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Toxicity of Trace Metal Mixtures to American Flagfish (*Jordanella floridae*) in Soft, Acidic Water and Implications for Cultural Acidification

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Laboratory exposures over 1.3 generations showed that trace metal mixtures played a dominant role in the reproductive failure of American flagfish (*Jordanella floridae*) in soft (6.0 $mg \cdot L^{-1}$ as $CaCO_3$ total hardness), acidified (pH 5.8) water. This finding may also apply for native fish species inhabiting culturally acidified waters. Reproductive failure was complete when pH 5.8 water contained a mixture of Al, Mn, Fe, Ni, Zn, Cu, and Pb at 57% of the concentrations found in acidified waters (or 0.57 ALC). Fry died within 6 d of exposure, adults transferred into the treatment ceased spawning, and hatching of transferred eggs was reduced. The onset of steady spawning activity was delayed by addition of smaller amounts (0.09–0.27 ALC) of metals, but reductions in size of juvenile fish did not persist to maturity. At pH 5.8 with no metals, no effects on any stage of the life cycle were observed. Subsequent testing of fry showed that lethality of a mixture of Al, Zn, and Cu was equivalent to that of all seven metals. The threshold acute LC50 for flagfish fry at pH 5.8 occurred with the simultaneous presence of Al, Zn, and Cu at 29, 5, and 2.3 $\mu g \cdot L^{-1}$, respectively, or with 95 μg $Al \cdot L^{-1}$ alone. Future research on trace metal stress in culturally acidified waters should consider Zn and Cu, in addition to Al.

Les expositions en laboratoire de 1,3 génération d'American flagfish (*Jordanella floridae*) ont montré que des mélanges de métaux à l'état de trace jouaient un rôle dominant dans l'infécondité de ce poisson dans des eaux acidifiées (pH 5,8) et douces (dureté totale 6,0 $mg \cdot L^{-1}$ en $CaCO_3$). Cette découverte vaudrait aussi pour des espèces indigènes habitant des eaux acidifiées par des cultures. L'infécondité etait totale dans de l'eau de pH 5,8 contenant un mélange de Al, de Mn, de Fe, de Ni, de Zn, de Cu et de Pb concentré à 57 % des valeurs trouvées dans les eaux acidifiées (ou 0,57 ALC). Le frai mourait en moins de 6 jours d'exposition, les adultes soumis à ce traitement cessaient de frayer et moins d'oeufs exposés éclosaient. Le début de la pleine activité de frai a été retardé par l'addition de petites quantités (0,09–0,27 ALC) de métaux, mais les réductions dans la taille des poissons juvéniles n'ont pas persisté jusqu'à la maturité. Avec un pH 5,8 et en l'absence de métal, on n'a observé aucun effet à aucun stade du cycle de vie. D'autres analyses du frai ont montré que la létalité d'un mélange de Al, de Zn et de Cu était équivalente à celle d'un mélange des sept métaux. À un pH de 5,8, le seuil de la CL50 aiguë pour le frai du flagfish a été atteint dans un mélange de 29,5 et de 2,3 $\mu g \cdot L^{-1}$ de Al, de Zn et de Cu respectivement ou dans 95 $\mu g \cdot L^{-1}$ de Al seul. D'autres recherches sur l'effet des métaux à l'état de trace dans des eaux acidifiées par des cultures devraient porter sur le Zn et le Cu, en plus de l'Al.

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There is extensive documentation of recruitment failures and the subsequent demise of fish populations in culturally acidified waters (Beamish and Harvey 1972; Beamish 1974, 1976; Schofield and Trojnar 1980; Muniz and Leivestad 1981; Mills 1984). Successful reproduction of some species cannot be ensured in soft waters experiencing long-term depressions of pH to below 6.5 (Fromm 1980), but metal toxicity has been suggested as a possible additional stress (Beamish 1976; Harvey and Lee 1982).

Recent research has focussed primarily on the toxic effects of aluminum in soft, acidic water. It is clearly toxic to fish (Driscoll et al. 1980; Brown 1983) and has been considered to be the controlling factor for fish distributions in acidified waters (Schofield and Trojnar 1980). In spite of this, the common condition in most waters showing some degree of acidification-related stress on fisheries is depressed pH and elevated levels of other metals in addition to Al. Impacted lakes in the Sudbury and La Cloche mountain regions of Ontario have elevated levels of Al, Mn, Zn, Ni, and Cu (Beamish 1976; Keller et al. 1981; Kelso and Gunn 1984). Acidified waters in Scandinavia have elevated levels of Al, Zn, and Pb (Henriksen and Wright 1978; Muniz and Leivestad 1981) and fishery impacts have been documented (Jensen and Snekvik 1972). Elevated levels of Al, Mn, and Zn are found in acidified lakes of the northeastern United States from which population losses have been reported (Baker 1982). The controlled acidification of Lake 223 in Ontario increased the waterborne concentrations of Al, Mn, and Zn (Schindler and Turner 1982), and both

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fathead minnows (*Pimephales promelas*) and lake trout (*Salvelinus namaycush*) experienced recruitment failures (Mills 1984). Conversely, in situ bioassays (i.e. Gunn and Keller 1984) did not demonstrate recruitment failure of lake trout in the presence of depressed pH and elevated levels of inorganic, monomeric Al, which is the lethal form of that metal (Driscoll et al. 1980). Thus, research to date has not conclusively linked recruitment failures of fish to depressed pH and elevated levels of Al.

