Toxicity of Trace Metal Mixtures to Alevin Rainbow Trout (*Oncorhynchus mykiss*) and Larval Fathead Minnow (*Pimephales promelas*) in Soft, Acidic Water

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Hickie, B.E., N.J. Hutchinson, D.G. Dixon, and P.V. Hodson. 1993. Toxicity of trace metal mixtures to alevin rainbow trout (*Oncorhynchus mykiss*) and larval fathead minnow (*Pimephales promelas*) in soft, acidic water. Can. J. Fish. Aquat. Sci. 50: 1348–1355.

The acute lethality of a fixed-ratio mixture of Al, Mn, Fe, Ni, Zn, Cu, and Pb (75:60:60:12:12:6:6 $\mu g \cdot L^{-1}$ = 1.0 acid lake concentration or ALC, representative of Ontario lakes acidified to pH 5.8) was examined with alevin rainbow trout (*Oncorhynchus mykiss*) and larval fathead minnow (*Pimephales promelas*). All testing was done in extremely soft, acidic water (2.5 mg $Ca \cdot L^{-1}$; pH 4.6–5.8). For the acid-tolerant trout alevins (144-h LC50 = pH 4.32), median lethal metal mixture levels at pH 5.8 were 5.0 ALC. Toxicity of the mixture increased at lower pHs, with a median lethal threshold of 1.0 ALC at pH 4.9. A mixture of Al, Zn, and Cu was equivalent in toxicity to the full mixture; mixture toxicity was caused by Cu alone at pH 5.8 and by Al alone at pH 4.9. For the acid-sensitive fathead minnow larvae (144-h LC50 = pH 5.54), the mixture of metals typical of lakes acidified to pH 5.8 was lethal (LC50 = 0.84 ALC); again, toxicity was associated with Al, Cu, and Zn. This research implies that Cu could be an important factor contributing to the demise of acid-sensitive fish at pHs above those associated with increased Al solubility and toxicity.

On a pris des alevins de la truite arc-en-ciel (*Oncorhynchus mykiss*) et des larves du tête-de-boule (*Pimephales promelas*) pour évaluer la létalité aiguë d'un mélange aux proportions constantes d'Al, de Mn, de Fe, de Ni, de Zn, de Cu et de Pb (soit 75 : 60 : 60 : 12 : 12 : 6 : 6 $\mu g \cdot L^{-1}$), ce qui correspond à 1,0 fois la concentration dans un lac acide (ou CLA), qui est représentative de la situation dans les lacs de l'Ontario qui sont acidifiés jusqu'à pH 5,8. Tous les essais se sont déroulés dans une eau extrêmement douce et acide (2,5 mg $Ca \cdot L^{-1}$; pH 4,6 à 5,8). Dans le cas des alevins de truite tolérants au pH acide (CL50 144 h = pH 4,32), le niveau létal médian du mélange de métaux à pH 5,8, etait de 5,0 CLA. Plus le pH était bas, plus la toxicité du mélange augmentait; un seuil médian de létalité de 1,0 CLA a été obtenu à pH 4,9. Un mélange d'Al, de Zn et de Cu était équivalent, en toxicité, au mélange complet; la toxicité a été obtenue par le Cu seulement à pH 5,8, et par l'Al seulement a pH 4,9. Dans le cas des larves du tête-de-boule sensibles au pH acide (CL50 144 h = pH 5,54), le mélange typique de métaux des lacs acidifiés à pH 5,8 était létal (CL50 = 0,84 CLA); une fois encore, la toxicité était associée à l'Al, au Cu et au Zn. Ces travaux pourraient indiquer que le Cu peut constituer un facteur important dans la mort des poissons sensibles au pH acide à des pH superieurs à ceux associes à la solubilité accrue et à la toxicité de l'Al.

Received December 11, 1989
Accepted February 9, 1993
(JA398)

Reçu le 11 décembre 1989
Accepté le 9 février 1993

The toxicity of trace metals to aquatic biota in soft, acidic waters has been documented in the literature describing the impacts of cultural acidification on Ontario lakes (Spry et al. 1981; Campbell and Stokes 1985). In particular, Al is mobilized in acidic waters (LaZerte 1984). Since the chemistry and toxicity of Al are highly pH dependent (Burrows 1977; Driscoll et al. 1980; Holtze and Hutchinson 1989), many studies have addressed its role in acidifying systems. Metals other than Al have properties of mobilization, speciation, bioaccumulation, and toxicity that also vary with pH (Campbell and Stokes 1985). Although Howarth and Sprague (1978) and Bradley and Sprague (1985) reported that in terms of free metal ion

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(but not total metal) the toxicity of Cu and Zn to fish decreases at low pH, Hutchinson and Sprague (1989) suggested that joint action with H^+ increased the toxicity of Cu and Zn at low pH for acid-sensitive species. Their interpretation suggests that Cu and Zn will be most toxic to acid-sensitive aquatic life in the extremely soft Precambrian waters most sensitive to acidification (Neary and Dillon 1988). In addition, base metals such as Cu and Zn are frequently extracted and smelted in Precambrian areas, leading to elevated levels in the surrounding waters (McFarlane and Franzin 1978; Munkittrick and Dixon 1988). It is therefore important to understand both the individual and joint toxicity of such metals in soft, acidic waters.

