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THE EFFECT OF CALCIUM AND MAGNESIUM RATIOS ON THE TOXICITY OF COPPER TO FIVE AQUATIC SPECIES IN FRESHWATER

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Abstract—While it is generally accepted that water hardness affects copper toxicity, the major ions that contribute to water hardness (calcium [Ca] and magnesium [Mg]) may affect copper toxicity differently. This is important because the Ca:Mg ratio in standard laboratory-reconstituted waters often differs from the ratio in natural surface waters. Copper toxicity was assessed for five different aquatic species: rainbow trout (RBT), fathead minnow (FHM), *Ceriodaphnia dubia*, *Daphnia magna*, and an amphipod (*Gammarus* sp.) under different Ca:Mg ratios (4:0, 3:1, 1:1, 1:3, and 1:4 mass basis) at a common hardness (180 mg/L as $CaCO_3$) and alkalinity (120 mg/L as $CaCO_3$). Copper toxicity increased at lower Ca:Mg ratios for RBT but increased at higher Ca:Mg ratios for *D. magna*. Fathead minnows (<24 h old) were more sensitive to copper in 1:1 Ca:Mg waters compared to 3:1 Ca:Mg waters. The toxicity of copper did not vary under different Ca:Mg ratios for *Gammarus* sp., *C. dubia*, and 28-d-old FHM. The effect of Ca:Mg ratios on copper toxicity changed for *D. magna* in softer water (90 mg/L as $CaCO_3$) compared with hard water studies.

Keywords—Acute exposure Copper Calcium Magnesium Water hardness

INTRODUCTION

Water hardness, alkalinity, pH, and natural organic matter are known to affect the toxicity of copper to aquatic organisms [1–3]. It has also been demonstrated that other factors, specifically the relative concentrations of calcium and magnesium [2,4] as well as other ions, such as sodium [2], can influence copper toxicity. Total water hardness is used as a surrogate to estimate ameliorating affects for metal toxicity [1]. However, the relative ratios of Ca and Mg (the major contributors to water hardness) can vary greatly from one water body to another (R.J. Erickson, unpublished data) and may not affect metal toxicity equally. For example, Ca was more protective of copper or cadmium toxicity than Mg to salmonids [4,5]. Therefore, it is important to understand not only how metal toxicity is influenced by water hardness but, more important, how the relative concentrations of Ca and Mg can affect the toxicity of metals in freshwaters. Understanding this relationship is especially critical because standard reconstituted laboratory dilution waters used for toxicity tests do not have Ca:Mg ratios similar to natural waters. Reconstituted laboratory dilution waters are comprised of equal quantities of calcium sulfate (as $CaSO_4 \cdot 2H_2O$) and magnesium sulfate (as anhydrous $MgSO_4$) [6,7], resulting in a Ca:Mg ratio of approximately 1.1:1 (0.7:1 on a molar basis), whereas the Ca:Mg ratio in natural waters ranges from 1.6:1 to 8:1 (1:1–5:1 on a molar basis) with an average of 3.3:1 (2:1 on molar basis) (R.J. Erickson, unpublished data).

Studies were conducted to determine the effect of different Ca and Mg ratios (4:0, 3:1, 1:1, 1:3, and 1:4 on a mass basis) in laboratory reconstituted hard water (180 mg/L as $CaCO_3$) on the acute toxicity of copper to select aquatic laboratory test organisms. Studies were conducted with rainbow trout (*Oncorhynchus mykiss*), fathead minnows (*Pimephales promelas*, <24-h- and 28-d-old fish), cladocerans (*Ceriodaphnia dubia* and *Daphnia magna*), and amphipods (*Gammarus* sp.) because of their sensitivity to copper in the copper criteria database [1,8] and their general importance in aquatic toxicity testing [7]. An additional set of Ca:Mg studies (4:0, 3:1, and 1:1) was also conducted with *D. magna* in laboratory-reconstituted moderately hard water (90 mg/L as $CaCO_3$).

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MATERIALS AND METHODS

Culture methods

The laboratory water used in testing was reconstituted from American Society of Testing and Materials (Philadelphia, PA, USA) Type I laboratory water (Milli-Q®; Millipore, Bedford, MA, USA). Source water to the Milli-Q system was obtained from Horsetooth Reservoir surface water (treated by filtration [sand, 0.2 and 0.05 μm] and ultraviolet radiation) or tap water (Fort Collins, CO, USA). All organisms cultured or held at the laboratory were maintained either in processed Horsetooth Reservoir water (HT) or in waters reconstituted from Milli-Q water. Organisms were not acclimated to the reconstituted waters in which they were going to be tested, except for *D. magna* used in the hard water (180 mg/L) experiments. *Daphnia magna* used in these experiments were cultured in reconstituted waters of a similar hardness (1:1 Ca:Mg ratio) and alkalinity prior to testing.