The possibility of joint toxicity of hydrogen ion and trace metal mixtures in acidified waters should not, therefore, be overlooked. Studies done in neutral pH waters have clearly shown that trace metal mixtures decrease reproductive success of fish (i.e. Cu, Cd, and Zn, Eaton 1973; McFarlane and Franzin 1978) and interact to produce acute lethality of juvenile fish (i.e. Cu, Mn, and Zn, Lewis 1978; Cu, Cd, and Zn, Finlayson and Verrue 1982). In addition, Wong et al. (1978) reported that As, Cd, Cr, Cu, Fe, Pb, Hg, Ni, Se, and Zn, each at the "safe" concentration recommended in the Great Lakes Water Quality Objectives, were very toxic to algae when all were present in a mixture.

These studies illustrate a clear potential for trace metal mixtures to have sublethal effects on fish in acidified lakes, especially considering the low levels of total hardness that characterize such waters. The amplifying effect of low hardness on lethality of Zn and Cu is well established (Mount 1966; Howarth and Sprague 1978; Chakoumakos et al. 1979; Borgmann 1983) but few studies have evaluated the toxicity of metal mixtures at concentrations near sublethal thresholds, and none at low pH in soft water.

The purpose of our study was to make a direct assessment of the possible role of trace metal mixtures in recruitment failure of fish in acidified soft water. Chronic exposure of the American flagfish (*Jordanella floridae*; Goode and Bean) over a full reproductive cycle in the laboratory was selected as the most effective exploratory approach, as such experiments have provided the most reliable information on chronic effects of toxicants on fish (McKim 1977). Although the flagfish is not native to Canadian soft waters, its responses to trace metal toxicity are similar to those of native species (Fogels and Sprague 1977; McKim 1977). Its responses to acidification in previous studies (Craig and Baksi 1977) have occurred well within the pH range at which native species have declined in Ontario Lakes (Harvey 1979).

Our test pH of 5.8 was chosen to represent a H^+ concentration at which some reproductive failure had been previously documented for flagfish (Craig and Baksi 1977) and for native species such as fathead minnows (Mount 1973; Mills 1984), brook trout (*Salvelinus fontinalis*) (Menendez 1976), lake trout (Ontario Ministry of the Environment 1978; Mills 1984), and other species (Harvey 1979). A necessary prerequisite was identification of the trace metals of potential toxicological significance and their concentrations in acidified lakes.

Specific objectives of our study were (a) to separate the effects of H^+ on fish reproduction from those of trace metal mixtures at low pH in soft water (b) to identify the life stages most sensitive to H^+ and metals stress, (c) to determine which of the metals found in acidified waters had the greatest toxicity, and (d) to assess the impact of rapid deterioration of water quality on selected life stages of fish (i.e. pH depressions and/or metal elevations accompanying snowmelt or rainstorms) (Jeffries et al. 1979; Dillon et al. 1984; Gunn and Keller 1984).

Materials and Methods

The methods used for the test of full life-cycle exposure were those established by Spehar (1976) and Holdway and Sprague (1979). Constant toxicant concentrations were maintained in a flow-through system during 3 mo while fry were raised to maturity and their reproductive performance assessed. Second-generation fry were then raised to age 42 d under the same experimental conditions. Duplicate exposures were run for first-generation fish and a single exposure for the second generation. Upon completion of continuous exposure, acutely lethal exposures of fry, reproducing adults, and eggs allowed determination of (a) the effects of sudden exposure of these life stages to extreme conditions, (b) the influence of previous exposure history on responses to lethal conditions, and (c) the metals of most toxicological significance to fry.

The concentrations of metals expected in waters acidified to pH 5.8 were determined using existing data from lakes in sensitive areas of Ontario (Muskoka–Haliburton, P. J. Dillon, Ontario Ministry of the Environment, Dorset Research Centre, Dorset, Ont. P0A 1E0, pers. comm.; Sudbury and La Cloche mountain region, Ontario Ministry of the Environment 1978; Algoma region, J. Kelso, Great Lakes Biolimnology Laboratory, Department of Fisheries and Oceans, Sault Ste. Marie, Ont. P6A 2B3, pers. comm.). Concentrations of Al, Mn, and Zn showed highly significant ($p < 0.000001$) negative correlations with pH, and correlation coefficients of −0.63, −0.60, and −0.40, respectively, were obtained for sample sizes of 113, 63, and 317. Concentrations of 75, 60, and 12 $\mu g \cdot L^{-1}$ at pH 5.8 were calculated from these regression lines for Al, Mn, and Zn, respectively, and were confirmed by comparison with data from Baker (1982). Fe, Ni, Cu, and Pb showed less significant correlations with pH ($p > 0.001$, $-0.2 < r < 0.14$), $n = 228$, 314, 318, and 296, respectively) but all are present in acidified waters and may either be toxic themselves or act to produce joint toxicity with H^+ and other trace metals. Their concentrations were thus averaged for all lakes, and mean values of 60, 12, 6, and 6 $\mu g \cdot L^{-1}$, respectively, used to represent the concentrations expected at pH 5.8. Other metals, such as Cd and Hg, are known to be present in acidified waters but insufficient data on their concentrations prevented proper consideration. Accordingly, a mixture of Al, Mn, Zn, Fe, Ni, Cu, and Pb, at the concentrations established for a lake at pH 5.8, was considered one acid lake concentration or 1.0 ALC of metals and used as the basis for metals dosage in the experiments (Table 1).

We assessed effects of three different concentrations of the seven-metal mixture at pH 5.8 and compared these with effects in neutral (pH 7.3) and acid controls (pH 5.8). The concentration ratios of the seven metals remained relatively constant at the three treatment levels, and only the overall metals concentrations (or ALCs) differed. These averaged 0.09, 0.27, and 0.57 ALC for each treatment, respectively, above background levels of the seven metals established in controls.