Two approaches can be taken in examining the toxicity of mixtures. A mechanistic approach can be used to examine the relative toxic contributions of mixture components (usually two toxicants) when those components are combined in varying ratios. Interactions can then be described in terms of whether toxicity is additive, greater than additive (synergistic), or less than additive (antagonistic). Alternatively, an applied approach can be used where the concentrations of the components of the mixture are fixed, often in a ratio defined to mimic a specific case or situation. This approach treats a complex mixture as one entity, both conceptually and statistically, and allows for a rapid determination of both the toxicity of the whole mixture and (by selective elimination) of the contribution of the specific components.

Hutchinson and Sprague (1986) used the latter approach to examine the contribution of metal mixtures to the demise of fish species in culturally acidified waters. The mixture they examined included Al, Mn, Fe, Ni, Zn, Cu, and Pb in a concentration ratio based on levels measured in a variety of lakes acidified to pH 5.8 in southern Ontario. The mixture of metals at those concentrations (Table 1) was termed an acid lake concentration or 1.0 ALC unit. If all metal concentrations were 10% of their respective Ontario means, the mixture would contain 0.1 ALC. If the concentrations of all metals in the mixture were 10-fold higher than their Ontario concentrations, fish were exposed to 10 ALC. Therefore, ALC units could be treated in the same way as concentrations of individual metals when median lethal concentrations (LC50s) were calculated and compared statistically. The ALC unit was defined relative to pH 5.8 to approximate the pH of reproductive failure for sensitive cyprinids in soft water (Matusek et al. 1990).

Hutchinson and Sprague (1986) showed that an environmentally realistic ALC metal mixture played an important role in the reproductive failure of flagfish (*Jordanella floridae*) at pH 5.8. Exposure to 0.57 ALC equivalent at pH 5.8 over a full life cycle resulted in complete reproductive failure, while exposure to pH 5.8 alone had no effect. Subsequent acute lethality tests with flagfish fry were used to identify the active metals in the mixture. Lethality of the full ALC mixture could be explained by a mixture of Al, Zn, and Cu; the remaining metals (Fe, Mn, Ni, and Pb) made little if any contribution to toxicity. While Al was an important toxic component of the mixture, it alone did not account for the toxicity of the ALC mixture. A significant proportion of toxicity remained attributable to joint action of Al with Zn, Cu, and/or H^+ (Hutchinson and Sprague 1989).

The intent of the present study was to extend the findings of Hutchinson and Sprague (1986, 1987, 1989) to fish species inhabiting acid-sensitive waters in Ontario. Alevins of rainbow trout (*Oncorhynchus mykiss*) and larvae of fathead minnow (*Pimephales promelas*) were tested in conditions similar to those used by Hutchinson and Sprague (1986, 1987, 1989). Early life stages were used, since they are generally regarded as those most sensitive to metal stress (McKim 1985; Van Leeuwen et al. 1986). Three null hypotheses were tested: firstly, 1.0 ALC of metals would not be acutely toxic to rainbow trout or fathead minnow larvae at pH 5.8; secondly, metal toxicity would not vary with the pH of the test water; and thirdly, any toxicity was not related to interactions, but to Al alone. Taken together, these studies apply the approach and results of Hutchinson and Sprague (1986) to the more specific case of species that inhabit Ontario lakes that are sensitive to acid loading. If fish species disappear from these lakes as acidity increases, is it due to acid alone or does metal toxicity contribute?

TABLE 1. Summary of metal concentrations ($\mu g \cdot L^{-1}$) comprising the ALC mixture. Examples are also given for 0.1 ALC(7) and 1.0 ALC(Al,Zn,Cu). The metals not included in the subset would be present at ambient levels and would not be used in calculating ALC units.

Metal	1.0 ALC(7)	0.1 ALC(7)	1.0 ALC(Al,Zn,Cu)
Al	75	7.5	75
Mn	60	6.0	—
Fe	60	6.0	—
Ni	12	1.2	—
Zn	12	1.2	12
Cu	6	0.6	6
Pb	6	0.6	—

Materials and Methods

General Considerations

This study was conducted at the Dorset Research Centre of the Ontario Ministry of Environment and Energy. Hypotheses were tested through a series of 144-h static-exposure acute-lethality (LC50) tests with toxicant renewal at 48 h. Initial tests were designed to determine the relative toxicity of the ALC mixture and pH to rainbow trout alevins. Subsequent tests were conducted to identify the toxic components of the ALC mixture and their interaction with H^+ concentration. Based on results from these tests and the work of Hutchinson and Sprague (1986), a limited number of toxicity tests were conducted for comparison using larval fathead minnow, a species known to be very sensitive to acidification (McCormick et al. 1989).

Throughout this paper, the complete mixture of seven metals is referred to as an ALC(7). Tests involving subsets of metals are referred to by the components added, so that a test solution with only Al, Zn, and Cu added is referred to as ALC(Al,Zn,Cu). The concentrations of the metals within a subset remain the same as in the ALC(7) mixture; metals excluded from the subset were present at background concentrations only and were not considered in calculating ALC units (Table 1).

Design

Initial lethality tests (144-h LC50s) were conducted with rainbow trout alevins to define their H^+ tolerance and the toxicity of the ALC(7) metal mixture over a range of pHs (4.6, 4.9, 5.2, and 5.8). Following the findings of Hutchinson and Sprague (1986), a lethality test was conducted with the ALC(Mn,Fe,Ni,Pb) subset to determine whether it could be eliminated from further consideration as a toxic component. Subsequent tests with the ALC(Al,Zn,Cu) subset were completed to confirm that it was equitoxic to the ALC(7) metal

TABLE 2. Summary of water quality parameters for the dilution water (1:1 mixture of lake water and deionized water) used in lethality tests.