Rainbow trout used during these studies were obtained from Lost River Trout Hatchery (Mackey, ID, USA) or Clines Trout Hatchery (Boulder, CO, USA). Fish were delivered as swim-up fry and held in flowing HT culture water at approximately 12°C (Table 1) until testing (minimum 14 d after receipt). Trout were fed trout chow (Zeigler Brothers® Salmon Starter; Gardners, PA, USA) ad libitum twice daily during holding. All trout were juvenile stage (postlarval, swim-up stage), had absorbed their yolk sacs, and were actively feeding (>20 d) at the surface before they were selected for testing. The average weights of rainbow trout (RBT) used in the studies ranged from 0.23 to 0.49 g.

Fathead minnow (FHM) fry were obtained from in-house

Table 1. Water quality characteristics (mean ± standard deviation) for laboratory culture water and reconstituted laboratory waters used in Ca:Mg studies

Treatment[a]	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Initial pH	Total recoverable concn. (mg/L)			
				Ca	Mg	K	Na
Culture water							
Horsetooth[b]	28 ± 4.4	25 ± 1.7	7.4 ± 0.2	8.9	1.5	0.8	2.6
180 hardness							
4:0 (4:0)	177 ± 2.6	115 ± 3.1	8.2 ± 0.2	68.0 ± 4.3	0.02 ± 0.01	4.27 ± 0.20	61.9 ± 14
3:1 (1.8:1)	178 ± 2.3	116 ± 2.3	8.2 ± 0.1	44.8 ± 1.1	15.5 ± 0.50	4.35 ± 0.21	55.6 ± 2.3
1:1 (0.7:1)	180 ± 1.8	115 ± 2.9	8.2 ± 0.3	28.5 ± 0.52	25.9 ± 1.6	4.37 ± 0.13	56.3 ± 3.5
1:3 (0.2:1)	179 ± 4.2	114 ± 6.4	8.3 ± 0.0	11.8 ± 0.14	36.0 ± 0.21	4.23 ± 0.03	53.0 ± 1.1
1:4 (0.16:1)	176	114	8.3	10.0	37.0	4.17	51.7
0:4 (0:4)	180	116	8.2	—	—	—	—
90 hardness							
4:0 (4:0)	90	56	8.0	38.4	0.05	2.29	29.2
3:1 (1.8:1)	90	59	8.0	23.8	8.52	2.15	29.6
1:1 (0.7:1)	92	59	7.5	17.0	12.7	2.22	28.7

[a] Ca:Mg ratios are presented on a mass basis and on a molar basis parenthetically.
[b] Total recoverable and dissolved copper concentrations in Horsetooth culture water were 9.0 and 6.0 μg/L, respectively.

cultures. Brood stock were maintained in 37.8-L (10-gallon) aquaria with each aquaria having a male:female ratio of approximately 2:5. Adult organisms were fed frozen adult brine shrimp (San Francisco Bay Brand, Newark, CA, USA) ad libitum three times daily. Spawning tiles with eggs were removed daily and placed in Fungus Guard™ (Jungle Laboratories, Cibolo, TX, USA) for 24 to 48 h, and then spawning substrates with eyed eggs were placed in aerated hatching trays filled with reconstituted moderately hard water (hardness and alkalinity of 90 and 60 mg/L as $CaCO_3$, respectively). Less-than-24-h-old minnows were collected and used in three rounds of studies. For 28-d-old fish, approximately 500 <24-h-old FHM were maintained in HT water (similar to adults) and fed trout chow and brine shrimp nauplii ad libitum three times daily. Fish (28 d old) used for testing averaged 11.2 mm long (range 9–13 mm) and weighed 13.4 mg (range 4.86–26.1 mg).

Ceriodaphnia dubia were obtained from in-house cultures reared following U.S. Environmental Protection Agency (U.S. EPA) procedures [7]. Monocultures of gravid *C. dubia* were isolated daily to ensure that enough neonates could be produced that would be <24 h old and were within 8 h of age. Organisms were fed 0.2 ml of a yeast:trout chow:cereal leaves (YTC)/algal suspension [7] daily in moderately hard reconstituted water. Organisms were maintained at 25 ± 1°C during culturing.