To start the life-cycle experiment, each aquarium was supplied with 19 flagfish, 8–10 d old and feeding exogenously. These were first-generation offspring of wild parents imported from Florida. Fish were fed five times daily throughout the experiment, using age-specific diets (Holdway 1978) consisting of mixtures of Wardley's "Liquid Fry," live brine shrimp nauplii, frozen brine shrimp, powdered trout chow, or dry flake food.

Survival, size, and deformities were determined at age 42 d,

TABLE 1. Summary of metal concentrations ($\mu g \cdot L^{-1}$) for life-cycle and transfer experiments. Concentrations labelled "1.0 ALC" were those estimated as typical of the mixture of metals in a lake at pH 5.8 (1.0 acid lake concentration). Remaining values are the arithmetic average concentrations actually measured in test tanks during the experiment, the mixtures being characterized overall as fractional ALCs. Standard deviations are given in parentheses.

1.0 ALC	Al 75	Mn 60	Fe 60	Ni 12	Zn 12	Cu 6	Pb 6
Acid + 0.57 ALC	52	23	24	7	9	5	3
	(14)	(6.4)	(5.5)	(3.3)	(5.6)	(1.2)	(2)
Acid + 0.27 ALC	21	9	4.5	<5	5	2	2
	(6.1)	(2.2)	(1.9)	(—)	(1.5)	(3.9)	(1.1)
Acid + 0.09 ALC	10	3	4	<5	2	2	2
	(5.9)	(1)	(5)	(—)	(0.9)	(1.4)	(1)
Acid control	4	1	4	<5	3	2	2
	(6)	(0.4)	(5)	(—)	(1.4)	(1.3)	(1.1)
Neutral control	5	2	4	<5	3	2	1
	(2)	(0.7)	(3.3)	(—)	(2.9)	(0.7)	(0.7)

when fish were thinned to 15 per tank, at age 77 d, when breeding colonies of two males and four females were established, and at age 132 d, when egg counts were completed. Standard lengths of all fish were determined at age 42 and 77 d by photographing them against a standard measure on a white background. Standard lengths and wet weights of all fish were measured at 132 d, after assessment of egg production.

Egg counts were made daily for 25 d following the first egg production in each tank, and time to first and to steady spawning (≧20 eggs for four consecutive days) was noted. Hatching success and time to hatch were determined on 10–30 eggs collected from each tank on each of the first 4 d of steady spawning and incubated in floating nylon baskets in the same aquaria in which they were spawned. Survival and deformities in second-generation fry were assessed at 4, 6, and 42 d posthatch. Measurements of standard length and wet and dry weights (105°C for 24 h) were made on 15 fish from each tank at age 42 d.

Upon completion of continuous exposure (age 132 d), breeding colonies of adults from controls and the acid + 0.27 ALC exposure were transferred to acid + 0.57 ALC and their spawning activity assessed for 20 d. Eggs from each of the controls and the two low-metals exposures were moved to treatments of lower pH and/or higher metal levels within 3 h of spawning, and hatching success was determined (see Table 5). Fry from neutral and acid controls and the acid + 0.27 ALC treatment were reared to age 8–9 d and their survival assessed in the acid + 0.57 ALC treatment. These procedures allowed determination of (a) the effects of sudden exposure to extreme conditions and (b) the influence of previous exposure history on responses to lethal conditions.

Dilution water for these experiments was a mixture of reverse osmosis water (Culligan Aqua-Clear M-1000) mixed with well water to obtain a total hardness of 6.0 $mg \cdot L^{-1}$ as $CaCO_3$. Water for the neutral controls was not further adjusted and average pH was 7.27 (95% c.i. = ±0.02, n = 318). pH of the water for the rest of the tanks was continuously monitored (Radiometer PHM61 pH meter) and signals fed into a controller (Radiometer TTT80) which added 0.013 M H_2SO_4 into a reservoir through a magnetic valve. Marriotte bottles were used to deliver three separate concentrations of the seven-metal stock solution to the acidified water and to add small amounts of NaOH for further pH control. Average pH in each tank, based on daily observations, ranged from 5.81 to 5.84 (95% c.i. = ±0.07, n = 249–324) except for the acid + 0.57 ALC tanks where it averaged 5.87 ± 0.07 (n = 82). Flow into each of the all-glass, 25-L aquaria was 130 $L \cdot d^{-1}$, giving a 90% molecular replacement time of 11 h (Sprague 1973).

Stock solutions of metals were made up in each Marriotte bottle at least 24 h prior to use. Metals were added in the sulfate form for all except Pb, which was added as nitrate. The concentration of metal in each treatment or control was measured 50–52 times over the course of the experiment, except in the acid + 0.57 ALC treatment which had only 15 measurements because of complete mortality at an early stage. Samples were stored in linear-polyethylene bottles which had been bathed in 0.8 M HNO_3 for 24 h and rinsed 10 times in double-deionized water before use. Samples were preserved in 0.016 M HNO_3 ("Ultrex" brand). Concentrations of total Cu and Zn were analyzed by differential-pulse anodic stripping voltammetry in 0.1 M sodium acetate buffer on an E.G.&G. P.A.R.C. model 174A polarographic analyzer with a model 303 static mercury-drop electrode. All other metals were analyzed by atomic absorption spectrophotometry using a Pye-Unicam model SP1950 A.A.S. with a model SP9-01 flameless atomizer, or a Varian Techtron A6 with a CRA-90 carbon rod atomizer.