Parameter	Units	Mean ± SEM (n)
Alkalinity	$mg \cdot L^{-1}$	3.6 ± 0.4 (16)
Calcium	$mg \cdot L^{-1}$	2.5 ± 0.1 (17)
Chloride	$mg \cdot L^{-1}$	3.6 ± 0.1 (16)
DOC	$mg \cdot L^{-1}$	2.0 ± 0.1 (17)
Magnesium	$mg \cdot L^{-1}$	1.0 ± 0.1 (17)
Potassium	$mg \cdot L^{-1}$	1.3 ± 0.2 (17)
Sodium	$mg \cdot L^{-1}$	2.0 ± 0.1 (16)
Sulfate	$mg \cdot L^{-1}$	10.5 ± 0.4 (16)
Total Hardness	$mg \cdot L^{-1}$	10.3 ± 0.3 (17)
Conductivity	$\mu S \cdot cm^{-1}$	46.1 ± 1.4 (17)
True colour	TCU[a]	7.5 ± 0.2 (10)
Metals		
Al	$\mu g \cdot L^{-1}$	12.2 ± 1.8 (7)
Mn	$\mu g \cdot L^{-1}$	60.6 ± 2.9 (7)
Fe	$\mu g \cdot L^{-1}$	136.6 ± 14.9 (7)
Ni	$\mu g \cdot L^{-1}$	1.3 ± 0.2 (6)
Zn	$\mu g \cdot L^{-1}$	9.6 ± 0.7 (7)
Cu	$\mu g \cdot L^{-1}$	1.6 ± 0.3 (7)
Pb	$\mu g \cdot L^{-1}$	2.9 ± 0.6 (8)

[a]TCU = true colour unit.

mixture at pH 4.9 and 5.8. The remaining toxicity tests defined the individual contributions of Al, Zn, Cu, and H^+ to the toxicity of the ALC(7) mixture at pH 4.9 and 5.8. The results of tests with individual metals (Al, Zn, or Cu) are expressed both as total concentration (allowing comparisons with the literature) and as ALC units (allowing results to be interpreted relative to the toxicity of the mixture). Several tests were repeated to determine whether alevin trout displayed any shifts in tolerance over the duration of the 4-wk testing period. Tests were run in blocks designed to ensure that any tolerance shifts would not interfere with the interpretation of results. Tests with fathead minnow larvae were limited to defining tolerance and the toxicity of the ALC(7) and ALC(Al,Zn,Cu) mixtures at pH 5.8.

Test Water

All fish were held in water from St. Mary's Lake, Dorset, Ont. The water was treated prior to use by sand filtration, UV sterilization, and filtration through a 20-μm cellulose filter. St. Mary's lake is typical of many acid-sensitive Precambrian Shield lakes except for Na^+ and Cl^- levels that are slightly elevated by local road salting (Hutchinson et al. 1989). To reflect ion levels more typical of shield lakes, all toxicity tests were run using a 1:1 mixture of lake water and lake water deionized by reverse osmosis. The chemical composition of this "dilution water" is given in Table 2. St. Mary's lake is also typical of other lakes in having background levels of metals that vary from the ALC-defined ratio (Tables 1 and 2). This made it difficult to produce exposure mixtures exactly matching the ALC ratio, particularly those below 2.0 ALC. To account for this, LC50s were calculated using ALC units based on the total measured concentrations of those metals that were added in each test. The actual ALC levels for each treatment were calculated as the geometric mean of individual metal concentrations after conversion to ALC units. For example, a solution containing 162 $\mu g\ Al \cdot L^{-1}$, 14 $\mu g\ Cu \cdot L^{-1}$, and 22 $\mu g\ Zn \cdot L^{-1}$ would have a calculated ALC(Al,Zn,Cu) of 2.09 units (i.e., geometric mean of 2.16, 2.32, and 1.83 ALC, respectively).

Source of Fish

Eyed rainbow trout eggs were obtained from a hardwater source at the aquaculture unit of Sir Sandford Fleming College, Lindsay, Ont. The eggs and alevins were held in incubator trays receiving water at a rate of 3 $L \cdot min^{-1}$ at 6–7°C. The trout lethality tests were conducted over a 4-wk period from 2-d posthatch and ending prior to swim-up. The average test temperature was 8.6°C (range ±0.5°C).

Fathead minnow larvae were produced from a breeding culture maintained at Dorset. Larvae were collected within 24 h of hatch and held in floating nylon mesh cages until used in tests starting at 48–72 h posthatch. Prior to testing, larvae were fed four times per day with live brine shrimp nauplii (*Artemia* sp.) that had been rinsed in fresh water. Fathead minnow were cultured and tested at 22°C (range ±0.5°C): They were not fed during lethality tests.

Test Methods

All lethality tests used five treatment levels plus a dilution water control. The five test levels were set according to a geometric progression (e.g., 1, 2, 4, 8, and 16 ALC) and usually encompassed an order of magnitude in metal (ALC) or H^+ concentration. For tests of H^+ toxicity, the controls were kept at the average ambient pH of 6.5, while in tests with metals the control was adjusted to the test pH. Of seven lethality tests conducted with fathead minnow at pH 5.8, five were rejected owing to excess control mortality (greater than 20%). Additional controls consisting of dilution water and St. Mary's lake water at pH 6.5 established that mortality was associated with H^+ stress rather than other factors.

