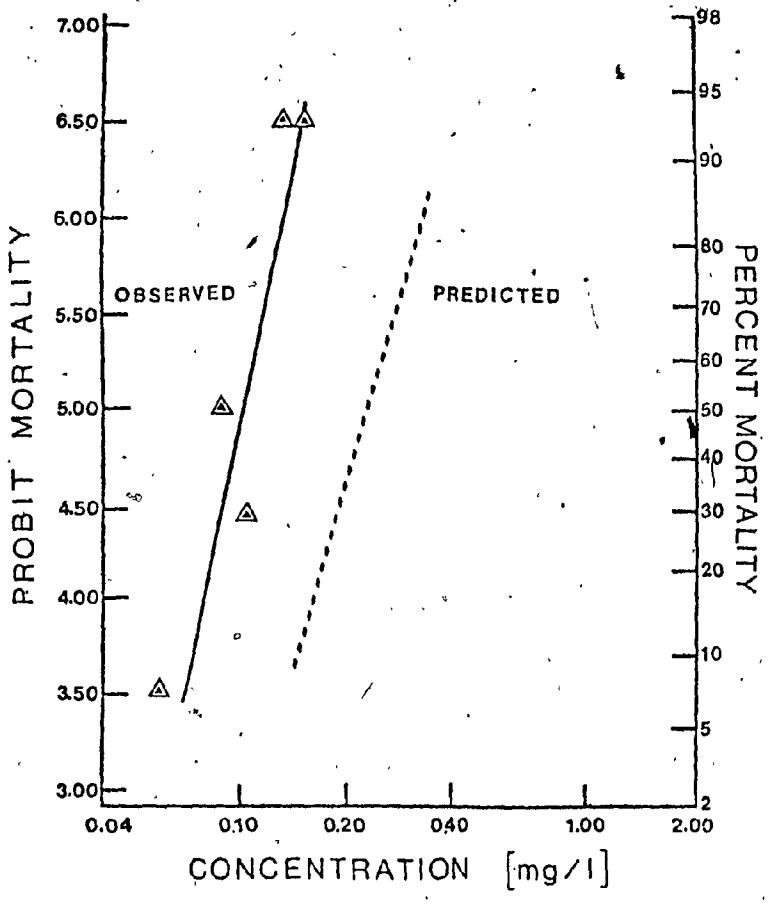

Figure 17. Observed lethal response curve for zebrafish exposed for 96 hours to mixtures of copper and zinc in moderately hard or "tap" water. The curve for copper ("tap") as a discrete toxicant is depicted for reference. Dose-response co-ordinates which are predicted for the mixture by the model of concentration-addition are found at the intersections of vertical lines dropped from observed co-ordinates and the extrapolated lethal response curve for copper

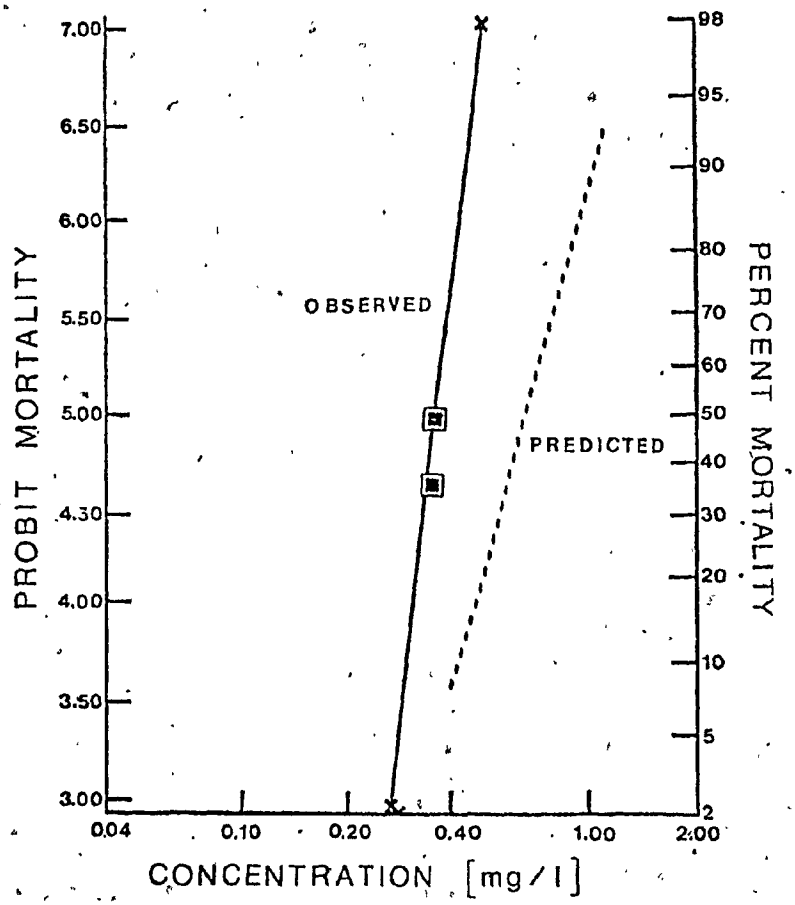

Figure 18. Observed lethal response curve for zebrafish exposed for 96 hours to mixtures of copper and zinc in very hard water. The curve for copper (very hard) as a discrete toxicant is depicted for reference. Dose-response co-ordinates which are predicted for the mixture by the model of concentration-addition are found at the intersections of vertical lines dropped from observed co-ordinates and the extrapolated lethal response curve for copper

Table 17. Lethal response data for zebrafish exposed to mixtures of copper and zinc. Responses predicted in accordance with the model of Response-Addition

