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REDUCED LETHALITY OF Al, Zn AND Cu MIXTURES TO AMERICAN FLAGFISH BY COMPLEXATION WITH HUMIC SUBSTANCES IN ACIDIFIED SOFT WATERS

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Abstract—Acute lethality of Al/Zn/Cu mixtures to fry of American flagfish (*Jordanella floridae*) was reduced by factors of 2.1 to 14.5 in water of pH 5.8 from four lakes of the Canadian Shield, compared to lethality in synthetic soft water. Test waters ranged from clear to dark brown but were otherwise similar. LC50s for total Al/Zn/Cu were correlated with several measurements of humic substances in the lakes, including total organic carbon (TOC), absorbance at 310 nm (A_{310}), color, and Secchi depth. Dialysis techniques showed that toxicity of Al and Cu was reduced by complexation with high-molecular-weight (MW) (>1,000) humic substances in the colored water. Zn was present mainly as low-MW forms and effects of complexation on its lethality were not resolved. Regressions predicted that survival of early life-stages of fish is doubtful in acidified, soft-water lakes containing less than 2.2 mg/L of TOC or in which $A_{310} < 0.016$. Changes in trace-metal lethality associated with TOC were greater than those associated with changes in total hardness.

Keywords—Fish Trace metals Humic substances Complexation Acidification

INTRODUCTION

Complexation with organic compounds is a well-known modifier of trace-metal bioavailability and toxicity to aquatic organisms. Copper toxicity, for example, is reduced in the presence of the synthetic chelating agents NTA and EDTA [1], sewage effluent [2] and purified humic acid [3]. While some individual metals such as copper have been extensively studied, there have been few assessments of complexation of metals in mixtures [1,4].

Trace-metal toxicity has recently emerged as an important determinant of fish survival in culturally acidified waters [5,6]. Fish mortalities have been related to the toxicity of H^+ and Al [7,8], but acidified waters may also contain elevated concentrations of Mn and Zn [8,9]. In previous work we showed that Al, Zn, and Cu levels that were typical of acidified lakes were acutely lethal to fry of American flagfish (*Jordanella floridae*) when present simultaneously in a mixture at pH 5.8 in synthetic soft water and that fry mortality was the critical response governing reproductive success [6]. A pH of 5.8, in the absence of metals, had no observed effect on the fish.

Humic substances are ubiquitous components of fresh water and their concentrations vary widely. Any moderation of Al/Zn/Cu lethality to fish by humic substances would make complexation an important determinant of future fish distributions in culturally acidified waters. Complexation with organic substances in natural waters is known to reduce toxicity of Al [10] and Cu [5] but Zn lethality was not influenced by organic complexation in one study by Zitko et al. [5].

Previous attempts at predicting metal lethality in natural waters have been unsuccessful [11,12]. Valid relationships predicting concentration of lethal Al species in acidic waters have been developed but are dependent on complex speciation techniques [13]. The literature on Cu complexation is lengthy but predictions of lethality are based either on measurements of free Cu^{++} ion concentrations [14] or on precise quantification of humic acid content [3]. The need is thus apparent for a simple measure of humic substance concentration

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which would serve as a surrogate for complex speciation techniques in estimating metal lethality to aquatic organisms.

Winner [3] showed a consistent relationship between humic acid concentration and Cu toxicity to *Daphnia pulex*. He suggested that since (a) humic substances were the dominant form of organic carbon in most nonpolluted waters, and (b) their influence on Cu toxicity was proportionally greater than that of water hardness, that some measure of total organic carbon (TOC) might prove useful in establishing water-quality criteria for trace metals. Correlations of natural organic carbon content of fresh waters with measurements of color and ultraviolet absorbance are well established [15,16] and suggest their utility in assessing metal toxicity.

We tested four Canadian Shield lake waters of different organic carbon content to assess the degree of Al/Zn/Cu detoxification in each type of water and to compare this to lethality of the same metal mixture determined previously in synthetic soft water. The tests were designed to determine the relationships between humic substance content (as measured by total organic carbon, ultraviolet absorbance, color, and Secchi depth), concentrations of low-molecular-weight (MW) forms of Al, Zn, and Cu, and their lethality to fry of flagfish in soft water acidified to pH 5.8. Our first purpose was to investigate the extent to which natural concentrations of humic substances would modify the previously established lethality of metals. Our second purpose was to explore several simple measurements of humic substances for their utility in describing such detoxification.

MATERIALS AND METHODS

Four soft-water lakes in south-central Ontario were chosen to cover the range of organic carbon content expected in most Canadian Shield waters. Their colors ranged from clear (Blue Chalk Lake) to dark brown (Brandy Lake) but they were otherwise similar in basic characteristics (Table 1). The four lakes, in order of increasing color, were Blue Chalk, Walker, and Chub (lat. 45° 12–23′ N; long. 79° 5′ W) and Brandy Lake (lat. 45° 6′30″ N; long. 79° 32′ W). Water was collected through the ice on two occasions and once from open water, and brought to the laboratory for use in tests.