Stock solutions of each metal (reagent grade) were made up in 1% (v/v) nitric acid from the sulfate salts, except for Pb in which lead nitrate was used. For tests with individual metals, the stock solutions were used directly in making the series of test concentrations. For tests involving metal mixtures, the stock solutions were used to make a cocktail of the desired metals in the ratio defined by the ALC unit. This cocktail was then diluted appropriately to make the series of test levels.

Test solutions were prepared and their pH adjusted with dilute KOH or HNO_3 at least 16 h prior to use, allowing time for CO_2 levels and metal speciation to equilibrate. Test solutions were made up in 2- or 1-L linear polyethylene beakers for trout or fathead minnow, respectively. Immediately before use, the pH of test solutions was readjusted to within 0.05 pH unit of nominal. During the tests, pH was checked twice daily and adjusted if necessary. The average measured pH in all tests was within 0.1 pH unit of nominal (SD ± 0.10). All solutions were replaced after 48 h.

Tests were started by randomly assigning an exposure cup (100-mL linear polyethylene beaker with nylon mesh bottom) containing 10–12 fish to each test beaker. Observations for mortality were made at 3, 8, 24, 32, and 48 h and every 24 h thereafter until the end of the test at 144 h. Death was defined as a lack of movement of operculae, pectoral fins, and the heart under 4–10× magnification.

Water Analysis

Metal levels and reference chemistry of the dilution water were determined for one control beaker from each block of tests (Table 2). Total metal concentrations were measured for each exposure level at 48 and 144 h. Samples were stored in acidwashed polystyrene (Fe, Mn) or linear polyethylene (Al, Zn, Cu, Ni, Pb) jars and acidified to 1% (v/v) with trace-metal-grade HNO_3. Total metal concentrations were determined by graphite furnace atomic absorption spectrophotometry (AAS) (Al), preconcentration and flame AAS (Zn, Cu, Ni, Pb), or colorimetry (Fe, Mn). Overall, average measured values ranged from 85 to 111% of nominal values for all metals except Pb, which averaged 152% of nominal and showed high variability at low concentrations. All remaining analyses were performed using standard techniques (Locke and Scott 1986). Analytical blanks appropriate to the design were completed in conjunction with all chemical analyses.

Dialysis techniques were used to estimate the degree of high-molecular-weight metal complexation at pH 4.9 and 5.8 (LaZerte 1984; Hutchinson and Sprague 1987). A separate test series of six beakers was used at each pH, each with a different concentration of the ALC(7) mixture. Dialysis bags (Spectrapor 6, 1.8 × 20 cm) with a nominal molecular weight cutoff of 1000 were prepared, cleaned according to manufacturer's instructions, and then filled with double-distilled water. One bag was placed in each beaker for 36 h, after which each metal was measured in the beaker water and the dialysate to separate total from dialysable forms.

Statistical Treatment

Incipient lethal levels (144-h LC50s) were calculated using the Spearman–Karber method (Hamilton et al. 1977) from fish mortality data and mean measured pH or metal concentrations (expressed as ALC units). The measured ALC levels for each treatment were calculated as the geometric mean of individual metal concentrations converted to ALC units. Only those metals added to the treatments were used to calculate ALC exposure levels, since the remaining metals were present in all treatments, including controls. The LC50s for the ALC(7) tests were also calculated using only Al, Zn, and Cu concentrations to examine possible bias in comparing LC50s for the ALC(7) and ALC(Al,Zn,Cu) tests. The resulting modified LC50s are denoted as ALC(7^1). Correction for control mortality was not required in tests with trout. In tests with fathead minnow, tests were rejected if control mortality exceeded 20%. Corrections for control mortalities below this level were not made, since control conditions (pH 5.8) were considered to be marginal for survival. Pairwise comparisons of LC50s were based on a standard error of the difference at a 5% probability level (Sprague and Fogels 1977).

The toxic interaction of pH and metal mixtures to trout was assessed by a linear regression of $\log_{10}$ LC50s for the ALC(7^1) mixture and the ALC(Al,Zn,Cu) subset plotted against exposure pH, and median lethal pH (in the presence of background metal levels). Geometric mean regression was used, since error was associated with both the LC50 estimates and the H^+ measurements (Halfon 1985).

Results

Fathead minnow larvae were more sensitive to pH than rainbow trout alevins under comparable test conditions (Table 3). The fathead minnow 144-h LC50 of pH 5.54 represented a 14- to 20-fold lower H^+ concentration than the LC50s of pH 4.39 and 4.24 for rainbow trout. The two tests with alevins, conducted on days 2 (pH 4.24) and 11 (pH 4.39), revealed a significant increase (30%) in sensitivity to H^+. The pH LC50s

TABLE 3. H^+ toxicity (median lethal pH) to rainbow trout alevins and fathead minnow larvae in the presence of the background metal levels given in Table 2.

	Median lethal pH	95% confidence limits
Rainbow trout	4.24	4.17–4.31
	4.39	4.34–4.44
Fathead minnow	5.54	5.37–5.72

were all determined in the presence of background metal levels calculated to be equivalent to 0.41 ALC (geometric mean of all metal data from controls, Table 2).