Other chemical variables were measured daily in each tank. Average characteristics of the test water (with 95% c.i.) were as follows: temperature, 25.1 ± 0.42°C; dissolved oxygen, 7.6 ± 0.17 $mg \cdot L^{-1}$; total hardness, 6.4 ± 0.4 $mg \cdot L^{-1}$ (as $CaCO_3$) in all but the acid + 0.57 ALC treatment (5.0 ± 0.32 $mg \cdot L^{-1}$); total inflection point (TIP) alkalinity (Gran 1952), 15.0 ± 0.10 $mg \cdot L^{-1}$ (as $CaCO_3$) in the neutral controls and 2.0 ± 0.9 $mg \cdot L^{-1}$ in all other tanks. A photoperiod of 16 h light: 8 h dark was maintained, with 0.5-h transitions between dark and light periods included in the light portion. Radiant intensity at the water surface was 1.8–4.0 $W \cdot m^{-2}$, supplied from fluorescent and incandescent lighting.

The active metals in the mixture were determined by comparing the lethality of seven metals with that of fewer metals or individual metals, always at pH 5.8. These tests of acute lethality were run simultaneously in groups of two, three, or four, with one reference bioassay of seven metals for each series. Groups of 10–15 flagfish fry, age 2–3 d, were exposed in 100-mL Nalgene beakers with nylon mesh bottoms, floating

TABLE 2. Percentage mortality and size of flagfish at different ages following continuous exposure to acid and metals. LC50s are expressed in ALCs with 95% confidence limits given in parentheses. Standard lengths are given at 42 and 77 d since weights could not be taken. At 132 d, dry weights and lengths showed the same trends as the wet weights given. Standard deviations of size measurements are given in parentheses.

	Percent mortality				Size			
	First generation		Second generation		First generation			Second generation
					Length (mm)		Weight (mg)	Length (mm)
	42 d	42–132 d	4 d	42 d	42 d	77 d	132 d	42 d
Neutral control	10	0	5	22	15.9 (2.7)	28.5 (4.2)	2190 (912)	14.7 (2.1)
Acid control	5	0	3	18	16.4 (3.0)	24.0[c] (5.0)	2332 (978)	13.2[c] (2.9)
Acid + 0.09 ALC	5	0	7	24	17.0 (2.0)	24.0[c] (3.1)	2052 (1057)	13.6[c] (2.5)
Acid + 0.27 ALC	25	0	9	38	15.1 (2.1)	23.4[c] (3.0)	1579 (543)	11.8[c,e] (2.6)
Acid + 0.57 ALC	100[a]	—	100[a,d]	—				
LC50	0.34 (0.31–0.36)		0.38[b] (0.36–0.40)	0.33 (0.30–0.35)				

[a]Significantly greater than both controls ($p < 0.05$).
[b]Significantly different from 42-d LC50s ($p < 0.05$).
[c]Significantly smaller than neutral controls ($p < 0.05$).
[d]For fry transferred from neutral control.
[e]Significantly smaller than first-generation fry ($p < 0.05$).

in 2-L Nalgene beakers. These fish were spawned and hatched in soft (6.0 $mg \cdot L^{-1}$ as $CaCO_3$) water at a pH of approximately 7.3.

Five exposures were used in each test of acute lethality, plus a control with no added metals. Toxicant was made up from stock solutions and the pH adjusted to 5.8 at least 12 h before each test started. Toxicant was replaced at 48 h and exposures continued until lethal thresholds were reached at 96–120 h. Fry were not fed during tests. Observations were made nine times, in a logarithmic series from 4 to 120 h, and mortalities recorded when movement of the pectoral fins, operculum, and heart could no longer be detected with a dissecting microscope. Water baths held exposure temperatures at 25.0°C (SD = ±0.06) and manual addition of 0.016 M HNO_3 or KOH resulted in an average pH of 5.81 ± 0.02, based on measurements made at each observation time. Test water was from the same source as in the chronic tests, with average total hardness of 6.0 ± 1.7 $mg \cdot L^{-1}$, dissolved oxygen of 7.3 ± 0.29 $mg \cdot L^{-1}$, and TIP alkalinity of 1.0 ± 0.3 $mg \cdot L^{-1}$.

Water samples for analysis of total metals were taken twice from each beaker in each test and preserved and stored in the same manner as for the test of chronic exposure. Total metal concentrations were determined by flameless AAS, using a Scintrex AAZ-2 with tungsten filament atomization and Zeeman modulation for Al, Mn, and Pb, or a Varian A5 with a CRA-90 carbon rod atomizer and no background correction for Zn and Cu. Concentrations of Ni and Fe were not measured, but were estimated from ratios of nominal to measured concentrations determined in the test of chronic toxicity.

All statistical comparisons between treatments were initiated using one-way analysis of variance with pooled replicates if sample sizes were unequal, or two-way ANOVA with replicates considered as the number of observations for equal sample sizes. Any deviations from basic procedures are evident in the appropriate results section. Treatment effects were compared with acid and neutral controls using Dunnett's test (Steele and Torrie 1960). A protected least significant difference (LSD) test was used to determine significant differences in lethality of different metal combinations. Bartlett's test or plots of residuals were used to confirm homogeneity of variance and data were transformed before analysis if necessary.

Median lethal concentrations (LC50s) were calculated by the Spearman–Karber method (Hamilton et al. 1977), using data on fish mortalities and measured metal concentrations in ALC. Tests in which control mortality exceeded 20% were discarded as invalid. Lower levels of control mortality were corrected, using Abbott's formula, prior to LC50 calculation.