Water hardness	Mean assayed copper concentration (mg/l ± SD)	Mean assayed zinc concentration (mg/l ± SD)	Observed % mortality in 96 hours	Predicted % mortality if r = -1	r = 0	r = +1
Soft	0.020 ± 0.01	5.05 ± 0.18	7	< 1	< 1	< 1
	0.023 ± 0.01	5.29 ± 0.23	7	< 1	< 1	< 1
	0.026 ± 0.01	5.72 ± 0.16	0	< 1	< 1	< 1
	0.030 ± 0.01	6.27 ± 0.23	14	< 1	< 1	< 1
	0.034 ± 0.01	6.68 ± 0.16	0	< 1	< 1	< 1
	0.041 ± 0.01	7.52 ± 0.36	36	< 1	< 1	< 1
	0.046 ± 0.02	8.18 ± 0.33	21	< 1	< 1	< 2
	0.070 ± 0.01	9.45 ± 0.56	100	< 6	< 6	< 5
	0.090 ± 0.02	10.58 ± 0.25	93	<16	<15	<11
	0.120 ± 0.02	13.52 ± 0.76	100	<61	<53	<38
"Tap"	0.019 ± 0.00	3.47 ± 0.31	0	< 1	< 1	< 1
	0.019 ± 0.00	2.79 ± 0.29	7	< 1	< 1	< 1
	0.025 ± 0.01	3.90 ± 0.21	0	< 1	< 1	< 1
	0.036 ± 0.01	3.84 ± 0.30	50	< 1	< 1	< 1
	0.045 ± 0.02	4.68 ± 0.18	29	< 1	< 1	< 1
	0.055 ± 0.01	6.88 ± 0.26	93	< 1	< 1	< 1
	0.059 ± 0.04	5.42 ± 0.32	93	< 1	< 1	< 1
	0.090 ± 0.02	8.27 ± 0.21	100	< 1	< 1	< 1
	0.094 ± 0.00	9.76 ± 1.36	100	< 3	< 3	< 2
Very hard	0.064 ± 0.01	5.89 ± 0.51	0	< 1	< 1	< 1
	0.074 ± 0.01	7.28 ± 1.11	0	< 1	< 1	< 1
	0.104 ± 0.00	9.70 ± 0.99	50	< 1	< 1	< 1
	0.108 ± 0.01	9.26 ± 0.68	36	< 1	< 1	< 1
	0.181 ± 0.01	12.64 ± 0.98	100	< 1	< 1	< 1
	0.184 ± 0.01	11.29 ± 0.98	100	< 1	< 1	< 1

Supra-Additive Synergism

The lethal toxicity of mixtures of copper and zinc at each water hardness was found to be greater than that predicted for concentration or response-addition. In order to be able to represent non-additive response data relative to that predicted for concentration-addition, we computed α/α_1, the relative "enhancement factor" for each mixture (Table 18). Results of enhancement factor computations suggest that the degree of supra-additivity increases relative to concentration-addition between soft and tap water, and remains relatively stable between tap and very hard water. The difference in the enhancement of toxicity of mixtures to that expected for concentration-addition at the respective water hardnesses was greatest in tap water, less in very hard, and least in soft water (Figure 14). A possible explanation for this phenomenon is offered in the Discussion.

Fish Tissue Analysis

Mean wet weights of fish subjected to tissue analysis are recorded in Table 19. All fish used were obtained from the same weight class. Values of mean metal content, as determined by atomic absorption spectrophometry, in µg element (copper, zinc, calcium, magnesium, and sodium) per gm dry tissue weight of gills excised from fish surviving the 48 hour exposure to various concentrations of copper and/or zinc, are listed in Table 20. Metal content of exposed whole body fish is listed in Table 21. Variances in heavy

Table 18. Computation of relative potency factor, α/α_1, used as a measure of the enhanced effect (ratio of observed/predicted values) of the toxic mixture of copper and zinc at each of three water hardnesses (Anderson & Weber 1975a, b)

Observed response (probits)	α	α_1	α/α_1
Soft			
3.53	0.098	0.072	1.37
3.53	0.098	0.078	1.26
3.93	0.111	0.094	1.18
4.64	0.137	0.118	1.16
4.21	0.120	0.129	0.93
6.47	0.236	0.198	1.19
			$\bar{x} = 1.18 \pm 0.16$
"Tap"			
3.53	0.106	0.056	1.90
5.00	0.178	0.088	2.02
4.43	0.146	0.108	1.35
6.47	0.298	0.147	2.03
6.47	0.298	0.132	2.26
			$\bar{x} = 1.91 \pm 0.34$
Very Hard			
7.00	1.313	0.487	2.70
5.00	0.675	0.364	1.85
4.64	0.598	0.356	1.68
3.00	0.347	0.269	1.29
			$\bar{x} = 1.88 \pm 0.59$

α = antilog of the abscissal value obtained from lethal response curves of copper, and corresponding to the magnitude of the observed response (see equations 24, 26, and 28).

α_1 = total concentration of mixture expressed additively in terms of copper.

Σ (mean assay level of copper and zinc as equipotent level of copper).

Table 19. Mean wet weights (mg) of zebrafish exposed to sublethal levels of copper, zinc or a mixture at each of the three water hardnesses prior to wet acid digestion

Heavy Metal	Soft	"Tap"	Very Hard
Copper	0.33 ± 0.05	0.31 ± 0.05	0.30 ± 0.07
Zinc	0.37 ± 0.09	0.33 ± 0.06	0.31 ± 0.05
Copper-zinc	0.34 ± 0.06	0.36 ± 0.04	0.30 ± 0.06
Control	0.34 ± 0.03	0.33 ± 0.06	0.31 ± 0.08

Table 20. Mean metal content of fish gills excised from fish surviving the 48 hour exposure to various sublethal concentrations of copper and/or zinc at 3 water hardnesses (ug element/g dry tissue weight)