At pH 5.8, the LC50 (95% CI) of the ALC(7) mixture for fathead minnow was 0.84 (0.57–1.12) ALC (Table 4). Rainbow trout were nearly sixfold more tolerant of the ALC(7) mixture with an LC50 of 4.98 (4.29–5.78) ALC. The first null hypothesis, i.e., that a metal mixture representative of a lake at pH 5.8 would not be lethal, was accepted for rainbow trout (LC50 >> 1.0 ALC) but rejected for fathead minnow (LC50 < 1.0 ALC).

Toxicity tests with the ALC(7) and ALC(Al,Zn,Cu) subset demonstrated that mixture toxicity varied with test pH and that the subset was equivalent to the full mixture in potency (Table 4). The toxicity of the ALC(7) metal mixture to rainbow trout varied inversely with test pH, ranging from 4.98 ALC at pH 5.8 to 1.17 ALC at pH 4.6, leading to rejection of the second null hypothesis. This pattern was also evident in tests conducted with the ALC(Al,Zn,Cu) subset at pH 5.8 and 4.9. At pH 5.8, the ALC(7) LC50 of 4.98 did not differ significantly from the ALC(Al,Zn,Cu) subset LC50 of 5.60 conducted in the same experimental block (day 1). Both of these values were significantly higher than the ALC(Al,Zn,Cu) LC50 of 2.81 conducted on day 25, demonstrating that there was a significant twofold increase in sensitivity over the duration of the study. Tests conducted at pH 4.9 also showed no difference in toxicity between the full ALC(7) mixture and the ALC(Al,Zn,Cu) subset. Similarly, the tests conducted with larval fathead minnow showed that the ALC(7) and ALC(Al,Zn,Cu) subset were equitoxic at pH 5.8. The ALC(Mn,Fe,Ni,Pb) subset did not contribute to mixture toxicity; exposures of up to 20 ALC equivalents at pH 5.8 were nonlethal to trout. Taken together, these results demonstrate that toxicity of the ALC metal mixture could be attributed to components of the ALC(Al,Zn,Cu) subset under all test conditions.

Since the ALC(Mn,Fe,Ni,Pb) subset was shown to be nontoxic, and background levels of Fe and Mn were elevated, LC50 values for the ALC(7) tests were recalculated using only Al, Zn, and Cu data to better reflect their contribution to mixture toxicity. The resulting ALC(7^1) LC50 estimates (Table 4) were lower than ALC(7) values on average, although not significantly, and in no way altered the pattern or interpretation of results. The influence of pH on mixture toxicity was demonstrated by the following geometric mean regression (Fig. 1):

$$\log_{10}\text{LC50 (ALC)} = 0.72\ \text{pH} - 3.56 \quad (r^2 = 0.94,\ n = 9,\ p < 0.001)$$

which combined toxicity data for the equitoxic ALC(7^1) metal mixture and ALC(Al,Zn,Cu) subset plotted against test pH, and median lethal pH plotted against the background metal levels (0.31 ALC; based on geometric mean of Al, Zn, and Cu data). This equation showed that a pH of 4.94 would be required to produce an LC50 of 1.0 ALC for rainbow trout alevins under

TABLE 4. 144-h LC50s of the ALC(7) mixture and mixture components to rainbow trout alevins and larval fathead minnow over a range of pH. For tests with single metals, LC50s are also given as total metal concentrations. ALC(7^1) are the ALC(7) test results with the LC50s recalculated using only the measured Al, Zn, and Cu ALC levels. The trout tests were conducted in four time blocks over a 4-wk period. The superscripts a, b, c, and d indicate, respectively, tests that started 2–3, 11–12, 16–19 or 23–26 d posthatch.

ALC component	Nominal test pH	LC50 (ALC units)	95% confidence limits	LC50 ($\mu g \cdot L^{-1}$)
Rainbow trout				
ALC(7)	5.8	4.98[a]	4.29–5.78	—
	5.2	2.00[b]	1.83–2.19	—
	4.9	1.32[b]	1.17–1.49	—
	4.6	1.17[b]	0.92–1.50	—
ALC(7^1)	5.8	4.66	4.06–5.78	—
	5.2	1.99	1.83–2.17	—
	4.9	1.09	0.90–1.29	—
	4.6	1.01	0.73–1.40	—
Mn, Fe, Ni, Pb	5.8	>20.0[a]	—	—
Al, Zn, Cu	5.8	5.60[a]	4.57–6.83	—
	5.8	2.81[d]	2.27–3.47	—
	4.9	1.28[c]	1.07–1.52	—
Al	5.8	>14.4[d]	—	>1050
	4.9	1.17[c]	1.10–1.25	88
	4.9	1.21[d]	1.04–1.41	91
Cu	5.8	2.95[b]	2.08–3.47	18
	4.9	3.10[c]	2.33–4.13	19
Zn	4.9	36.10[c]	28.05–46.46	433
Fathead minnow				
ALC(7)	5.8	0.84	0.57–1.12	—
ALC(7^1)	5.8	0.70	0.49–1.53	—
Al, Zn, Cu	5.8	1.10	0.82–1.50	—