Daphnia magna (<24 h old) were also obtained from in-house cultures for all studies. Approximately 30 adult organisms were cultured in 2 L of reconstituted hard water (hardness and alkalinity ~180 and 120 mg/L as $CaCO_3$, respectively) and maintained at a temperature of 20 ± 1°C. Organisms were fed a 3:5 YTC:algal mixture daily at a rate of 8 ml/1 L of culture water. The YTC was prepared as described in U.S. EPA [7] except that the trout chow was not digested prior to use. Adult organisms were transferred to new culture media (~3:1 new:old media) three times weekly.

Amphipods (*Gammarus* sp.) were obtained from Environmental Consulting and Testing (Superior, WI, USA). Organisms (adults of mixed age) were maintained in a mixture of reconstituted hard water and water in which the organisms were shipped (hardness and alkalinity of 62 and 61 mg/L as $CaCO_3$, respectively). Organisms were fed YTC (*C. dubia* diet) and Tetramin® (Tetra Sales, Blacksburg, VA, USA) flake food ad libitum daily during holding. The average weight of the organisms used for testing was 24.5 mg (range 8.71–44.5 mg).

Reference toxicant tests (positive control test with sodium chloride) were conducted with each batch of testing organisms at the Fort Collins Environmental Toxicology Laboratory (Fort Collins, CO, USA). Organism responses were within historical control limits, which represent ±2 standard deviations from the historical laboratory mean median lethal concentration (LC50).

Experimental design

Test methods were in accordance with appropriate U.S. EPA and American Society for Testing and Materials guidance [6,7,9]. The laboratory water used in Ca:Mg testing studies was prepared in a similar fashion as described in U.S. EPA [6,7], except the amounts of calcium (as $CaSO_4 \cdot 2H_2O$), magnesium (as anhydrous $MgSO_4$), and sodium bicarbonate ($NaHCO_3$) were altered to achieve a target hardness (180 mg/L as $CaCO_3$), alkalinity (120 mg/L as $CaCO_3$), and Ca:Mg ratio (4:0, 3:1, 1:1, 1:3, and 1:4) (Table 1). All Ca:Mg ratios are presented on a mass basis unless otherwise indicated.

All testing was conducted using static (48-h tests) or static renewal (96-h tests) procedures with a 16:8-h light:dark photoperiod. For 96-h tests, solutions (~90–100%) were renewed after 48 h of exposure. During test renewal, organisms were transferred to freshly prepared test solutions. Organisms in all tests were exposed to six experimental copper concentrations (using a 50 or 60% dilution series) in each Ca:Mg laboratory water. Negative control treatments (unspiked dilution water) were conducted concurrently with each test. The biological endpoint measured for all acute toxicity tests was death (defined as no visible movement or any response to gentle prodding with a blunt probe).

Rainbow trout studies were conducted as 96-h static renewal tests at 12 ± 1°C, in 5 to 9 L of test solution (loading rate ≤0.58 g/L) with two replicates of 10 fish each (Table 2). Fish were held in HT water at 12 ± 1°C and were not fed for 48 h prior to testing. In the initial set of studies, a 0:4 Ca:Mg treatment was included; however, control organisms in this treatment had significant mortality (90% at 48 h). Because of this response, the 0:4 Ca:Mg treatment was replaced with a 1:4 Ca:Mg treatment.

Fathead minnow studies were conducted as 48-h static (two

Table 2. Experimental design and median lethal copper concentrations (LC50s) measured during Ca:Mg studies