Results

Life-Cycle Experiment

Survival

Mortality of first- and second-generation fry was rapid and complete in the acid + 0.57 ALC exposure. First-generation fry died within 10 d and no second-generation fry (age 8–9 d) survived 2 d of exposure following transfer from the neutral control. Survival was not affected in the two lower metal exposures. Mortalities of 25 and 38% in the acid + 0.27 ALC treatment at 42 d of the first and second generations, respectively, were not significantly greater than those in controls, some such mortality being usual in experiments of this type (Table 2). Few or no deaths occurred in the remaining test conditions from 42 d until termination at 132 and 42 d of the first and second generation, respectively.

Previous exposure history (spawning to 8 d post-hatch) of fry influenced their resistance time in the acid + 0.57 ALC exposure. Fry transferred from the acid + 0.09 ALC treatment

TABLE 3. Summary of effects of continuous exposure to acid and metals on reproduction in flagfish. Total fry mortality at acid + 0.57 ALC prevented assessment of effects in that treatment. All given values are mean response with standard deviation in parentheses.

	Time to steady spawning (days of exposure)			Egg production (eggs·female^{-1}·d^{-1})			Time to hatch (d)	Percent hatched			Spawning success after transfer to acid + 0.57 ALC	
	A	B	Avg.	A	B	Avg.		A	B	Avg.	No. spawnings	Eggs·female^{-1}·d^{-1}
Neutral control	73	76	74.5 (2.1)	26.8 (15.7)	30.8 (21.1)	28.8 (18.5)	5.9 (0.4)	68.2 (18.3)	80.0 (10.3)	74.1 (15.1)	2	0.03
Acid control	76	76	76.0 (0.0)	20.9 (18.6)	32.1 (20.8)	25.0 (20.3)	5.8 (0.5)	71.8 (18.1)	93.2 (5.6)	82.5 (16.9)	5	5.5
Acid + 0.09 ALC	80	80	80.0[a,b] (0.0)	20.3 (14.8)	23.1 (14.4)	21.5 (14.3)	6.1 (0.6)	84.2 (8.8)	88.9 (3.1)	86.5 (6.6)	—	—
Acid + 0.27 ALC	78	79	78.5[a] (0.7)	18.4 (12.0)	39.2 (24.2)	29.4 (23.8)	6.3 (0.5)	75.5 (16.9)	62.5 (18.3)	69.0 (17.7)	4	1.7

[a]Significantly different from neutral control.
[b]Significantly different from acid control.

TABLE 4. Hatching success for eggs spawned in one treatment (rows) and incubated in a different treatment (columns). Eggs were transferred within 4 h of spawning. Controls for each transfer were cases where spawning and incubation environments were identical. Values given are mean percentage hatching success with one standard deviation given in parentheses.

Spawning environment	Neutral control	Acid control	Acid + 0.09 ALC	Acid + 0.27 ALC	Acid + 0.57 ALC
Neutral control	75.9 (27.5)	76.1 (26.3)		57.1 (36.2)	42.8[a] (39.7)
Acid control		85.8 (8.3)		79.2 (17.5)	80.2 (20.8)
Acid + 0.09 ALC			91.2 (4.6)	—	69.9[a] (18.6)
Acid + 0.27 ALC				92.0 (4.4)	62.6[a] (18.6)

[a]Significant reduction ($p < 0.01$) over controls.

died within 3 d compared with 2 d for those transferred from neutral controls. Fry transferred from the acid + 0.27 ALC treatment resisted lethal exposures for 6 d. Two males out of 15 adult fish (age 132 d) died 5 d after transfer to acid + 0.57 ALC from less severe treatments.

The 42-d LC50 for first-generation fish was 0.34 ALC (95% c.l. = 0.31, 0.36), as determined from percentage mortalities of pooled replicates. The 42-d LC50 calculation for second-generation fry included 100% mortalities of fry transferred into the acid + 0.57 ALC treatment, as none were, of course, available from continuous exposure. Its value of 0.33 ALC (95% c.l. = 0.30, 0.35) was not significantly different from that for first-generation fish ($p > 0.05$; standard error of the difference) (Sprague and Fogels 1977). The 96-h LC50 for second-generation fry, as determined from percentage mortalities in pooled subsamples, was 0.38 ALC (95% c.l. = 0.36, 0.40), which is close to, but significantly different from, the 42-d threshold of acute lethality.

Size

Treatment effects on first-generation fish varied with age and length of exposure. At 42-d, no significant differences in size of fish from controls or treatments existed. At maturity (77 d), fish from the pH 5.8 controls and both acid + metals treatments were significantly smaller ($p < 0.05$) than those from the pH 7.3 controls. Comparisons within affected treatments were not significant, however (Table 2). Reduced size in these fish was not maintained with continued exposure, as neither standard length nor wet and dry weights were significantly affected at the end of the spawning period (132 d).

Standard lengths of 42-d-old, second-generation fish from the acid + 0.27 ALC treatment were significantly less than those from the pH 7.3 control but no other comparisons were significant. Second-generation fish exposed to acid or acid + metals were significantly smaller than those of the same age (42-d) from the first generation but had been exposed from hatch instead of from age 8–9 d.

Spinal deformities were observed infrequently in the continuous exposure experiment. Scoliosis (20° spinal bend) was seen in 1 of 151 fish that hatched in the neutral control but that fish did not survive. A 90° spinal bend was seen in 1 of 166 fish in the acid controls and this individual survived. Four percent of the fry in the acid + 0.27 ALC treatment had scoliosis; severity of spinal bends ranged from 20 to 180°. Only half of these fish survived 6 d. Scoliosis (45–180°) was observed in the acid + 0.27 and 0.57 ALC treatments in the transfer experiments. The incidence of these deformities could not be quantified but was judged to be of minor significance because all fish died in the acid + 0.57 ALC treatment.