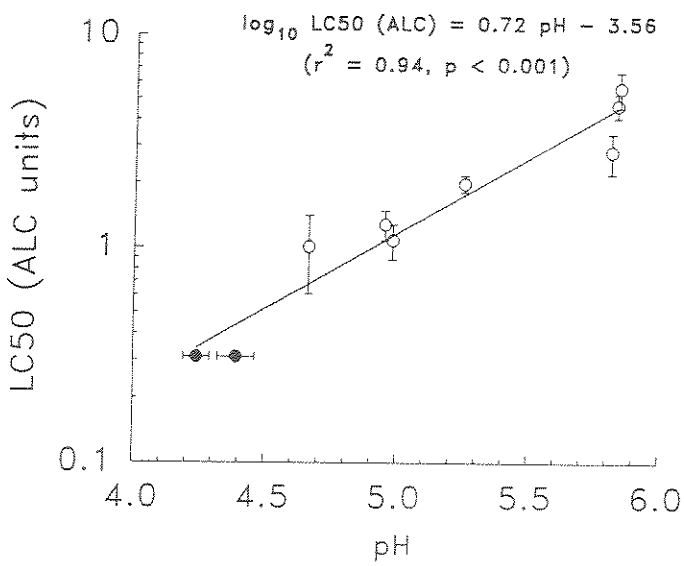

FIG. 1. Effect of pH on the lethality (144-h LC50) of metal mixtures to rainbow trout alevins in soft, acidic water. The geometric mean regression describes the effect of pH on the LC50s for ALC(7^1), ALC(Al,Zn,Cu) (○), and the median lethal pH values (●). The pH LC50s are plotted against the background metal (Al,Zn,Cu) level of 0.29 ALC. All LC50s are given with their 95% confidence limits.

our test conditions. The linear relationship also suggests additive action of H^+ and metal stress across the range of test conditions we used.

The remaining tests determined the toxicity of the individual components of the ALC(Al,Zn,Cu) subset to rainbow trout alevins at pH 5.8 and 4.9 (Table 4). At pH 5.8, Al toxicity was very low, with the highest exposure level of 14.4 ALC (1050 $\mu g \cdot L^{-1}$) producing only 20% mortality. Al toxicity was significantly greater at pH 4.9 than pH 5.8, at which point the two tests conducted with Al yielded LC50s equivalent to those for ALC(7), ALC (7^1), and ALC(Al,Zn,Cu). The LC50 for Cu at pH 5.8 (2.95 ALC, 18 $\mu g \cdot L^{-1}$) was not significantly different from the ALC(Al,Zn,Cu) LC50 (2.81 ALC) conducted as part of the same experimental block (started on day 25), but was significantly lower than the LC50s for ALC(7), ALC(7^1), and ALC(Al,Zn,Cu) conducted in earlier experimental blocks. There was no significant difference in Cu toxicity between pH 5.8 and pH 4.9. At pH 4.9, the LC50 for Cu (3.10 ALC, 19 $\mu g \cdot L^{-1}$) was significantly higher than the LC50s for ALC(7), ALC(7^1), and ALC(Al,Zn,Cu). The LC50 for Zn was only determined at pH 4.9, but was sufficiently high (36.10 ALC equivalents, 433 $\mu g \cdot L^{-1}$) that it could be eliminated from consideration as a toxic component. Overall, the third hypothesis was only partially supported, i.e., the relative importance of Al in the mixture varied with pH. At pH 4.9, Al was shown to be the primary toxic component for trout, while Cu was

TABLE 5. Percentage of total metals in the ALC(7) mixture capable of passing through dialysis tubing (1000 molecular weight) at pH 4.9 and 5.8. Values are the mean ± SEM of four or five samples in which total metal concentrations were similar to the 144-h LC50s for trout at each pH level.

	% dialysed metal	
Metal	pH 4.9	pH 5.8
Al	27.0 ± 2.2	5.7 ± 0.9
Mn	71.8 ± 4.0	79.0 ± 4.8
Fe	27.0 ± 2.0	12.7 ± 2.4
Ni	77.4 ± 7.4	88.0 ± 3.9
Zn	87.0 ± 2.0	90.6 ± 3.9
Cu	61.2 ± 2.5	60.3 ± 6.2
Pb	99.0 ± 7.4	44.0 ± 6.9

shown to be the primary toxic component at pH 5.8. At intermediate pH levels, toxicity is likely due to some degree of joint action between Cu and Al, and at pH 4.9 to the joint action of H^+ and Al.

The pH-dependent changes in the toxicity of the ALC(7) mixture and its toxic components were reflected by changes in metal availability as determined by dialysis (Table 5). The increase in Al toxicity between pH 5.8 and pH 4.9 was matched by an increase in the dialysable fraction from 5.7 to 27%. Expressing the trout LC50s for Al alone as dialysed metals gave values of 24.3 and >59.9 $\mu g \cdot L^{-1}$ (20% mortality) at pH 4.9 and 5.8, respectively. The toxicity to trout of both Cu alone and the dialysable fraction remained constant over the same pH range. The LC50s expressed as dialysable Cu were 11.4 $\mu g \cdot L^{-1}$ at pH 4.9 and 9.5$\mu g \cdot L^{-1}$ at pH 5.8. Dialysed Al and Cu levels associated with the ALC(7[1]) and ALC(Al,Zn,Cu) LC50s for fathead minnow were somewhat lower than for trout, ranging from 2.99 to 4.70 μg Al$\cdot L^{-1}$ and from 2.53 to 3.98 μg Cu$\cdot L^{-1}$, respectively, possibly being indicative of joint metal–H^+ stress at pH 5.8. These toxic levels of dialysable metals are similar to those reported by Hutchinson and Sprague (1987) for flagfish at pH 5.8 (5.2–5.6 μg Cu$\cdot L^{-1}$; 26–60 μg Al$\cdot L^{-1}$). Of the remaining metals in the ALC mixture, the dialysable proportion of Zn, Ni and Mn was similar at pH 4.9 and 5.8, while more Fe and Pb dialysed at pH 4.9 than at pH 5.8.