Water hardness	Concentration of metal (mg/l) in exposure tanks		Mean metal content of fish gills (ug element/g dry tissue weight ± S.D.)				
	Copper	Zinc	Copper	Zinc	Calcium	Magnesium	Sodium
Soft	0.057	--	49 ± 8	390 ± 48	24,200 ± 850	1,650 ± 140	5,790 ± 390
Tap	0.057	--	25 ± 3	290 ± 8	23,800 ± 550	1,540 ± 70	5,650 ± 990
Very Hard	0.057	--	25 ± 5	350 ± 25	33,500 ± 2,580	1,770 ± 120	5,680 ± 530
Soft	--	6.60	12 ± 5	740 ± 84	25,200 ± 1,870	2,310 ± 100	6,310 ± 430
Tap	--	6.60	13 ± 4	600 ± 70	25,900 ± 1,910	1,640 ± 50	5,990 ± 380
Very Hard	--	6.60	14 ± 4	390 ± 47	33,400 ± 3,030	1,710 ± 70	5,700 ± 460
Soft	0.025	3.90	36 ± 9	680 ± 28	26,900 ± 190	1,750 ± 110	5,770 ± 460
Tap	0.025	3.90	27 ± 2	550 ± 206	27,500 ± 1,180	1,700 ± 82	5,700 ± 480
Very Hard	0.025	3.90	17 ± 4	460 ± 1	30,800 ± 1,960	1,770 ± 170	5,820 ± 230
Soft	0	0	15 ± 2	340 ± 28	21,800 ± 240	1,760 ± 18	5,670 ± 400
Tap	0	0	14 ± 1	380 ± 37	25,500 ± 1,420	1,720 ± 94	5,930 ± 480
Very Hard	0	0	18 ± 4	310 ± 21	26,000 ± 1,750	1,890 ± 100	5,830 ± 400

Table 21. Mean metal content of fish whole body from fish surviving the 48 hour exposure to various sublethal concentrations of copper and/or zinc at 3 water hardness (µg element/g dry tissue weight)

Water hardness	Concentration of metal (mg) in exposure tanks		Mean metal content of fish whole body (µg element/g dry tissue weight ± S.D.)				
	Copper	Zinc	Copper	Zinc	Calcium	Magnesium	Sodium
Soft	0.057	--	35 ± 4	540 ± 5	24,200 ± 4,190	2,250 ± 360	4,290 ± 770
Tap	0.057	--	13 ± 1	500 ± 64	22,500 ± 2,760	1,970 ± 260	4,760 ± 340
Hard	0.057	--	7 ± 0	460 ± 50	23,300 ± 3,380	2,150 ± 310	4,200 ± 210
Soft	--	6.60	6 ± 0	610 ± 50	16,100 ± 490	2,200 ± 490	3,970 ± 1,110
Tap	--	6.60	5 ± 1	550 ± 35	17,200 ± 360	2,000 ± 110	4,640 ± 1,180
Hard	--	6.60	5 ± 0	340 ± 40	14,100 ± 1,710	1,960 ± 390	3,580 ± 90
Soft	0.025	3.90	13 ± 1	560 ± 63	12,200 ± 2,910	1,610 ± 90	3,660 ± 380
Tap	0.025	3.90	10 ± 3	600 ± 141	11,600 ± 1,300	1,960 ± 450	3,560 ± 500
Hard	0.025	3.90	9 ± 1	580 ± 23	12,800 ± 3,430	2,050 ± 510	3,570 ± 650
Soft	0	0	9 ± 2	380 ± 9	18,200 ± 2,270	2,080 ± 130	3,820 ± 330
Tap	0	0	11 ± 4	440 ± 79	16,700 ± 1,400	2,050 ± 310	4,380 ± 1,250
Hard	0	0	9 ± 3	440 ± 51	17,100 ± 3,240	2,100 ± 250	4,000 ± 560

metal content between samples were often high (see Standard Deviations listed in Tables 20 and 21). Mean metal concentrations of gills and whole bodies of fish subjected to the various exposure regimes were compared using a test for differences between the means. A summary of trends in elemental concentration in fish tissue, based on mean values, follows.

Metal Bioconcentration in Gill Tissue

Copper concentrations in gills of copper-treated fish decreased by 49% between samples from soft to tap water regimes. No significant change in concentrations was noted between tap and very hard water (at $p = 0.05$). Copper concentrations in gills of copper-zinc-treated fish decreased by 25% from soft to tap water regimes, and 53% from tap to very hard water. Zinc concentrations in gills of zinc or copper-zinc-treated fish decreased, with increasing hardness, by 48% and 32%, respectively. ("Increasing water hardness" refers to an increase in hardness from soft to very hard except where specific increases are indicated.) Copper concentrations in gills of zinc-treated fish, and zinc in copper-treated fish did not significantly change (at $p = 0.05$) with increasing water hardness. Copper levels in controls were similar to those found in zinc-treated fish, and zinc levels similar to those in copper-treated fish (at $p = 0.05$).

Calcium concentrations in gills of all fish tested generally increased with increasing water hardness. However, wide standard deviations were observed for each sample lot. Magnesium and sodium

concentrations in gills of all fish tested did not significantly change (at $p = 0.05$) with increasing water hardness. Elemental levels in control fish were similar to those found in exposed fish (at $p = 0.05$).

Metal Bioconcentration in Whole Bodies

Copper concentrations in copper and copper-zinc-treated fish decreased by 80% and 31% respectively with increasing water hardness. Zinc concentrations in zinc and copper-zinc-treated fish decreased by 45% and 11% respectively. Copper concentrations in zinc-treated fish did not significantly change (at $p = 0.05$) with increasing water hardness. Copper levels in the control fish were marginally greater than levels found in zinc-treated fish, and zinc levels were similar to levels in copper-treated fish ($p = 0.05$).

Calcium concentrations in all fish tested did not significantly change (at $p = 0.05$) with increasing water hardness. However, wide standard deviations were observed within each sample lot. Calcium levels were greater in the copper-treated fish than in the zinc-treated, which in turn were greater than in the copper-zinc treated fish. Concentrations of magnesium and sodium in all fish tested were relatively stable with increasing water hardness. Elemental levels in control fish were roughly equivalent to those found in the experimental fish (at $p = 0.05$).

Comparison of Gill and Whole Body Residues

Copper concentrations in fish gills were generally higher than

in whole body for all fish tested ($p = 0.05$). Zinc concentrations in fish gills were lower than in whole body for copper-treated fish, and the same in copper-zinc-treated and control fish. Zinc levels in gill samples were higher than in whole body for zinc-treated fish ($p = 0.05$).