Reproduction

The first spawning occurred after 67 d (neutral controls) and

TABLE 5. Toxic contributions by different metals in the original mixture of seven. Values given are mean LC50 with one standard deviation given in parentheses. Underlining joins mixture LC50s judged not significantly different ($p > 0.05$) by a protected LSD test.

	7 Metals	Al−Zn−Cu	Al	Mn	Mn−Fe−Ni−Pb
N	5	6	5	2	2
Avg. LC50	0.39	0.38	1.26	4.50	4.32
(ALCs)	(0.09)	(0.11)	(0.68)	(0.87)	(0.00)

74 d (acid + 0.27 ALC) of exposure. Such activity is inconclusive, however, and time to steady spawning was considered a more dependable criterion. Time to steady spawning of neutral (74.5 ± 2.1 (SD) d) and acid controls (76.0 ± 0.0 d) was not significantly different (Table 3). Steady spawning was significantly delayed in the acid + 0.09 ALC treatment (80.0 ± 0.0 d) compared with both neutral and acid controls. Spawning in the acid + 0.27 ALC treatment was delayed (78.5 ± 0.7 d) when compared with neutral controls, but not when compared with acid controls.

Spawning, once started, was very consistent; eggs were obtained from all tanks on at least 23 days of the 25-d observation period. Egg production ranged from 21.5 to 29.4 eggs·female^{-1}·d^{-1} (Table 3) but no significant effect of treatment on egg production was demonstrated.

A striking disruption of spawning occurred in the acid + 0.57 ALC treatment when three breeding colonies were transferred to it. Over 20 d, fish never achieved steady spawning and, at best, spawned on only 5 days. Within this meagre range of production, fish transferred from the acid control performed best (5.5 eggs·female^{-1}·d^{-1}) and those from neutral controls worst (0.03 eggs·female^{-1}·d^{-1}, Table 3). Nontransferred control fish continued to produce 18−34 eggs·female^{-1} on 17 days of the same 20-d period.

Neither time from spawning to hatch nor percentage hatching success varied significantly with treatment. Elapsed time between egg collection and total hatching varied from 5.8 (SD = ±0.5) to 6.3 ± 0.5 d and percentage hatching success ranged from 69 ± 17.7 to 86.5 ± 6.6 (Table 3). No first-generation fish survived to reproduce in the acid + 0.57 ALC treatment. Hatching success was therefore assessed by transfer of eggs from other treatments to acid + 0.57 ALC within 4 h of spawning. Controls for these transfers were eggs that had been spawned and incubated in equivalent exposure conditions. A X^2 transformation was applied to stabilize variance. Results of *t*-tests showed that incubation in the high metals treatment reduced hatching success significantly ($p < 0.01$) for eggs transferred from neutral controls and the two lower-metals treatments, but not for eggs transferred from the acid controls (Table 4). No significant reductions occurred for eggs incubated in any other treatment. The higher probability level ($p < 0.01$ vs. $p < 0.05$) was used as protection from a nonsignificant ANOVA, as hatching success was obviously lower in the acid + 0.57 ALC treatment (J. Hines, Department of Mathematics and Statistics, University of Guelph, Guelph, Ont. N1G 2W1, pers. comm.). The most convincing evidence of lethality of the acid + 0.57 ALC treatment was that all fry that did hatch were dead within 8 d. Encapsulation of the heads of hatched fry by the chorion was observed frequently in this treatment.

Toxic Metals in the Mixture

Aluminum, Zn, and Cu were the most potent of the seven metals. Six lethal tests done with mixtures of these three metals at pH 5.8 gave an average LC50 of 0.38 ALC (95% c.i. = ±0.11) which was almost identical to the LC50 of 0.39 ± 0.09 ALC obtained for the seven-metal mixture (Table 5). Concentrations of Al, Zn, and Cu at the mixture LC50 were 29.0, 4.6, and 2.3 μg·L^{-1}, respectively. A one-way ANOVA showed an overall significant effect of metals combination on $\log_{10}$ LC50 ($p < 0.001$). Aluminum on its own had an average LC50 of 1.26 ± 0.68 ALC (=95 μg·L^{-1}), a value three times greater than, and significantly different from, its LC50 concentration in the mixtures of three and seven metals. Aluminum alone was thus nonlethal to flagfish fry at the concentrations expected in lakes at pH 5.8. Aluminum lethality was unaccountably variable. The ratio of the highest to the lowest LC50 was 4.0 although procedures and equilibration times were identical in all tests. Other combinations were of more consistent lethality, with ratios ranging from 1.0 to 2.7. The combination of Mn, Fe, Ni, and Pb had an average LC50 of 4.32 ± 0.0 ALC, which was not significantly different from the LC50 for Mn (4.5 ± 0.87 ALC). These metals thus had 1/10 of the lethal potency of the seven-metal and Al−Zn−Cu mixtures. Comparison with these combinations, and with Al alone, were significantly different.

Discussion

Total mortality of first-generation fry in the acid + 0.57 ALC treatment, plus abrupt cessation of spawning, reduced hatching success, and total mortality of second-generation fry transferred into it, demonstrate that joint toxicity of H^+ and metal mixtures at pH 5.5−6.0 may have significant deleterious effects on fish reproduction in culturally acidified waters. Reproduction was not affected by H^+ itself, even though pH 5.8 is within the pH range previously considered to be detrimental to flagfish (Craig and Baksi 1977) and sensitive native species (Fromm 1980).