Discussion

These toxicity tests demonstrated that a mixture of Al, Mn, Fe, Ni, Zn, Cu, and Pb was acutely toxic to both rainbow trout alevins and fathead minnow larvae when added to soft, acidic water. There were, however, considerable differences in sensitivity due to both species and test pH. The first null hypothesis, i.e., that metal levels typical of acid lakes (1.0 ALC) would not be acutely toxic to fish at pH 5.8, was supported by the data for rainbow trout alevins, but not by those for fathead minnow larvae. Using 1.0 ALC as a numerical descriptor of typical metal levels in acid lakes, a simple comparison of confidence limits about LC50s demonstrates that most rainbow trout alevin mortality occurred at metal concentrations greater than 1.0 ALC (Table 4). Only at pH levels below 5.0 did the range of LC50s encompass 1.0 ALC. In contrast, the limited data from tests with fathead minnow did not support the hypothesis. The confidence limits of LC50s were lower than or encompassed 1.0 ALC, indicating that average metal concentrations in acid lakes would be lethal to some fish species. The sensitivity of fathead minnow to trace metals in soft, acidic water is comparable with the sensitivity of flagfish found by Hutchinson and Sprague (1986, 1987, 1989) under similar conditions.

The fathead minnow is known to be among the most acid sensitive of fish species. Palmer et al. (1989) demonstrated the extreme sensitivity of early fathead minnow life stages to H^+ and Al in soft water (6.2 $mg \cdot L^{-1}$ Ca; 21.6 $mg \cdot L^{-1}$ total hardness). A 96-h exposure of larval fathead minnow to pH 5.5 resulted in 85% mortality. The addition of 50 μg Al$\cdot L^{-1}$ (0.7 ALC equivalent) resulted in 100% mortality. At their next pH level of 6.5, exposures of larvae to up to 400 μg Al$\cdot L^{-1}$ were nonlethal. Mount (1973) found reduced egg production and hatchability of fathead minnow chronically exposed to pH 5.9. McCormick et al. (1989) found reduced larval survival at pH 6.0 plus 15 μg Al$\cdot L^{-1}$ and predicted recruitment failure of the species at pH 5.5. Mills et al. (1987) recorded recruitment failure of fathead minnow in a Precambrian Shield lake that was experimentally acidified to pH 5.93, with species extinction occurring at pH 5.64. Rahel and Magnuson (1983) found that fathead minnow were limited to lakes of pH ≥6.5 in a survey of 138 Wisconsin lakes of pH 4.0–9.2. In Ontario, the proportion of lakes supporting the species was reduced below pH 6.0, while the species was absent from lakes of pH <5.2 (Matusek et al. 1990). It is obvious that pH 5.9–6.1 marks the threshold of recruitment success for this species. It is also the pH range that favours reduction of the proportion of the most toxic Al species (inorganic monomeric Al including Al^{3+}).

Since the baseline conditions (2.5 mg Ca$\cdot L^{-1}$; pH 5.8, 55% of median lethal H^+ concentration) used in the present study were clearly stressful to fathead minnow larvae, the additional stress of minute levels of metals was catastrophic. There is a clear need to examine the interaction of pH, Ca concentration, and environmental levels of metals on the distribution of this sensitive species. Other species, such as the common shiner (*Luxilus cornutus*, formerly *Notropis cornutus*), are also very sensitive to acid stress (Holtze and Hutchinson 1989; Matusek et al. 1990), and their present distribution may reflect the combined stresses of acid and metals.

Test pH had a significant effect on the lethality of the ALC metal mixture to trout in this study, leading to rejection of the second hypothesis. The observed inverse relationship between mixture toxicity and H^+ concentration (Fig. 1) can be explained by the actions of H^+ as both a direct toxicant and as a factor modifying metal speciation, bioavailability, and hence, toxicity. This relationship is consistent with models describing the joint action of H^+ and free metal ions with respect to gill–metal binding and toxicity (Pagenkopf 1983). As H^+ concentrations increase, fewer metal ions are required to bring about a toxic effect at the gill surfaces. Eventually, the H^+ concentration reaches a level at which it is toxic in its own right. At sublethal pH levels, H^+ acts jointly with aqueous metal ions at the gill surface (Pagenkopf 1983), leading to ionoregulatory stress (loss of Na^+ and Cl^-). The most likely mechanism of Al toxicity in acidified waters is that of joint action with H^+ at gill surfaces, with resultant loss of Na^+ and Cl^-, at a pH that is stressful in the absence of Al (Holtze and Hutchinson 1989). This mechanism would reduce the apparent toxicity of Al as pH increased to less stressful levels. Although several workers have reported maximum Al toxicity at pH 5.5–6.3 (Driscoll et al. 1980; Neville 1985), their conclusions required oversaturated Al test solutions, conditions that do not reflect in situ conditions (LaZerte 1984; Hutchinson et al. 1987).