Flagfish fry (age 2–3 d posthatch) were obtained from a laboratory population of breeding adults. Eggs were spawned and hatched at 25°C in soft, neutral water (5.0 mg/L as $CaCO_3$, pH approx. 7.3) and fry were kept in the same conditions until used in tests.

Toxicity tests used five concentrations of the Al/Zn/Cu mixture in each lake water, plus a control of unmodified lake water. The metals were added in concentration ratios of 12.5:2:1, approximating the relationship we determined previously for acidified waters [6]. Metals already present in each lake were accounted for when calculating exposure concentrations. Two liters of each desired concentration, as determined in range-finder tests, were made up in exposure beakers of linear poly-

Table 1. Average chemical characteristics of the four lakes used in tests of lethality and metal complexation[a]

	Blue Chalk Lake	Walker Lake	Chub Lake	Brandy Lake
pH	6.36 ± 0.05	6.3 ± 0.08	5.48 ± 0.22	6.5 (6.1–7.0)
Ca (mg/L)	2.9 ± 0.1	3.3 ± 0.1	2.9 ± 0.1	3.9 (3.6–5.0)
Mg (mg/L)	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	1.2 (1.0–1.6)
SO_4 (mg/L)	7.7 ± 0.1	8.3 ± 0.2	9.8 ± 0.1	6.3 (4.9–7.1)
Total carbon (mg/L)	5.1 ± 0.5	6.3 ± 0.5	8.1 ± 0.4	12.3 (5.5–19.9)
Dissolved organic carbon (mg/L)	1.9 ± 0.03	3.8 ± 0.1	5.3 ± 0.1	10.4 (4.7–15.6)
Secchi depth (m)	6.5 ± 0.2	4.6 ± 0.3	2.5 ± 0.1	0.90 (0.7–1.0)
Aluminum (μg/L)[b]	7.3 ± 4	24.3 ± 13	103 ± 33	160 ± 24
Zinc (μg/L)[b]	13.0 ± 22	8.3 ± 13	21.7 ± 18	14.7 ± 15
Copper (μg/L)[b]	3.1 ± 1.9	3.7 ± 0.6	4.9 ± 2.7	4.0 ± 0.9
Manganese (μg/L)	39.7 ± 3	35.5 ± 5	53.4 ± 1	146 (30–559[c])
Iron (μg/L)	91.4 ± 8	68.2 ± 8	364 ± 22	1030 (86–5070[c])
Lead (μg/L)	3.3 ± 0.2	3.0 ± 0	3.0 ± 0	6 (<3–10[c])

[a] Data are from the Ontario Ministry of the Environment unless noted. Means ± 1 standard deviation are given except for Brandy Lake, where MOE data are given as mean and range.

[b] Data measured directly in metal-free controls at the time of the study.

[c] Denotes a maximum hypolimnetic value (5–6.2 m), July 1982.

ethylene, using a stock solution of metal sulphates in 0.15-N nitric acid. The pH in each exposure beaker was adjusted to 5.8 with dilute KOH or HNO_3 and the beakers were transferred to a 25°C water bath 12 h prior to testing. Groups of 12 fish were randomly assigned to linear-polyethylene exposure cups (100 ml with nylon mesh bottoms) floating in the exposure beakers. 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. Lethal thresholds were reached at 96 h in all but the first test, in which they were reached at 120 h. Fish were not fed during tests and toxicant was not renewed as previous results showed no significant difference in Al/Zn/Cu LC50s for flagfish fry tested under continuous-flow and static-exposure conditions [6]. Three sets of toxicity tests were carried out, each using the same four lake waters and the procedures outlined above.

Average pH in all tests was 5.78 (SD = ±0.09), based on readings made at each observation time prior to adjustment with dilute KOH and HNO_3. Temperature averaged 24.8 ± 0.7°C for the same observation times. Dissolved oxygen, measured twice per beaker in each test, averaged 7.2 ± 0.3 mg/L, or 88% of air saturation (Table 2). Total hardness at the start of each test varied from 8 to 13 mg/L (as $CaCO_3$) and total inflection-point alkalinity [17] from 0.8 to 4.6 mg/L (as $CaCO_3$) in the four lake waters (Table 2).