Calcium concentrations in fish gills were generally higher than in whole body samples for all fish tested. Magnesium concentrations were lower in gills of copper, copper-zinc-treated, and control fish, and similar in zinc-treated fish to whole body estimates. In contrast, sodium concentrations were generally higher than in whole body for all fish tested ($p = 0.05$).

DISCUSSION

The forms and their respective concentrations of copper and zinc, in aqueous solution, were not modified to any appreciable degree by calcium and magnesium ions at concentrations representative of the soft and tap water regimes (Table 4). However, the pulse polarographic analyses were not performed at hardness levels which could be equated to the very hard water regime. Nevertheless, according to Peter Hodson (Burlington Inland Waters Directorate, personal communication), there is no apparent reason to expect this pattern to change between the highest hardness tested and the very hard water examined in the toxicity bioassays, i.e. 306 mg/l (as $CaCO_3$).

There are, to our knowledge, no other reports of such analyses with which to relate our findings. However, a comparable study in the literature was performed by the less sensitive electrochemical technique (Laitenen, 1975) of anodic stripping voltammetry. O'Shea and Mancy (1978) found that the forms of copper, cadmium, and lead, at low levels, did not change between media which lacked the alkaline cations and those which contained 40 mg/l calcium or 24 mg/l magnesium. It was concluded, therefore, that water hardness cations, calcium and magnesium, do not affect the lethality of copper, zinc, and their mixtures through promotion of chemical complexation in the ambient environment over the hardness range studied. This conclusion may not apply should alkalinity and pH vary among hardness regimes (Howarth, 1976;

Waiwood, 1977; and Chakoumakos et al, 1979). However, in the present study, the environmental factors were constant.

Having dismissed chemical interactions as a possible cause of the altered potency of the heavy metals with hardness, we have assumed that the observed results may have been a consequence of specific physiological interactions; i.e. physiological interactions mediated by the organism. Other researchers have arrived at a similar conclusion that calcium ions may protect fish from the toxic effects of heavy metals by acting internally rather than neutralizing metals in surrounding water (Matthiessen and Brafield, 1973). They inferred that calcium acted at gills to lessen the adverse effects of zinc on fish.

Two pieces of evidence in our studies have suggested that the physiological interactions, involving hardness cations, lead to an alteration of the potency of each heavy metal and their mixture (Figure 14), but not their respective modes of action:

Firstly, the various signs of poisoning, observed during toxicity tests, did not appear to change in type, but rather changed in severity (Table 5). For example, mucous production, which was generally high in fish exposed to zinc or copper-zinc mixtures, was observed to lessen with increasing hardness. In zinc-exposed fish, mucous production has been associated with other gill abnormalities, such as an extensive thickening and a general decrease of intracellular adhesion in the respiratory epithelium, distension of the mitochondria in the chloride cells, as well as a hyperplasia of these chloride cells. It has been

suggested that these dysfunctions, collectively, lead to an impairment of gaseous exchange, followed by death (Lloyd, 1960, 1962; Skidmore and Tovell, 1972; Matthiessen and Brafield, 1973). The most visible signs of copper poisoning were respiratory distress and peripheral hemorrhaging in the abdominal region. The severity of these signs also decreased with hardness. It is not known how representative these signs are of copper's critical mode of action.

The second item of evidence to support the assumption that calcium and magnesium affect the potency of heavy metals and their mixtures, and not their mode of action, is gained by examining characteristics of the lethal response curves. At similar ambient concentrations of either copper or zinc, the median time of death (LT_{50}) increased with water hardness (Figure 8). In addition, the slopes of the toxicity curves for copper were statistically parallel while those for zinc were only assumed to be parallel. (The zinc data were poor fits to the linear regression.) Thus no change in the mode of action of copper and zinc with water hardness was suggested. The same pattern held for the mixture (Figure 9).

Similarly, the dose-response curves for copper and zinc at these water hardness regimes would also appear to be parallel (Table 11, Figure 13). Note that the test for parallelism could not be applied to the zinc-very hard water data, because of the poor fit of the co-ordinates to a linear regression. The slopes of the dose response curves for mixtures of copper and zinc are assumed to be parallel although the data points are too few for statistical

analysis (Figure 15).

According to Bliss' (1939) rationale, such similarity between slopes of dose-response curves may be indicative of toxic agents with the same mode of action. The results then lend credence to the hypothesis that water hardness cations alter the potency of each heavy metal and their mixtures; but do not interfere with the respective modes of action.

Another report tends to support this conclusion. For example, similarity of slopes of regression lines (log median lethal time-log concentration) computed from Lloyd's (1961) empirical data, as well as a slight displacement of the regressions along the abscissa (concentration), suggest that water hardness modifies the potency, but not the mode or rate of action of copper and zinc mixtures.

To determine whether a constant relationship between hardness cation concentrations (here in fixed proportions) and the potency of heavy metals, held over the hardness range tested, linear regressions were fitted to hardness-potency co-ordinates (Figure 14). For both copper and zinc, and their mixtures, linear regressions were found to be poor representations of the data distribution (Table 12) (at $p = 0.20$ and 0.90, respectively). This pattern could not have been influenced by alkalinity, pH, or the relative proportion of calcium to magnesium between hardness regimes, because, as previously mentioned, each of these modifying factors was constant. On the other hand, sodium and chloride levels were not consistent between hardness regimes (Table 3a).

Tabata (1969), in studies with Daphnia, examined sodium as a modifying agent of heavy metal toxicity. He found that sodium ions significantly decreased the lethal potency of copper and zinc solutions. Through extrapolation from Tabata's data, the level of sodium present in our soft water system may be accredited with depressing copper and zinc's potencies by 57 and 21%, respectively (Table 22). Furthermore, there is evidence that chloride ions may also lessen, at least in soft waters, the toxicity of heavy metals (Anon, 1958; Lloyd, 1960; and Tabata, 1969). The 1958 Water Pollution Research Board of London (Anon, 1958) showed that the toxicity of zinc salts to sticklebacks in soft water was reduced by the addition of $CaCl_2$ to a greater extent than an equimolar addition of $CaCO_3$. The chloride anion, more so than the carbonate ion, would appear to be the antagonistic factor. Lloyd (1960) found a similar relationship to exist for the toxicity of solutions of zinc to rainbow trout. Thus, there may have been some effect of the higher concentration of the chloride ions, in the very hard water regime, that lessened the toxicity of the individual heavy metals. However, these findings cannot be easily extrapolated to our experiments. In addition, there is no quantitative data to deduce the degree of impact, if any, of chloride ions on heavy metal toxicity in our hard water systems. Consequently, we have assumed that the chloride ion interference, in the very hard water regime of our study, was minimal. No adjustments were made for possible modifying effects of the chloride ions within the very hard water regime.