Close examination of the toxic contributions of the various metals in the ALC mixture indicates that all metal toxicity can be attributed to Al at pH 4.9 and to Cu at pH 5.8. These observations lead to rejection of the third hypothesis, i.e., that Al was the only mixture component contributing to toxicity. In soft water, the toxicity of total Cu shows little change with pH, while toxicity of the ionic and hydroxide forms increases slightly with pH (Howarth and Sprague 1978). This would partially explain the change from Al to Cu toxicity at pH 5.8. Although the toxicity of dissolved Zn also increases with pH (Bradley and Sprague 1985), its low toxicity (LC50 = 36 ALC equivalents at pH 5.9) precludes any role in toxicity of the metal mixture. Since Al, Zn, Cu, and H^+ are all ionoregulatory stressors, joint action at low pH and removal of H^+ stress at high pH would interact to produce the observed results. The same findings were reported by Hutchinson and Sprague (1989).

For rainbow trout alevins, the pH-induced shift from Al to Cu toxicity was due to changes in Al toxicity, since there was no significant change in the toxicity of Cu (Table 4) or in its bioavailability based on dialysis (Table 5) between pH 4.9 and 5.8. There was, however, an approximately 12-fold decrease in toxicity of total Al between pH 4.9 and pH 5.8. Part of this may be attributed to reduced H^+ stress. A pH of 4.98 represents a H^+ concentration that is 22% of the LC50 ($10^{-4.98}/10^{-4.32}$), while a pH of 5.84 represents just 3% of the lethal H^+ concentration ($10^{-5.84}/10^{-4.32}$, Tables 3 and 4), a level that is unlikely to produce even sublethal stress. Of more importance, however, were the changes in Al toxicity relating to its reduced solubility at pH 5.8. At pH 4.9, the Al 144-h LC50 averaged 90 $\mu g \cdot L^{-1}$ (Table 4). At pH 5.8, more than 1050 $\mu g \cdot L^{-1}$ were required for Al alone, or an average of 420 $\mu g \cdot L^{-1}$ from the two tests with the ALC(Al,Zn,Cu) mixture (Table 4). Both values are well above the theoretical solubility of Al at that pH (Burrows 1977). This is reflected in the low concentrations of soluble Al as measured by dialysis at pH 5.8 (5.7% × 420 $\mu g \cdot L^{-1}$ = 24 $\mu g \cdot L^{-1}$, Table 5), although some of the nondialysed Al would have formed organic complexes and so was soluble, but not available. The LC50 at pH 4.9, expressed as toxic, inorganic monomeric Al (dialysed, LaZerte 1984; Hutchinson and Sprague 1989), was 24 $\mu g \cdot L^{-1}$ for Al alone (27% × 1.54 ALC, Table 4). Low Al solubility at pH 5.8, equivalent availability of Cu at pH 4.9 and pH 5.8, and reduced H^+ stress at pH 5.8 thus combined to reduce the toxicity of the mixture as pH was raised (Fig. 1).

Al toxicity has been implicated as an important factor in the loss of fish from acidified systems (Driscoll et al. 1980; Campbell and Stokes 1985). However, our results suggest that Cu could contribute to impacts on fish populations and that this can occur at pH levels where Al toxicity and solubility are greatly reduced. With H^+ tolerant species such as rainbow trout, lethal levels of Cu were considerably higher than are usually observed in "pristine" lakes. For pH-sensitive species, such as the fathead minnow, however, environmentally realistic levels of Cu may be lethal in moderately acidified systems. Recent studies by our group (Welsh et al. 1993) have shown Cu to be extremely toxic to larval fathead minnow under test conditions similar to ours, with 96-h LC50s as low as 2.0 μg $Cu \cdot L^{-1}$ at a pH of 5.6 and a dissolved organic carbon concentration of 0.2 $mg \cdot L^{-1}$. Unfortunately, few studies of metal toxicity in the extremely soft waters characteristic of acidified systems have been published. Further research on the toxicity of Cu and other metals to fathead minnow and other acid-sensitive species, such as the zooplankter *Daphnia galeata mendotae* (LC50 = pH 5.85, Keller et al. 1990), in soft, acidic waters is warranted.

These results demonstrate that of the metals tested, only Al and Cu exert toxicity on fish at levels typical of acidified lakes in Ontario. Toxicity will also increase as pH decreases. Furthermore, the large differences in sensitivity to Cu between larvae of fathead minnow and alevins of rainbow trout suggest that some species could be eliminated by Cu toxicity at pHs above those associated with increased Al solubility and toxicity. At the lethal pH for fathead minnow, the levels of Cu normally present in the control treatments were very close to the 1–5 μg $Cu \cdot L^{-1}$ that killed fathead minnow at higher pHs as reported here and by Welsh et al. (1993). Is H^+ sufficiently concentrated to be lethal, or is this simply the pH at which Cu ions become toxic? These concentrations are below the mean levels measured in some acidified Ontario lakes and very close to the routine analytical detection. As such, care is needed to avoid accidental contamination of test solutions and water samples. Under these circumstances, research and monitoring of acid effects on aquatic ecosystems may overlook the contribution by Cu. Reports of acid effects should be treated with caution if waterborne metal levels are not measured simultaneously with pH and acidity, and in conjunction with a rigorous program of analytical quality assurance.

Acknowledgements

This work was supported in part by a contract from the Department of Fisheries and Oceans to D.G.D. We thank P. Dillon, G. Mierle, and K. Holtze for providing access to facilities at the Dorset Research Centre, Ontario Ministry of Environment and Energy (OMEE), and the staff of the Dorset and Rexdale chemistry laboratories of OMEE for coping with the increased analytical load. Dr. J.B. Sprague kindly provided a breeding population of fathead minnow.

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