The significant effects of continuous exposure in the acid + 0.09 and 0.27 ALC treatments were reduced size and increased time to steady spawning. Although these represent the most sensitive responses, they do not appear to be of major importance to successful recruitment. Failure to induce significant developmental abnormalities in the present study suggests the presence of genetic factors, interspecific differences, or unidentified environmental stresses in cases where deformities have been observed in the field (i.e. Beamish 1976). Total mortality of fry in the acid + 0.57 ALC treatment indicates that survival of newly hatched flagfish is the process most critical to continued population viability. This is a consistent finding for many toxicants and several species of fish (McKim 1977).

It appears that pH 5.5−6.0, and the associated metals levels, marks a threshold of reproductive success for many fishes. For example, significant effects on flagfish reproduction were observed when water from Lake Panache (Sudbury, Ont.) was acidified to pH 6.1, and continued viability was unlikely at pH 5.5 (Craig and Baksi 1977). Fathead minnows experienced recruitment failures when Lake 223 was acidified to below pH 6.0 (Mills 1984), and Rahel and Magnuson (1983) reported that black crappie (*Pomoxis nigromaculatus*) and smallmouth bass (*Micropterus dolomieui*) were absent from lakes of pH less than 5.7 and 5.5, respectively. Our conclusions for flagfish would thus seem to apply also to sensitive fish species that are native to northern acidified waters.

Separation of the effects of H^+ from those of elevated trace metals at low pH has not been achieved in previous studies. Mount (1973) and Menendez (1976) obtained bioassay water from river systems and did not report trace metal concentrations. Craig and Baksi (1977) reported detrimental effects on flagfish reproduction at pH 6.0 and lower in water from Lake Panache. Trace metal concentrations (micrograms per litre) in Lake Panache (Craig and Baksi 1977, see text-table), however, are even higher than those which killed all fry, reduced hatch, and interrupted spawning in our study:

	Al	Mn	Fe	Ni	Zn	Cu	Pb
Lake Panache	>55	—	16	33	13	6	<6
Acid + 0.57 ALC	52	23	24	7	9	5	3

Thus, elevated concentrations of both H^+ and trace metals were contributing to the observed effects in Lake Panache water.

Mills (1984) reported recruitment failure of fathead minnows when Lake 223 was experimentally acidified to pH 5.6. Although concentrations of Al and other trace metals were judged to be well below lethal levels, average summer epilimnetic concentrations of Al, Zn, and Cu were 20, 4, and 1−2 $\mu g \cdot L^{-1}$ 2 yr earlier when the same lake was at pH 6.08 (Schindler et al. 1980b). Concentrations of Zn, Al, Mn, and Fe showed substantial (three- to eight-fold) increases when enclosures in Lake 223 were acidified to pH 6.0 in other experiments (Schindler et al. 1980a), and Zn reached concentrations of 80−130 $\mu g \cdot L^{-1}$. By comparison, significant reductions in survival of fry of flagfish and fathead minnows occurred with 85 and 295 $\mu g\ Zn \cdot L^{-1}$, respectively, in single-metal exposures at pH 7−8 and total hardness of 44−46 $mg \cdot L^{-1}$ (Spehar 1976; Benoit and Holcombe 1978). Metal enrichment, joint metal toxicity, low total hardness, and the similarity between concentrations of Al, Zn, and Cu present in Lake 223 and at the LC50 in our study all suggest that trace metal lethality contributed to the recruitment failures in Lake 223.

Concentrations of trace metals in the acidic lakes of the La Cloche Mountain region (Beamish 1976; Kelso and Gunn 1984) exceed those in the present study and could therefore have contributed to the declines in fish populations recorded there. Hulsman et al. (1983) recorded high mortality of eggs of walleye (*Stizostedion vitreum*) and yolk-sac larvae of rainbow trout (*Salmo gairdneri*) during in situ exposures in acidified (pH 4.6−5.4) streams in the same area. Concentrations of Al, Mn, Zn, and Ni were elevated by factors of 2.6−6 compared with control sites (pH 6.0−6.6). Concentrations of Al, Mn, and Zn are frequently elevated in other acidified lakes where impacts on fisheries have been documented (Henriksen and Wright 1978; Muniz and Leivestad 1981; Baker 1982). Thus, the common condition in most lakes showing some degree of acidification-related stress on fisheries is the presence of elevated concentrations of trace metal mixtures and H^+.

The 96-h LC50 of Al−Zn−Cu for flagfish fry in synthetic soft water at pH 5.8 was 0.38 ALC. This corresponds to the simultaneous presence of Al, Zn, and Cu at 29, 4.6, and 2.3 $\mu g \cdot L^{-1}$, respectively. To date, Al has received the most attention as a toxic co-contaminant in acidified waters (Schofield and Trojnar 1980; Muniz and Leivestad 1981; Baker 1982; Brown 1983) in spite of an absence of toxicity testing of the other trace metals which are present, either in mixtures or individually (Baker 1982). The threefold increase in Al−Zn−Cu lethality over that of Al alone (LC50 = 1.26 ALC or 95 $\mu g \cdot L^{-1}$) in the present study indicates that Al will not be the only metallic toxicant of importance if acidified lakes contain Zn and Cu as well. Future attempts to describe or to predict the sensitivity of fish populations to acidification should therefore consider the toxicity of trace metal mixtures, and not just Al, in addition to considerations of total alkalinity, Ca^{2+} concentration, and pH. Our results show that Mn, Fe, Ni, and Pb are not of significant toxicity at the concentrations found in acidified lakes of at least one region suffering cultural acidification.