Table 22. "Adjustment" of soft water lethal response data for copper or zinc, for the presence of sodium. Documented increases in toxic potencies of copper and zinc with decreases in sodium are predicted according to empirical data of Tabata (1969).

Heavy metal	Copper	Zinc
Observed LC_{50}	0.154	14.62
Observed regression	Y = 11.308 + 7.724x (33)	Y = -4.070 + 7.724x (34)
Predicted[1] LC_{50}	0.066	11.55
Predicted[1] regression	Y = 14.117 + 7.724x (35)	Y = -3.207 + 7.724x (36)
Increase in toxic potency[1]	57%	21%

[1]Predicted on basis of increase in toxic potency which occurs when sodium levels are decreased (adjusted) from 64.0 ppm to 12.7 ppm.

Hardness-potency data, adjusted for sodium's presumed effects, are linearly distributed (at $p = 0.05$) for both copper and zinc (Table 23). This correlation to a linear regression suggests a causal relationship between hardness cations and heavy metal potency. The respective "fitted" lines are compared to those computed for each of copper and zinc by Tabata (1969), whose studies were on Daphnia, and to that formulated for copper by Chakoumakos et al (1979) who studied Salmo clarki, the cutthroat trout (Table 24). According to a Student's "t"-test, there is no significant difference (at $p = 0.05$) between the slopes of all three regression equations for copper over the hardness range studied. The same comparison applies to zinc. Table 25 lists linear regressions representing zinc-hardness coordinates for this study (i.e. fitted regressions) and Tabata's. A Student's "t"-test indicates that the slopes of these lines are not significantly different. This consistency, between exposures, in the quantitative relationship of hardness cations with either copper or zinc's potency suggests a commonality in calcium or magnesium's modifying action.

The degree between hardness' effects on copper and zinc's potencies differs, as observed through comparing slopes of their respective lines (Figure 14). The data show that water hardness cations have a possibly greater influence in altering the potency of copper. Copper dissolved in the adjusted soft water is 3.63 times more potent than copper in moderately hard water and 10.18

Table 23. Correlations between Log 96-hr LC_{50} of copper or zinc and Log water hardness for observed lethal response data. LC_{50}'s predicted on the basis of an increase in toxic potency, with a decrease in sodium levels from 64 to 12.65 mg/l(according to Tabata's (1969) data) are used in the estimation of the "adjusted" correlation coefficient

Heavy Metal	Copper	Zinc
observed correlation coefficient	0.926	0.933
significance (p)	0.20	0.20
predicted correlation coefficient	0.992	0.990
significance (p)	0.02	0.02

Table 24. Regression equations relating Log LC_{50} of copper to Log water hardness

Author	Aquatic organism	Test	Equation	
Tabata, 1969	Daphnia sp.	24-hr LC_{50}	Y = -2.640 + 0.442x	(37)
Chakoumakos et al (1979)	Salmo clarki	96-hr LC_{50}	Y = -2.029 + 0.616x	(38)
Horovitch (present study)	Brachydanio rerio	96-hr LC_{50} ("adjusted" according to the presence of sodium)	Y = -2.362 + 0.861x	(39)

Table 25. Regression equations relating Log LC_{50} of zinc to log water hardness

Author	Aquatic organism	Test	Equation	
Tabata, 1969	Daphnia sp.	24-hr LC_{50}	$Y = -0.710 + 0.551x$	(40)
Horovitch (present study)	Brachydanio rerio	96-hr LC_{50} ("adjusted" according to the presence of sodium)	$Y = 0.664 + 0.290x$	(41)

times more potent than copper in very hard water. In comparison, potency values derived from the zinc lethal response data are 1.53 and 2.19, respectively. This difference in relative potency between copperand zinc suggests that the apparent physiological interactions between hardness cations and each heavy metal are not quantitatively the same.

Certain evidence to support this claim is gained from an analysis of data from Zitko and Carson (1976). Zinc, at a concentration of 14 μ moles/litre, in a solution of 344 μ moles/litre of calcium, caused the death of 80% of a test population of Atlantic salmon alevins in 37 hours. Comparatively, the same concentration of zinc, in a solution of 288 μ moles/litre of magnesium, was not lethal during a 96-hour exposure period. In contrast, copper, at a concentration of 1.56 μ moles/litre in a solution of 364 μ moles/litre of calcium, caused the death of 50% of the test population in 26 hours. The same concentration of copper, in a solution of 337 μ moles/litre of maghesium, resulted in about 20% less time for the same percentage of mortality to occur. This evidence suggests that magnesium, on an equimolar basis, has a greater effect than calcium in altering zinc's lethal potency[1]. The opposite relationship appears to exist between these cations and copper's lethal potency. By extrapolation, our hardness regime, in which calcium prevails, would affect copper's toxicity more than that of zinc's. However, this conclusion is only valid

[1]See Sillen and Martell (1964) for further explanation.

should equal increments in either calcium or magnesium concentrations have proportionally the same effect. Zitko and Carson's (1976) data is not complete for magnesium; however, there is a suggestion that a much greater percentage change in calcium levels is required to cause a change in copper's potency than in zinc's. Obviously more research is required on this problem.