Content of humic substances in the lake waters was determined by ultraviolet spectroscopy for all tests; total carbon (TC) and total organic carbon (TOC) for the second and third sets of tests; apparent color for the third set; and Secchi depth at the time of water collection for the third set of tests. Average concentrations of dissolved organic carbon (DOC) in the lakes were obtained from the Ontario Ministry of the Environment (P. J. Dillon, personal communication) and used to characterize each lake water at the start. Ultraviolet absorbance was measured by buffering water samples to pH 6.88 with phosphate buffer [18] and scanning from 450 to 250 nm, using a Beckman Model 24 spectrophotometer and 10-mm quartz cells. Absorbance values were selected at 25-nm intervals for between-lake comparisons and stepwise regression was used to determine those giving the best description of mixture lethality. Carbon measurements utilized a CO_2-induced color change in phenolphthalein indicator following ultraviolet irradiation, determined on a Technicon Auto-Analyzer II system. Inorganic carbon was removed, where appropriate, by adding 1% (v/v) 1-N H_2SO_4 to samples and purging with nitrogen [19]. Apparent color was measured by visual comparison with Co/Pt standards. The dialyzable (MW <1,000) fraction of the total organic carbon was also determined

Table 2. Summary of water chemistry measurements made during tests of lethality of Al/Zn/Cu mixtures to flagfish fry in water from four study lakes[a]

	Synthetic water	Blue Chalk Lake	Walker Lake	Chub Lake	Brandy Lake
pH	5.81 ± 0.02	5.79 ± 0.10	5.78 ± 0.08	5.76 ± 0.08	5.78 ± 0.09
Temperature (°C)	25.0 ± 0.06	24.9 ± 0.7	24.9 ± 0.6	24.8 ± 0.8	24.7 ± 0.7
Dissolved oxygen (mg/L)	7.3 ± 0.3	7.2 ± 0.3	7.3 ± 0.8	7.2 ± 0.3	7.5 ± 1.4
Total hardness (mg/L as $CaCO_3$)	6.0 ± 1.7	8.0 ± 0.6	9.0 ± 0.6	8.0 ± 1.2	13.0 ± 0.0
Alkalinity (mg/L as $CaCO_3$)	1.0 ± 0.3	3.2 ± 1.2	3.7 ± 0.14	0.75 ± 0.0	4.6 ± 0.9
Apparent color (Co/Pt std.)	0	3	10	32	80
Absorbance (at 275 nm)	—	0.016 ± 0.003	0.055 ± 0.010	0.137 ± 0.013	0.305 ± 0.063
Total organic carbon (mg/L)	<0.3	1.9 ± 0.7	3.9 ± 0.8	4.9 ± 0.6	8.5 ± 1.3
Dialyzed organic carbon (mg/L)	—	1.5 ± 0.3	2.3 ± 0.7	2.8 ± 1.1	2.7 ± 0.6

[a] Values given are mean ± 1 standard deviation from three sets of toxicity tests except for apparent color and dialyzed organic carbon, which were measured once in the third set of tests. Values from tests done in synthetic water (see text) are given for purposes of comparison but were not included in statistical analyses.

for each lake water, using dialysis methods outlined below and the analytical techniques described for carbon.

Metal concentrations in the test water were determined as (a) total metals, and (b) low-MW ($<1,000$) complexes measured by equilibrium dialysis [20]. Water samples for analysis of total Al, Zn, and Cu were taken from each beaker at 48 h of the first two sets of tests and twice from each beaker in the third (24 and 72 h). Dialyzable metals were measured once in each beaker to coincide with the period of greatest mortality, between 36 and 72 h of each test. The molecular-weight cutoff (MWCO) of 1,000 was chosen to distinguish between complexed and noncomplexed metals [13, 20]. Dialysis bags (2.5 × 12 cm) were cut from Spectrapor-6 dialysis tubing, cleaned according to the manufacturer's instructions, and stored in 0.15-N HNO_3. Before use, each bag was rinsed 10 times in distilled, deionized water, filled with the same, sealed with plastic clips, and then placed in the exposure beakers for 36 h to allow equilibration of noncomplexed metals within and outside of the bag [20]. All metals samples were acidified to 1% (v/v) with reagent-grade HNO_3 and stored in linear-polyethylene sample bottles until analyzed. Sample bottles were bathed in 0.8-N HNO_3 for 24 h and rinsed seven times in distilled and deionized water before use.

Metal concentrations were measured on a Perkin-Elmer 5000 automated atomic absorption spectrophotometer (AAS) following preconcentration to 5% of the original sample volume by evaporation. Detection limits and background sensitivities were 3 μg/L and 10%, 1.0 μg/L and 7%, and 2 μg/L and 7% for Al, Zn, and Cu respectively and recovery of spiked samples is routinely 100 ± 10% for this method (B. Loescher, personal communication). Copper was analyzed for the third set of tests on a Scintrex AAZ-2 flameless AAS. Background correction was achieved by Zeeman modulation and the detection limit was 0.4 μg/L. Measured concentrations of total metals averaged 129, 155, and 126% of nominal values for Al, Zn, and Cu respectively. Since nominal concentrations were based on yearly averages for each lake (P. J. Dillon, personal communication) this variation likely resulted from seasonal variations in metal concentration at the time of water collection.