The metal concentrations used in this study are lower than those reported as lethal in previous studies (i.e. Baker 1981, Al; Eaton 1973, Zn and Cu) as well as being below the levels reported in most waters where reproduction is impaired (Beamish 1976; Baker 1982; Hulsman et al. 1983; Kelso and Gunn 1984). The effects revealed are clearly the result of joint action of Al, Zn, Cu, and H^+ and suggest that these toxicants were additive or close to additive in their effects. In addition, the extremely low total hardness (6.0 $mg \cdot L^{-1}$ as $CaCO_3$) and the absence of organic complexing agents in our test water (TOC < 0.5 $mg \cdot L^{-1}$) would both maximize metal toxicity (Howarth and Sprague 1978; Baker 1981; Borgmann 1983). Complexation with organic ligands in natural waters would reduce toxicity of Al (Baker 1981), Cu (Borgmann 1983), and Al−Zn−Cu mixtures (Hutchinson 1984), but not Zn (Zitko et al. 1973).

Speciation of Al, Zn, and Cu was not determined during our tests, as previous studies done with the same water source in our laboratory showed that toxic, free-aquo ions of Cu and Zn made up 95−100% of the totals for those metals at pH 5−6 (Howarth and Sprague 1978; Bradley and Sprague 1985a). Aluminum solubility is minimal at pH 5.8. Any dissolved Al in our tests (≈10 $\mu g \cdot L^{-1}$) likely existed as anionic monomers and the remainder as neutral, polymeric precipitates (Spry et al. 1981). Aluminum is toxic in both the inorganic, monomeric form (Driscoll et al. 1980) and when present in oversaturated solutions at pH 4.9−6.0 (Grahn 1981; Baker 1982). Our test water thus favoured the formation of lethal metal species. The chemistry of acidified natural waters would also favour the same metal speciation (Howarth and Sprague 1978; Bradley and Sprague 1985a) with the exception of Al precipitates (LaZerte 1984) and possible organic complexes of Cu and Al (Borgmann 1983; Driscoll et al. 1980). Specific modifying effects of pH, hardness, alkalinity, and complexation on Al−Zn−Cu lethality were approached in subsequent experiments (Hutchinson 1984).

Acclimation of fish to H^+ and trace metals is another factor that could influence interpretation of laboratory and field studies (Sprague 1985). Craig and Baksi (1977) reported decreased egg production of flagfish in a 20-d period following pH depression to below 6.0. The lack of effect on egg production seen in our study suggests that the fish had acclimated to acid and metals in the 77 d of continuous exposure preceding reproduction. In addition, only eggs transferred from the neutral control to the acid + 0.57 ALC treatment showed reduced hatching success; those transferred from pH 5.8 did not. Craig and Baksi (1977) may therefore have observed a short-term physiological stress that reduced egg production. Physiological acclimation of brown trout (*Salmo trutta*) to acid water has been demonstrated during 6 wk of exposure to pH 6.0 (McWilliams 1980), but Daye (1980) reported no resistance acclimation of embryos and alevins of Atlantic salmon (*Salmo salar*) following minimum exposures of 2 wk in water of pH 4.5–6.0. McWilliams (1980) concluded that failure of other investigators to induce acclimation to low pH resulted from too short an exposure time or because tests were done at lethal pH levels instead of sublethal ones.

Increased tolerance of juvenile rainbow trout has been reported following previous sublethal exposure to Al (Orr et al. 1986), Zn (Bradley and Sprague 1985b), and Cu (Dixon and Sprague 1981). Guthrie and Schofield (1982) reported resistance acclimation of embryos and sac-fry of brook trout to elevated concentrations of H^+ (pH = 4.5–5.0) and Al ($0.2-0.4\ mg \cdot L^{-1}$). In the present study, increased resistance time of fry exposed to acid + 0.57 ALC, after previous exposure to acid + metals (spawning to 8–9 d post-hatch), suggests an adaptive response to Al–Zn–Cu toxicity.

Responses after transfer to the acid + 0.57 ALC treatment suggest that short-term exposures of eggs, fry, and reproducing adults to H^+ and trace metals are critical to reproductive success in acidified waters. Elevated concentrations of H^+ and trace metals in lakes and streams during snowmelt and rainstorms have been recognized as such a potential short-term stress to fish (Jeffries et al. 1979; Dillon et al. 1984; Gunn and Keller 1984). Such episodic events preclude acclimation and would increase the potential for H^+ and metal toxicity to fish over that in chronically acidified waters.

Severe impacts of cultural acidification in Canada have been mostly confined to fish populations in the Sudbury and La Cloche mountain regions of Ontario, in lakes characterized by elevated concentrations of several trace metals besides Al. Beamish (1976) suggested the importance of pH as a master variable in these lakes, as it controlled the reproduction of fish through (a) elevation of trace metal concentrations, particularly Mn and Zn, and (b) joint action with the metals to kill fry. Since that time, the combined stresses of H^+ and inorganic, monomeric Al have not been shown to be sufficient to explain reproductive failures of fish in Sudbury area lakes (Gunn and Keller 1984). Surveys and controlled exposures, on the other hand, have shown recruitment failures in the presence of trace metal loadings which are similar to, or exceed, those in the present study (i.e. Beamish 1976; Craig and Baksi 1977; Yan et al. 1977; Hulsman et al. 1983; Mills 1984; Somers and Harvey 1984). Thus, we concur with the scenario proposed by Beamish (1976). Future attempts to describe or to predict the impact of cultural acidification on fish populations should consider the entire trace metal environment of acidified waters and should not be confined to the effects of Al and H^+ alone.

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