Species	Organism age	Ca:Mg ratio[a]	Water hardness/ alkalinity	Test duration (h)	LC50 (95% CI) total Cu (μg/L)	% Dissolved Cu
Rainbow trout	42 d posthatch	4:0	172/114	96	67.9 (55.5–82.9)	87
		3:1	178/116		53.9 (47.1–61.8)	84
		1:1	176/114		35.5 (31.3–40.4)	85
		1:3	176/119		18.1 (15.8–20.7)	96
		0:4	180/116		—[b]	—
	47 d posthatch	4:0	176/108		52.5 (45.2–61.0)	89
		3:1	180/113		46.2 (38.7–55.1)	90
		1:1	178/114		30.7 (25.7–36.6)	97
		1:3	180/110		17.9 (15.3–21.0)	93
		1:4	176/114		18.1 (15.6–21.0)	95
	45 d posthatch	4:0	179/116		37.3 (32.0–43.5)	86
		3:1	177/117		27.7 (23.3–32.9)	90
		1:1	180/115		21.2 (18.6–24.3)	90
Fathead minnow	<24 h old	4:0	178/115	48	—	—
		3:1	178/117		636 (572–706)	—
		1:1	180/111		747 (541–1031)	—
		3:1	180/117		647 (508–823)	—
		1:1	180/117		400 (296–541)	—
		4:0[c]	176/118	96	—	—
		3:1	176/116		837 (686–1021)[d]	—
		1:1	182/116		503 (418–607)[d]	—
	28 d old	4:0	176/118		442 (377–518)	—
		3:1	176/116		502 (421–599)	—
		1:1	182/116		434 (365–516)	—
Ceriodaphnia dubia	<24 h old	4:0	178/115	48	10.50 (9.62–11.47)	—
		3:1	178/117		17.47 (13.50–22.60)	—
		1:1	180/111		16.16 (13.85–18.87)	—
		4:0	178/117		7.92 (7.62–8.23)	—
		3:1	180/117		12.94 (11.60–14.43)	—
		1:1	180/117		14.49 (12.82–16.38)	—
		4:0	180/117		7.88 (6.61–9.39)	—
		3:1	182/117		9.14 (5.78–14.45)	—
		1:1	180/118		7.89 (6.15–10.14)	—
Daphnia magna	<24 h old	4:0	176/114	48	16.35 (14.27–18.74)	—
		3:1	176/111		20.97 (17.66–24.90)	—
		1:1	180/111		57.28 (48.66–67.44)	—
		4:0	180/117		21.55 (16.94–27.41)	—
		3:1	182/117		31.77 (26.90–37.51)	—
		1:1	180/118		42.68 (35.88–50.77)	—
		4:0	90/56		12.20 (10.27–13.88)	—
		3:1	90/59		16.93 (13.97–20.52)	—
		1:1	92/59		11.99 (10.06–14.29)	—
Gammarus sp.	Mixed	4:0	176/118	96	181 (94.24–348)	—
		3:1	176/118		103 (84.48–125)	—
		1:1	182/116		133 (94.7–187)	—

[a] Ca:Mg ratio presented on mass basis.
[b] Control survival was 10% at 48 h. See text for details.
[c] Consisted of only a control treatment.
[d] Data are for 48 h; 96-h values are provided in the text.

rounds for <24-h-old fish) and 96-h static renewal (one round for <24-h- and 28-d-old fish) tests at 25 ± 1°C in 200 or 300 ml of test solution (loading rate ≤0.45 g/L) with either two replicates of 10 fish each (96-h tests) or four replicates of five fish each (48-h tests). Organisms less than 24 h old were placed directly into test solutions, whereas 28-d-old organisms were held in HT culture water at test temperature for 48 h off-feed prior to test initiation. Fathead minnows were not fed during 48-h acute studies. However, in the 96-h acute toxicity studies, organisms were fed 0.1 ml (<24-h-old fish) or 0.2 ml (28-d-old fish) of concentrated newly hatched brine shrimp nauplii suspension per test chamber 2 h prior to test renewals on day 2.

Ceriodaphnia dubia studies were conducted as 48-h static tests at 25 ± 1°C in 50 or 80 ml of test solution with four replicates of five organisms each (Table 2). *Ceriodaphnia dubia* were placed directly into test solutions without any additional acclimation. Organisms were not fed during testing. *Daphnia magna* studies were carried out in a similar fashion, except studies were conducted at 20 ± 1°C. An additional series of studies was conducted at a target hardness and alkalinity of 90 and 60 mg/L, respectively.

Gammarus sp. studies were 96-h static renewal tests conducted at 17 ± 1°C in 300 ml of test solution with two replicates of 10 organisms each. Test solutions were renewed on day 2. Amphipods were held at 13 to 16°C without feeding for 48 h prior to test initiation, and organisms were not fed during testing.

Test solutions

Copper was introduced into each test as a reagent-grade chloride salt ($CuCl_2 \cdot 2H_2O$; Mallinckrodt, Paris, KY, USA). During RBT testing, up to four samples were collected from

each test treatment for analytical confirmation of nominal copper concentrations. Samples were collected and composited from treatment replicates. For 48-h acute studies, only two samples were collected from each test treatment, at test initiation and at test termination or when 100% mortality occurred in an individual treatment. For 96-h static renewal acute toxicity studies, samples were collected at test initiation, prior to test renewal (old solutions), just after test renewal (FHM and amphipod studies), and at test termination or when 100% mortality occurred in an individual treatment (trout studies).