Median lethal concentrations (LC50s) were calculated from data on fish mortality and measured metal concentrations using the Spearman–Karber method [21]. No corrections were made for control mortalities of 8.5 to 17% in 4 of the 12 tests because of possible toxicity from low-MW, metal-organic complexes present in unaltered lake water [22]. In each test, two LC50s were calculated for each metal as if it were acting independently, using measured concentrations of (a) total metal and (b) dialyzed metal. Each LC50 was then divided by the concentration of that particular metal which would be expected in a lake of pH 5.8 (75, 12, and 6 μg/L for Al, Zn, and Cu respectively [6]). This translated each single-metal LC50 into units of "acid lake concentration," or ALC. The three single-metal LC50s were then averaged to obtain one LC50, in units of ALC, which described toxicity of the mixture. Accordingly, for each of the three tests and each of the four lakes there was an LC50 for the mixture of total metals and one for the mixture of dialyzed metals.

Statistical comparisons between treatments (lakes) were initiated using analysis of variance or regression procedures. The LC50s (in ALC) were compared on the basis of lake (and hence humic content) and on form of the metal (dialyzed or total) using a two-way analysis of variance. A three-way ANOVA was used to compare concentrations (in μg/L) of the individual metals present at the mixture LC50 on the basis of lake, metal (i.e., Al, Zn, Cu) and form of the metal. The means for significant treatment effects were compared using a protected LSD test. This approach allowed comparisons to be made for the entire metal mixture, as well as for individual metals within the mixture. A two-way ANOVA was used to compare measurements of dialyzed and total organic carbon between lakes and significant treatment means compared as before. A probability level of $p < 0.05$ was used, but actual computed significance levels are presented in the text to illustrate the relative strengths of the observed relationships.

Relationships between the four measurements of humic content, absorbance at ultraviolet wavelengths, the LC50s for total Al/Zn/Cu, and between concentrations of dialyzed and total Al, Zn, and Cu at the mixture LC50s were described by linear regression, following $\log_{10}$ transformation of all variables. Stepwise regressions of total carbon, total organic carbon, Secchi depth, apparent color, and absorbance at 250, 275, 310, 325, 350, and 400 nm with mixture LC50s were used to select those giving the best description of lethality. A probability level of $p < 0.001$ was chosen to establish significance because of the relatively small sample sizes used ($n < 13$).

RESULTS AND DISCUSSION

The presence of humic substances significantly reduced the lethality of Al/Zn/Cu mixtures to flagfish fry in acidified, soft waters. Average LC50s for total metals in the three tests ranged from 0.78 ± 0.23 ALC in low-color Blue Chalk Lake to 5.52 ± 2.25 ALC in Brandy Lake, the darkest of the four (Table 3), and all between-lake comparisons were significantly different. Lethality was reduced by factors of 2.1, 4.2, 7.4, and 14.5 for Blue Chalk, Walker, Chub, and Brandy Lakes respectively, compared to an LC50 of 0.38 ± 0.11 ALC obtained by previous testing [6] in synthetic, soft water (TOC < 0.3 mg/L, Table 3). The overall treatment (lake) effect on mixture lethality was significant at $p < 0.0001$ when LC50s were expressed as total metals and at $p < 0.001$ when expressed as concentrations of dialyzed metals. Average LC50s for dialyzed metals were significantly lower ($p < 0.001$) but showed the same relationship to water color as those for total metals. They rose from 0.62 ± 0.09 ALC in Blue Chalk Lake to 2.42 ± 1.1 ALC in Brandy Lake and all between-lake differences were significant.

Three separate estimates of humic substance content were highly correlated in the four study lakes. Apparent color rose from 3 (Co/Pt) units in Blue Chalk Lake to 80 units in Brandy Lake with parallel increases in ultraviolet absorbance and concentrations of total organic carbon (Table 2). Pearson product-moment correlation coefficients were 0.94 ($p < 0.00001$) *for* A_{310} and TOC, 0.97 ($p < 0.013$) for TOC and color, and 0.99 ($p < 0.008$) for A_{310} and color.

Reductions in trace metal lethality were directly related to the humic substance content of the lake waters and were easily estimated by measurements of total carbon, total organic carbon, ultraviolet absorbance, apparent color, and Secchi depth. These estimates of humic substance content showed strong correlations ($r = 0.90$ to 0.99) with lethality of total Al/Zn/Cu at pH 5.8 (Fig. 1), when determined for $\log_{10}$-transformed data. Regressions on ultraviolet absorbance (at 250, 275, 310, and 325 nm), total carbon, and total organic carbon gave highly significant descriptions ($p < 0.0001$, $r^2 > 0.81$) of the lethality of total Al/Zn/Cu. Apparent color and Secchi depth were highly correlated with mixture lethality ($r^2 = 0.99$ and -0.94 respectively) but the regressions were not significant ($p > 0.002$) because the sample size was limited to only four.