According to the procedure outlined in Materials and Methods, copper-zinc mixtures were tested for concurrence with either the model of concentration-addition or response-addition (Tables 16 and 17). The results fitted neither (Table 18). Because the slopes of the respective dose-response lines were not significantly different we assumed that the two constituents, copper and zinc, were acting similarly when present together, but that some unknown kinetic and/or dynamic interaction was causing an enhanced response to that predicted. We elected to represent our response data for the mixtures relative to that expected for concentration-addition. The relative potency factors (Table 18) and isobologram (Figure 14) indicate the degree of enhanced response observed at each hardness regime. As in the orginal data for copper and zinc in pure solutions (Table 12), these data for their mixtures are not linearly distributed. Employing the same rationale, as previously discussed for sodium's "antagonistic" effect in the soft water, the co-ordinates for the LC_{50} of copper-zinc mixtures in soft water were adjusted (Table 26). Statistical tests showed that the distribution now approached linearity ($p = 0.10$) (Table 27). Furthermore, the potency at which the copper-zinc mixtures cause

Table 26. "Adjustment" of soft water lethal response data for a copper-zinc mixture, for the presence of sodium

Observed LC_{50}	0.133	
Observed regression	$Y = 11.794 + 15.559x$	(42)
Predicted[1] LC_{50}	0.095[2]	
Predicted[1] regression	$Y = 12.107 + 6.937x$[2]	(43)

[1]Predicted on basis of increase in toxic potency of copper or zinc, in pure solution, which occurs when sodium levels are decreased (adjusted) from 64.0 mg/l to 12.7 mg/l.

[2]By using the predicted potency of zinc relative to copper, i.e.:

$$\frac{\text{adjusted } LC_{50} \text{ copper}}{\text{adjusted } LC_{50} \text{ zinc}} = 0.0057,$$

convert mean assayed level of zinc to predicted equipotent level of copper. A predicted probit regression is then obtained using the observed 96 hour mortality and the "total copper + zinc expressed as copper" concentration.

Table 27. Correlation between Log 96-hr LC_{50} for a mixture of copper and zinc, and log water hardness for observed lethal response data. An LC_{50} is predicted on the basis of an increase in toxic potency, with a decrease in sodium levels from 64.0 to 12.7 mg/l (according to Tabata's (1969) data) and used in the estimation of the "adjusted correlation coefficient"

Observed correlation coefficient	0.62
Significance (p)	0.90
Predicted correlation coefficient	0.95
Significance (p)	0.10

50% death in test populations progressively decreases through the hardness range. This progression does not occur to the same degree over the hardness range, as would have been expected had the constituents met the criterion of concentration-addition, as represented by the copper potency-hardness isobol (Figure 14). Had zinc's isobol been selected arbitrarily for referencing concentration-addition, a similar pattern would have been observed. Because of this difference in slope, the displacement of the copper-zinc mixture relative to that expected for concentration-addition progressively increases through the hardness regimes. Specifically, the supra-additive effect in soft water would appear to be minimal, while in contrast, there is significant relative enhancement in the very hard water regime. This pattern offers an explanation why some authors concluded that copper-zinc mixtures were additive or nearly additive in soft waters, while others recorded synergisms in harder waters. Sprague (1964) found lethal mixtures to be slightly synergistic in short exposures (about 30 hours) at a hardness of 20 mg/1 (as $CaCO_3$). In a later study, Sprague and Ramsay (1965) found the same situation for short exposures, but reported strict addition, i.e. concentration-addition, for binary mixtures of these hevy metals at a hardness of 14 mg/1. Anderson and Weber (1975), who conducted multiple toxicity studies with copper and zinc at a hardness of 124 mg/1 computed an enhancement factor of 2.65 for the mixture relative to that expected for concentration-addition. At a comparable hardness of 128 mg/1 in the present study the relative enhancement factor was 1.91 (Table 18). In waters of a hardness

of 218 mg/l, Lewis (1978) found the toxicity of copper-zinc mixtures to be three to four times greater than expected on the basis of concentration-addition. The test organism used in these reported studies were Atlantic salmon juveniles, male guppies, and long fin dace, respectively. If species' differences are ignored, then it may be suggested that enhancement of toxicity relative to strict addition, increases with increasing hardness.

The relationship of the observed lethal potency of copper-zinc mixtures relative to that expected for concentration-addition, demonstrates a progressive increase in the degree of supra-addition with hardness. However, it should be emphasized that in absolute terms the potency of copper-zinc mixtures decreases with hardness. For example, a lethal mixture of copper and zinc at a set total concentration becomes progressively less potent with increasing hardness. Nevertheless, over the hardness range studied herein, the relative decrease in lethal potency of a mixture is less than that of either of the constituents, copper and zinc, in pure solution.

This pattern, whereby the potency of copper-zinc mixtures decreases with hardness, may not be solely due to the progressive increase in calcium and magnesium ions. Anderson et al (1979) and Weinstein (1978) have shown that the potency of a mixture can be a function of not only the total concentration but also the relative proportion of each constituent in the mixture. A comparison of the relative proportions of copper to the total concentrations of heavy metal mixtures shows that the proportionality range varies little between each water hardness regime tested (Table 16).

Therefore, we can eliminate the possibility that the apparent difference in potency of a copper-zinc mixture between water hardness was the consequence of a difference in the proportionality of constituents. The causal agents would seem to be calcium and magnesium.

At this point, then, the evidence submitted suggests that hardness cations, calcium and magnesium, interact physiologically with copper and zinc to decrease their individual and collective lethal potency. The relative effects, over the hardness range studied, seem to be greatest for copper, in pure solution, and least for copper and zinc, as a mixture.

We can perceive, in general terms, two mechanisms by which a modifying agent, i.e. calcium or magnesium, may decrease the potency of a toxicant. Firstly, the modifying agent might alter the toxicant's kinetics, eg. uptake or distribution, so that the dosage at the critical receptor sites, relative to the ambient concentration, is lessened. Secondly, the modifying agent might compete with a toxicant for critical binding sites or, alternatively, dampen the adverse response caused by a toxicant when bound to critical sites. The latter interactions involve the "dynamic" properties of a toxicant.