Test solutions were analyzed for total recoverable, dissolved, or acidified (no filtration or digestion, only acidified) copper [10]. All samples were acidified to 1% using concentrated, trace metals–grade nitric acid (Mallinckrodt). We determined that for our laboratory waters, total recoverable and acidified copper measurements were very similar (relative standard deviation 2.6 ± 1.8%, $n = 12$). Dissolved (filtration through a 0.45-μm polyethyl sulfone filter; Whatman, Clifton, NJ, USA) copper concentrations were measured only for RBT studies. Copper concentrations were determined using inductively coupled plasma atomic emission spectroscopy, graphite furnace atomic absorption, or direct aspiration flame atomic absorption spectroscopy.

Data analysis

Results of each of the individual acute tests were used to calculate time-dependent LC50 values. The LC50 values and associated 95% confidence limits were calculated using Spearman–Karber, trimmed Spearman–Karber, and probit analysis methods [11]. Test concentrations were $\log_{10}$ transformed prior to calculation of the LC50. The method selected for reporting the test results was determined by the characteristics of these data (the presence or absence of 0 and 100% mortality and the number of concentrations in which partial mortality had occurred) [7]. The binomial LC50 estimation method was also used when appropriate [12]. Trends among LC50 data were compared using least-squares regression analyses (Microsoft® Excel, Version 97 SR-2).

Differences among LC50s were compared using overlap of 95% CI in cases of only two treatments. In cases of three or more treatments, this was also used as a conservative method for determining differences among LC50s because of the increased chance for type I error. Because of this, most comparisons were done on a relative basis to determine trends in copper toxicity due to changes in Ca:Mg treatments.

RESULTS

Initial water quality characteristics for water treatments for test waters were similar among treatments at a given hardness except for the experimental variables (Table 1). Sodium concentrations were slightly higher in the 4:0 waters in 180-mg/L hardness studies as a result of one round of testing (FHM [96-h studies] and *Gammarus* sp.) because the alkalinity had to be slightly augmented by the addition of sodium bicarbonate.

Rainbow trout studies

Median lethal concentrations for RBT decreased with lower Ca:Mg ratios for each series of studies conducted (Table 2). Calcium had a greater effect on acute copper toxicity than magnesium; copper was more toxic in waters with higher magnesium concentrations (but a similar hardness and alkalinity) than in waters with high calcium concentrations. The greatest changes in copper toxicity occurred within the mid-Ca:Mg ratios, and the effect appeared to level off at higher and lower Ca:Mg ratios (Table 2).

Although RBT were more sensitive to copper in waters having higher Mg concentrations, we also observed that RBT could survive (for 96 h) in laboratory waters in which the Ca:Mg ratio was 4:0 but not when it was 0:4 Ca:Mg. Organisms tested in the 0:4 Ca:Mg treatment had significant mortality in the control treatment (90% at 48 h).

Fathead minnow studies

Less-than-24-h-old FHM were most sensitive to copper in 1:1 Ca:Mg waters compared with 3:1 Ca:Mg waters (Table 2). Studies were initially conducted with 4:0 Ca:Mg waters; however, control survival of <24-h-old FHM was significantly reduced (45 and 40% survival in the first and the third study, respectively) in these waters. In the third set of tests, 96-h LC50 (95% CI) for <24-h-old FHM were as follows: 3:1 Ca:Mg, 525.9 (461.3–599.6) μg/L, and 1:1 Ca:Mg, 335.7 (271.1–415.6) μg/L. As with the 48-h LC50s, FHM were more sensitive to copper in 1:1 Ca:Mg waters. No difference was observed in copper sensitivity for 28-d-old FHM under different Ca:Mg ratios (Table 2). In addition, <24-h-old FHM were not any more sensitive to copper than 28-d-old FHM (Table 2).

Cladoceran studies

Ceriodaphnia dubia sensitivity to copper was not consistently affected by Ca:Mg ratios, although some trends were observed among LC50s (Table 2). In the first two sets of studies, *C. dubia* were more sensitive to copper in the 4:0 Ca:Mg treatments than the 3:1 or 1:1 Ca:Mg waters. However, the last study showed no observable trend in copper sensitivity among Ca:Mg treatments (Table 2).

Daphnia magna sensitivity to copper was the reverse of that observed for RBT. *Daphnia magna* tested in hard water (hardness 180 mg/L) were more sensitive to copper in 4:0 Ca:Mg treatments and least sensitive to copper in 1:1 Ca:Mg waters (Table 2). No difference in copper sensitivity was observed among Ca:Mg treatments in moderately hard waters (hardness 90 mg/L) (Table 2).