Stepwise regression techniques showed that A_{310} gave the best description of mixture lethality. The equation

$$\log_{10} \text{LC50} = (0.65 \times \log_{10} A_{310}) + 1.176$$

was significant at $p < 0.00001$ and explained 88% of the variance in lethality of total Al/Zn/Cu (Fig. 1). For the set of nonoptical measurements, stepwise regression produced the equation

$$\log_{10} \text{LC50} = (1.145 \times \log_{10} \text{TOC}) - 0.395$$

which was significant at $p < 0.00002$ and explained 84% of the variation in lethality of the mixture (Fig. 1). Solving the above equations for an LC50 of 1.0 ALC showed that waters in which TOC exceeded 2.2 mg/L, or A_{310} exceeded 0.016, would require total Al, Zn, and Cu concentrations exceeding 75, 12, and 6 μg/L respectively at pH 5.8 (or 1.0 ALC) to kill 50% of the fry of those species of fish which were as sensitive as flagfish to H-ion and Al/Zn/Cu stress. Fish inhabiting lakes of less

Table 3. Lethality of a mixture of Al, Zn, and Cu to flagfish fry in four lake waters[a]

	Blue Chalk Lake	Walker Lake	Chub Lake	Brandy Lake
Dialyzed metals (LC50 in ALC)	0.62 ± 0.09	0.93 ± 0.04	1.43 ± 0.54	2.42 ± 1.10
Total metals (LC50 in ALC)	0.78 ± 0.23	1.59 ± 0.48	2.80 ± 0.44	5.52 ± 2.25
Dialyzed Al (concn. in μg/L)	26.3 ± 10.8	34.0 ± 19.0	35.7 ± 14.2	60.0 ± 17.1
Total Al (concn. in μg/L)	36.7 ± 11.6	95.7 ± 2.1	188 ± 38	486 ± 221
Dialyzed Zn (concn. in μg/L)	7.3 ± 2.5	17.7 ± 0.6	34.3 ± 15.5	64.7 ± 37.0
Total Zn (concn. in μg/L)	8.3 ± 2.5	21.2 ± 14.7	46.3 ± 10.1	74.3 ± 33.3
Dialyzed Cu (concn. in μg/L)	5.5 ± 1.7	5.5 ± 0.6	5.6 ± 1.1	5.2 ± 3.0
Total Cu (concn. in μg/L)	6.8 ± 2.8	10.1 ± 0.9	12.2 ± 1.3	23.4 ± 7.6

[a] The top part of the table gives the LC50s for the entire mixture as proportions of the metal levels expected in an acidified lake (*a*cid *l*ake *c*oncentration or ALC, see text). The bottom part of the table gives measured concentrations of individual metals which were present at the LC50 for the mixture. All values are mean ± 1 standard deviation from three sets of toxicity tests.

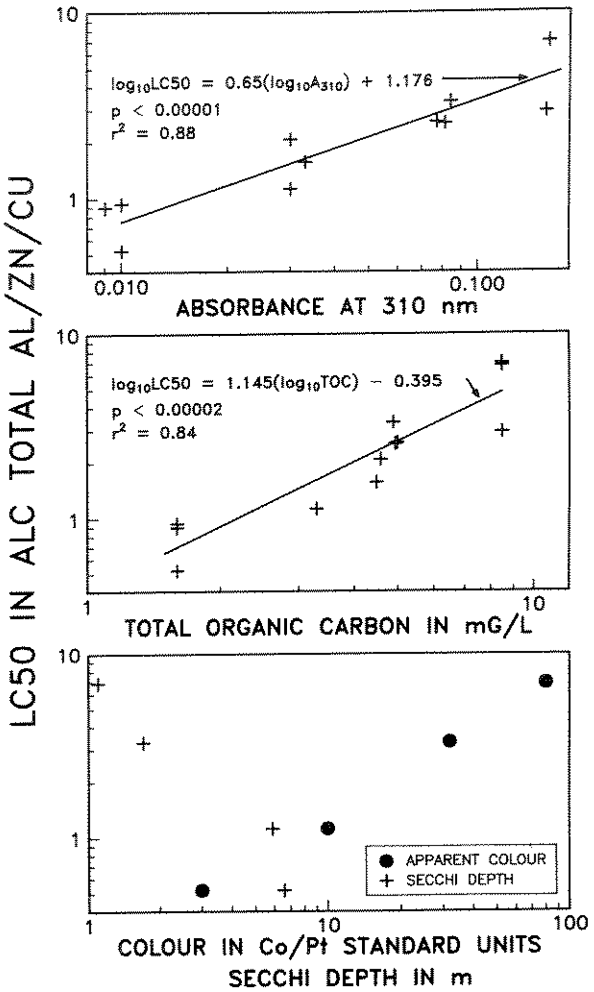

Fig. 1. Relationships between lethality of a mixture of Al/Zn/Cu and four estimates of humic substance content in the four study lakes. Regression lines are plotted for absorbance at 310 nm (A_{310}) and total organic carbon (TOC), which gave the best estimates of the LC50 for total Al/Zn/Cu.

color would be more at risk from trace metal lethality accompanying cultural acidification.