There is growing evidence in the literature that the gills are the critical organ site of the lethal action of many heavy metals, including copper and zinc (Carpenter, 1925; Dilling et al, 1926; Carpenter, 1927, 1930; Ellis 1937; Jones, 1938; Westfall, 1945; Lloyd, 1960; Baker, 1969; Skidmore, 1970; Burton et al, 1972;

Spear and Anderson, 1978). Not only do zinc and copper have a common critical organ, i.e. the gills, at which their respective lethal action is expressed, they share a capability to bind with protein's ligands. The affinity for such ligands, eg. OH, COOH, PO_3H_2, SH_2, NH_2, and imadazole groups, may differ between copper and zinc (Eisler and Gardner, 1973; Zitko and Carson, 1976). However, the general end effect of copper and zinc's toxic action may be the same. Thus, this standardization of structural and enzymatic proteins may explain the apparent similar action of copper and zinc as reflected by the similarity of the slopes of their respective dose-response lines (Anderson and Weber, 1975a). Note that this apparent commonality of effect for copper and zinc at high exposure levels does not refute the likelihood of each of these heavy metal's operating selectively and uniquely at sublethal levels.

Our preliminary bioconcentration study provides some support for this rationale. Changes in form and concentration of either copper or zinc were not assessed in the bioassay waters, but only in samples dissolved in soft, tap, or hard water. However, these simulations gave no reason to expect any such changes to occur. Table 20 shows that calcium levels do increase in gill tissues with hardness. Magnesium concentrations in gills vary widely in samples and thus no definite trends could be derived. However, sodium and chloride levels in gills do not change between the hardness regimes studied. Because at least calcium levels in gills change with hardness, there is justification for assuming that hardness' interactions with copper and/or zinc are physiological. A stoichastic relationship seems to exist.

Table 28 shows the bioconcentration factors, i.e. the level of copper and zinc in tissues relative to that available in the ambient media for each hardness and bioassay regime.[1] Copper's bioconcentration in gills after 48 hours exposure was approximately 49% less in "tap" than in soft water. The same comparison shows an 18% decrease for zinc. It would appear that hardness affects the kinetics, i.e. uptake, of copper more than zinc. However, this relationship does not hold for data representing the bioconcentration of copper and zinc between "tap" and very hard water regimes. The copper levels did not change. This is the only instance where copper's relative bioconcentration is less than zinc's with increasing water hardness. The percentage decrease in the relative bioconcentration of copper, between each water hardness regime, is greater than zinc's in whole body samples of fish exposed to pure solutions of copper or zinc, respectively. A similar pattern is observed in gill and whole body samples of fish exposed to mixtures.

The other interesting aspect of Table 28 is that the relative bioconcentration of copper and zinc is greater in both gill and body samples of fish exposed to their mixture than to pure solutions respectively. There is one exception to this trend; the relative bioconcentration of copper in whole body samples of fish in soft water was greater than that computed for the mixture. The absolute concentration of copper and zinc in gills of fish exposed to mixtures was not significantly different from the respective levels in fish exposed to pure solutions, at each water hardness (Table 20). Yet the respective concentrations of copper and zinc

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[1] Both in µg/g.

Table 28. Bioconcentration of copper and/or zinc in zebrafish gill and whole body tissue, relative to concentrations available in the ambient media for each hardness and bioassay regime. The exposure period was 48 hours for all bioconcentration studies

Tissue	Water hardness	Heavy Metal Bioconcentration Factor[1]			
		Individual		Mixture	
		Copper	Zinc	Copper +	Zinc
Gill	soft	0.86	0.11	1.44	0.17
	tap	0.44	0.09	1.08	0.14
	very hard	0.44	0.06	0.68	0.12
Whole body	soft	0.61	0.09	0.52	0.17
	tap	0.23	0.08	0.40	0.15
	very hard	0.12	0.05	0.36	0.15

[1] Bioconcentration factor = $\frac{\text{Heavy metal in fish } (\mu g/g)}{\text{Heavy metal in water } (\mu g/g)}$

in ambient pure solution were approximately one-half. The exposure period was the same for all of these bioconcentration studies.

Therefore at any given hardness level, it would appear that copper and zinc potentiate each other's uptake into the organism. Though these potentiated uptake rates are counteracted somewhat by increasing hardness, the end result would be as observed, a supra-additive effect relative to that predicted for concentration-addition between copper and zinc (Figure 14).

As much as these results point to kinetic interactions between hardness cations and the heavy metals, copper and zinc, and between copper and zinc, when present concurrently, there is at this stage, no reason to exclude the possibility of dynamic interactions. This question cannot be answered on the basis of the evidence collected herein.

Kinetic interactions of water hardness cations with copper and zinc cations are apparent determinants of the toxic potency of these heavy metals, singly and in mixtures. However, at any given level of hardness, copper and zinc interact reciprocally to potentiate their respective uptake rates. The end result of these combined forces is observed as a supra-additive form of multiple toxicity. Relative to that expected for concentration-addition, the degree of supra-additivity increases with hardness. In absolute terms, however, the enhanced potency of copper-zinc mixtures decreases with hardness. Further studies are necessary for a delineation of the specific mechanisms of interaction between heavy metals that lead to synergisms in response.

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APPENDIX I

COMPUTATIONS OF NOMINAL TOXICANT CONCENTRATIONS USED IN BIOASSAYS

1. Select the range of concentrations to be tested, based on published data, if available, in the literature.

2. Compute individual concentrations, equally spaced logarithmically, within this range, using the equation:

$$C_x = \text{antilog}\,[\log C_1 + (x - 1)(\log C_n - \log C_1)/(n-1)]$$

where n = number of concentrations to be determined

C_x = the concentration at position "x" in rank when all concentrations to be determined are listed as variables in order of increasing magnitude

C_1 = lowest concentration

C_2 = second lowest concentration

C_n = highest concentration

(Weinstein, 1978)

APPENDIX IIa

COMPUTER ANALYSIS,ON EFFECTS OF VARIABLE WET BODY WEIGHT OF ZEBRAFISH TO LETHAL LEVELS OF COPPER OR ZINC AT THREE DIFFERENT WATER HARDNESS LEVELS