It is clear that the observed reductions in metal lethality were achieved by complexation with humic substances in the study lakes. Our conclusion is substantiated by proportional increases in total-metal LC50s with TOC content in the four lake waters and the following documentation: (a) humic substances make up the bulk of TOC in unpolluted waters [4], (b) ultraviolet absorbance is a well-known measure of humic substance concentration [15,16,18] and was correlated with total metal LC50s in this study, and (c) humic substances and humic and fulvic acids have a well-documented affinity for metal ions [22,23]. The observed correlation between metal lethality and Secchi depth is further evidence, as water clarity in lakes of the Muskoka–Haliburton study region is controlled by TOC, and not by chlorophyll *a* (N. Yan, personal communication).

It is also apparent that the high-MW (>1,000) fraction of humic substances was responsible for the observed complexation. Concentrations of TOC rose from 1.9 mg/L to 8.5 mg/L across the study lakes (Table 2). These increases were significant and proportional to reductions in Al/Zn/Cu lethality. Concentrations of dialyzed organic carbon (MW < 1,000), on the other hand, ranged from 1.5 to 2.8 mg/L (Table 2) and no between-lake comparisons were significant.

There appeared to be two patterns of metal speciation within the mixture. Any influence of Al and Cu on mixture lethality resulted from toxic contributions of low-MW (<1,000) complexes which remained relatively constant and hence independent of the toxic contributions of total Al and Cu. For Zn, on the other hand, toxic contributions of low-MW forms rose with those of total Zn in each lake (Fig. 2). Attributing specific patterns to the toxicity of individual metals is not possible as single-metal exposures were not done. All interpretations therefore relate to the actions of individual metals within the Al/Zn/Cu mixture.

Total concentrations of each metal present at the mixture LC50 increased, of course, with the humic substance content of the lake water. Concentrations of dialyzed metals were consistently lower than those of total metals, but individual metals behaved differently in this respect. Average concentrations of total Al at the mixture LC50 ranged from 37 μg/L in Blue Chalk Lake to 486 μg/L in Brandy Lake and all between-lake differences were significant (Table 3). Concentrations of dialyzed Al at the mixture LC50 were significantly lower than those of total Al except in Blue Chalk Lake. Although dialyzed Al also tended to increase with the content of humic substances, the only significant comparisons were for those lakes at the two extremes of color (Blue Chalk Lake = 26.3 μg/L; Brandy Lake = 60 μg/L). Concentrations of

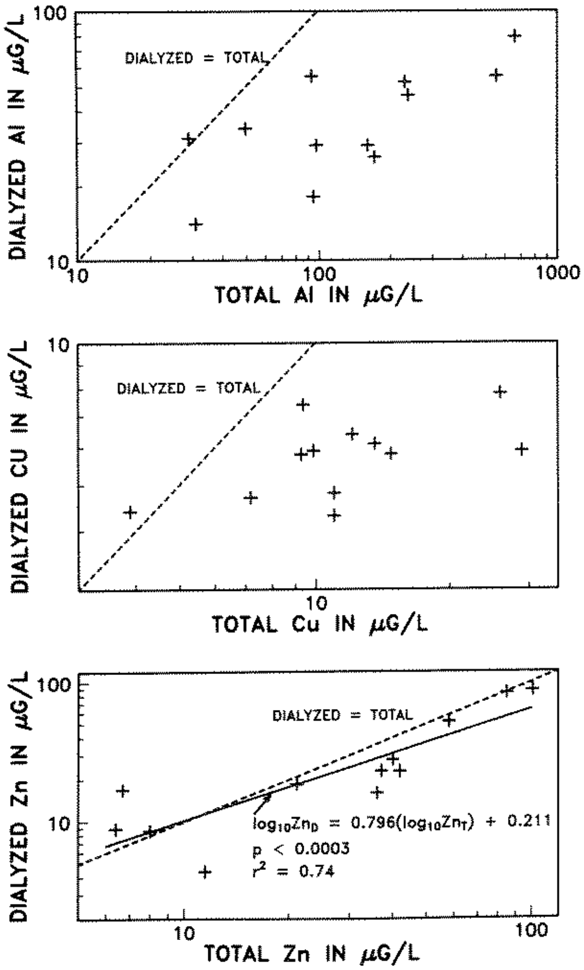

Fig. 2. Relationships between concentrations of dialyzed and total Al, Zn, and Cu which were present at the LC50 for mixtures of these metals in the four study lakes. The dashed line indicates a hypothetical relationship in which concentrations of the two forms of metal would be equal and the solid regression line indicates the significant relationship observed between dialyzed and total Zn.

dialyzed and total Al present at the mixture LC50 were not related to each other (Fig. 2) and regressions on $\log_{10}$-transformed data were not significant ($r^2 = 0.49$, $p < 0.011$).

The close agreement between concentrations of dialyzed Al present at the mixture LC50 in each lake suggests that any Al contribution to lethality was fully explained by that fraction. Concentrations of 26 to 60 μg/L dialyzed Al at the LC50 were nearly equivalent to the 29 μg/L total Al present at the mixture LC50 determined previously in synthetic soft water (TOC < 0.3 mg/L [6]). Such equivalence suggests that the dialyzed fraction consisted of inorganic, monomeric Al which is the dominant form in synthetic water and is lethal to fish [10,13], although the presence of some organic Al complexes is shown by the slight increase in dialyzed Al in Brandy Lake water (Table 3). Reduced Al lethality to fish in the presence of organic complexing agents agrees with previous reports of Al complexation with citrate, and with humic substances in Adirondack surface waters [8].

The influence of organic complexation on Cu lethality was similar to that for Al. Total Cu concentrations at the mixture LC50s for each of the study lakes were 6.8, 10.1, 12.2, and 23.4 μg/L, in the usual sequence from low-color Blue Chalk Lake to highly colored Brandy Lake (Table 3). The only significant differences, however, were those between Brandy Lake and the two low-color lakes. Concentrations of dialyzed Cu at the mixture LC50 were constant across the study lakes (5.2 to 5.6 μg/L; Table 3, Fig. 2). Significant differences in contributions of dialyzed and total Cu to the mixture LC50s were found only in the two high-color lakes, and there was no significant relationship between the two forms across all four lakes ($r^2 = 0.33$, $p < 0.052$; Fig. 2).

Equivalent concentrations of dialyzed Cu at the mixture LC50 show that contributions of this fraction to lethality were constant with humic content, and suggest that the low-MW fraction contained any lethal Cu species that were present. Increased Cu concentrations at the LC50s for total metals thus represent reductions in lethality through complexation with high-MW humic substances. These patterns agree with many previous studies showing moderation of Cu toxicity to aquatic organisms by complexation with natural humic substances [11], purified humic acid [3], and anthropogenic organic compounds [2,11]. The lethality of Cu/Zn mixtures to Atlantic salmon (*Salmo salar*) was reduced when humic acid was added to soft water in studies by Zitko et al. [4] and to brook trout (*Salvelinus fontinalis*) when NTA and EDTA were used [1].

Copper lethality to fish, with few exceptions, results from the actions of free ionic copper (Cu^{2+}) and copper hydroxides ($CuOH^+/Cu_2(OH)_2^{2+}$) (see reviews by Pagenkopf et al. [24], Florence [25] and Florence and Batley [26]). Although these studies have, for the most part, dealt with inorganic complexation, the same patterns are seen for the effects of organic complexation on Cu toxicity to rainbow trout [27] and coho salmon (*Oncorhyncus kisutch* [2]) and for phytoplankton and invertebrates as well as fish [3,14]. When Cu concentration is expressed as free Cu ion, toxicity is relatively constant, regardless of complexation [14]. Complexation thus reduces Cu toxicity and the more stable the complex, the lower is its toxicity [25]. Expressing Cu concentration as dialyzed Cu in our study also showed that its toxicity was constant, without the need for further prediction or measurement of Cu speciation.

Contributions of Zn to mixture lethality, on the other hand, could not be explained on the basis of constant contributions of low-MW forms in each lake. Concentrations of both forms of Zn at the LC50 for the mixture ranged from 7.3 to 74 μg/L in the four lakes (Table 3, Fig. 2). Within-lake comparisons showed no significant differences in concentration of the two forms at the mixture LC50 and ratios of dialyzed to total Zn ranged from 0.74 to 0.88. In addition, a highly significant ($p < 0.0003$; $r^2 = 0.74$) relation between LC50 contributions of dialyzed and total Zn was obtained. The slope of 0.80 (Fig. 2) relating lethality of the two forms is close to a hypothetical slope of 1.0, which would indicate total equivalence of each form at the mixture LC50.