Dose-response equations used in computer analysis:

$$Y = a_1 + b_1 \log_{10} C$$

$$Y = a_2 + b_2 \log_{10} (C/W^h)$$

where Y = probit of % mortality

a = intercept

b = slope

C = mean assayed toxicant concentration

W = mean wet weight of test organisms exposed to concentration C

h = a proportionality constant

APPENDIX IIb

RESULTS OF COMPUTER ANALYSIS

$$Y = a_1 + b_1 \log_{10} C \qquad Y = a_2 + b_2 \log_{10}(C/W^h)$$

Heavy metal	Water hardness			Heavy metal	Water hardness		
	Soft	"Tap"	Very hard		Soft	"Tap"	Very hard
Copper				Copper			
a	9.312	7.377	6.033	a	9.861	6.829	5.307
b	5.236	4.063	6.229	b	6.410	4.636	6.356
h	--	--	--	h	0.350	0.470	0.210
Zinc				Zinc			
a	-5.396	-4.081	-5.480	a	-4.596	-3.614	-1.742
b	8.970	7.275	7.459	b	7.658	7.144	6.281
h	--	--	--	h	0.440	-0.300	-0.830

APPENDIX IIc

SIGNIFICANCE OF DIFFERENCE BETWEEN CORRELATION COEFFICIENTS FOR DOSE-RESPONSE REGRESSIONS:

$$Y = a_1 + b_1 \log_{10} C \quad \text{and} \quad Y = a_2 + b_2 \log_{10} (C/W^h)$$

Heavy metal	Water hardness	r_{13}	r_{12}	r_{23}	t	critical t*
Copper	soft	0.934	0.969	0.965	0.754	3.182
	"tap"	0.935	0.962	0.972	0.998	2.365
	very hard	0.983	0.985	0.998	0.002	4.303
Zinc	soft	0.867	0.898	0.965	0.653	2.356
	"tap"	0.906	0.923	0.982	0.459	2.571
	very hard	0.802	0.885	0.906	0.583	3.182

where r_{13} = the correlation between Y in probit and $\log_{10} C$

r_{12} = the correlation between Y in probit and $\log_{10} (C/W^h)$

r_{23} = the correlation between $\log_{10} (C/W^h)$ and $\log_{10} C$

$$\text{and } t = \frac{(r_{12} - r_{13}) \sqrt{(N - 3)(1 + r_{23})}}{\sqrt{2(1 - r_{12}^2 - r_{13}^2 - r_{23}^2 + 2 r_{12} r_{13} r_{23}}}$$

* significant at the 0.05 probability level

APPENDIX III

FORMULA FOR CHI-SQUARE TEST FOR HETEROGENEITY

$$X^2 = \Sigma \frac{n\,(r - np)^2}{np\,(n - np)}$$

where n = number of fish

r = observed number of fish responding

np = expected number of fish responding

p = fitted probability

If critical X^2 is greater than the heterogeneity X^2 ($p > 0.05$), then there is a greater than 5% chance that the discrepancies between observed and expected responses could could be due to chance not to a poorly fitting line. Therefore, in this case, the computed regression is a good fit.

APPENDIX IV

CONSTRAINT OF BEST FIT PROBIT ANALYSIS
REGRESSIONS TO PARALLELISM

The slope "b" of any regression line:

$$Y = a + bx$$

can be calculated as follows: $b = sxx/sxy$

Where sxx = sum of squares for x and
sxy = sum of cross products

The intercept "a" of this regression line can be calculated as follows:

$$a = \bar{y} - b\bar{x}$$

To constrain any two lines:

$$Y_1 = a_1 + b_1 x_1 \quad \text{and} \qquad (1)$$

$$Y_2 = a_2 + b_2 x_2 \qquad (2)$$

to parallelism, calculate a common slope "bc" as follows:

$$bc = \frac{sxy_1 + sxy_2}{sxx_1 + sxx_2}$$

The new intercepts of lines (1) and (2), after these lines have been constrained to parallelism are:

$$a_1 = \bar{y}_1 - bc\,\bar{x}_1 \quad \text{and} \qquad (3)$$

$$a_2 = \bar{y}_2 - bc\bar{x}_2 \qquad (4)$$

APPENDIX V

COMPUTATION OF RELATIVE POTENCY FACTORS

If regression lines (3) and (4) are parallel, relative potency factors may be obtained by substituting the slopes and intercepts of equations for single toxicants "M" and "N":

$$Y_M = a_M + b_c \log_{10} C_M$$

$$Y_N = a_N + b_c \log_{10} C_N$$

where Y = response in probits

C = concentration of toxicant

a = intercept of regression functions for toxicity

b_c = common slope

into the following formula:

$$R_{M.N} = \text{antilog} \frac{[a_M - a_N]}{b_c}$$

where $R_{M.N}$ is the relative potency factor for the single toxicants, "M" and "N", at a given level of response, and where "M" represents the most toxic constituent of the mixture.

(Weinstein, 1978, modified from Anderson and Weber, 1975a)

Relative potency factors may also be computed as follows:

$$\text{Relative Potency Factor} = \frac{LC_{50} \text{ Toxicant "a"}}{LC_{50} \text{ Toxicant "b"}}$$

Where "a" is a more potent toxicant than "b"

APPENDIX VI

COMPUTATION OF RELATIVE POTENCY FACTOR (α/α_1), USED AS A MEASURE OF THE ENHANCED EFFECT OF A TOXIC MIXTURE

1. Tabulate assay levels of toxicant "M"* and toxicant "N", individually.

2. Determine relative potency of toxicant "N" to toxicant "M" (APPENDIX V) and tabulate.

3. Summate toxicant "M" and toxicant "N" (expressed as equipotent level of "M") = α_1.

4. Compute the antilog of the abscissal quantity determined from the lethal response curve of toxicant "M", and corresponding to the magnitude of the observed response = α.

5. The ratio of α/α_1 represents the enhanced effect of the toxic mixture beyond that predicted according to the model of concentration-addition.

* Toxicant "M" is more potent than toxicant "N".