This correspondence between lethality of the two forms of Zn can be interpreted in several ways. First, Zn may not have formed complexes with humic substances, beyond the 12 to 26% present as high-MW complexes at the mixture LC50. If so, all dialyzed Zn was inorganic and hence lethal. The low concentration (4.6 μg/L) of inorganic Zn present at the LC50 previously determined for Al/Zn/Cu in synthetic water [6] suggests, however, that if the dialyzed Zn in this study (7.3 to 64.7 μg/L, Table 3) was all inorganic that it should have caused more than 50% mortality. This was not the case. A more plausible explanation is that Zn preferentially (74 to 88%) formed complexes with low-MW humic substances and dialyzed as a Zn-organic complex with reduced or no contribution to mixture lethality. This does not rule out a potential contribution of Zn to mixture lethality. Borgmann [14] and Bradley and Sprague [28] concluded that Zn toxicity resides in the ionic form. Equilibrium chemistry dictates that residual ionic Zn will always be present regardless of complexation [29], and could therefore have contributed to mixture lethality.

Unfortunately, no clear understanding of Zn complexation emerges from a survey of available literature. Complexation of Zn with low-MW organics is supported by Jackson and Skippen [30], who concluded that fulvic acid showed preferential binding with Zn, instead of Cu, in the pH range 4 to 9 and that both metals were bound by humic acid. Fulvic acid has an average MW of 950 [31], and so could have carried Zn through the dialysis membrane in the present study. Florence [32] concluded that there was evidence for the association of Zn with organic matter, but no clear proof of the existence of soluble organocomplexes of Zn. Our results do not, therefore, clearly resolve any effects of complexation with humic substances on lethality of Zn. Further studies of organic complexation and Zn lethality to fish are clearly desirable.

Further acidification of surface waters can be expected to increase trace metal lethality in three ways. Previous laboratory studies showed that Al/Zn/Cu lethality to flagfish fry increased ($r = 0.96$, $p < 0.0002$; [9]) with H-ion concentration down to an LC50 of pH 4.82 (SD = ±0.03) for H ion alone. Metal-ligand stability constants vary directly with pH and so acidification of natural waters will lower metal/humic substance affinities, favoring the formation of toxic, free-ionic forms. This has been shown for organic complexes of Al [33], Zn [33], and Cu [34]. Finally, absolute concentrations of Al and Zn will increase as watersheds acidify [5,6]. Lethality of increased Al, Zn, and Cu levels can be estimated from results of the present study but the influence of pH on affinity of humic substances for metals and on metal lethality in colored waters cannot. Further experiments at several pH levels would validate the principles described here and widen their applicability.

In this study, we documented an LC50 increase of 0.72 ALC per mg/L increase in TOC. By comparison, similar toxicity tests in synthetic water found that LC50s increased by only 0.013 ALC for every mg/L increase in total hardness at pH 5.86, or by 0.026 at pH 6.85 [9]. Our findings are thus in accord with Winner's [3] conclusion that the humic content of water was far more important than water hardness in determining differences in Cu toxicity. He concluded that differences in toxicity should be predictable if humic concentrations could be measured and that it may prove possible to utilize a measure of total organic carbon (TOC) in lieu of more complex measurements of humic substances.

Prediction of Al/Zn/Cu lethality to flagfish fry was easily achieved in our study by direct measurement of TOC and by simpler measurements of lakewater absorbance at 310 nm. The lakes selected for our study spanned the range of color expected in most Canadian Shield lakes and so our conclusions should have broad applicability in other, similar systems. Ultraviolet absorbance is specific for humic substances and avoids the influence of other sources of organic carbon (i.e. chlorophyll *a*) provided anthropogenic inputs of TOC are minimal. Extreme concentrations of certain trace metals may, however, modify the TOC/absorbance relationships described here. Addition of 5 mg/L of Fe produced significant changes in the absorbance spectrum of humic acid [35]. Adding 1 mg/L of Al, Mn, or Fe enhanced the absorbance spectrum by 5 to 20% but did not alter its shape. Use of our relationships should be verified for waters containing >1.0 mg/L of Al, Mn, or Fe.

Acidified lakes in which the TOC concentration is below 2.2 mg/L, or A_{310} is less than 0.016, will be most susceptible to loss of fish populations through trace metal toxicity. In these conditions, the LC50 for Al/Zn/Cu at pH 5.8 was less than the metal levels expected in such lakes (75, 12, 6 μg/L Al, Zn, Cu respectively = 1.0 ALC). Although the flagfish is not native to northern waters, its relevance to studies such as ours has been conclusively established [6]. Variations in sensitivity between flagfish and native species (e.g., rainbow trout, *S. gairdneri*; [36]) are, in fact, less than those among native species responding to acidified waters [37]. Direct extrapolation of our findings to acidified waters is not advised but the relationships between humic substances and lethality described here are not likely to change with the test organism. Site-specific toxicity testing, using local waters and sensitive native species, would be a valuable addition to our findings before developing specific water-quality criteria incorporating simple estimates of trace metal complexation. Even without that, the relationships shown here, particularly those for TOC and A_{310}, could be used along with H-ion buffering capacity to distinguish lakes in which fish are most sensitive to acidification and concomitant elevations of metals.

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