Gammarus *sp. studies*

Gammarids were equally sensitive to copper in all three Ca:Mg treatments (Table 2), although the LC50s were slightly higher in the 4:0 and 1:1 Ca:Mg waters compared to the 3:1 Ca:Mg treatment.

DISCUSSION

We examined the influence of Ca:Mg ratios on copper toxicity by maintaining hardness, alkalinity, pH, and other selected ions (K^+, Na^+, and Cl^-) approximately constant. Our results indicate that the Ca:Mg ratio does not affect the toxicity of copper to these five laboratory test organisms in the same way. The sensitivity of RBT, <24-h-old FHM, and *D. magna* to copper changed under different Ca:Mg ratios, while the sensitivity of *C. dubia,* 28-d-old FHM, and *Gammarus* sp. was relatively unaffected. Both RBT and <24-h-old FHM were less sensitive to copper in waters having higher calcium concentrations, while *D. magna* exhibited greater sensitivity at higher Ca:Mg ratios. Welsh et al. [4] and Erickson et al. [2] also observed decreased copper toxicity at higher Ca:Mg ratios to salmonids and FHM, respectively. The Ca:Mg studies at a

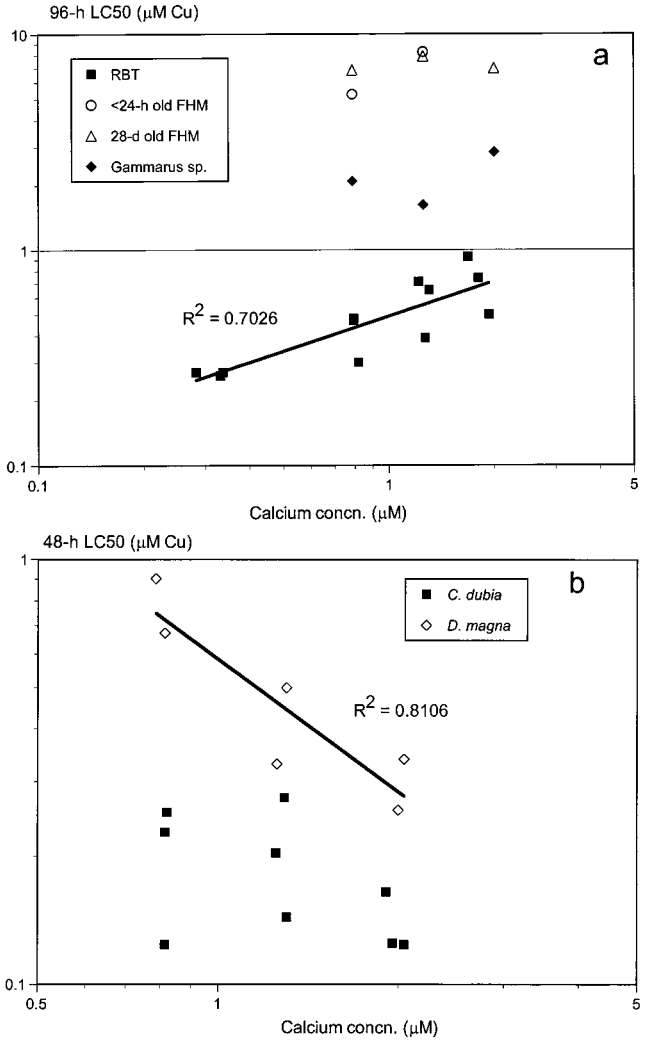

Fig. 1. Relationship between copper toxicity and calcium concentration (surrogate for Ca:Mg ratios because studies were conducted at the same hardness) with (**a**) rainbow trout (RBT), fathead minnows (<24-h- and 28-d-old FHM), and *Gammarus* sp. and (**b**) *Ceriodaphnia dubia* and *Daphnia magna.* Results for RBT, FHM, and *Gammarus* sp. are for 96-h studies, while values for *C. dubia* and *D. magna* are 48-h data. All studies were conducted at a hardness and alkalinity of 180 and 120 mg/L as $CaCO_3$, respectively.

lower hardness resulted in changes in organism sensitivity to copper. *Daphnia magna* tested at 180-mg/L hardness exhibited differences in copper sensitivity due to Ca:Mg ratios, while those tested in waters at 90-mg/L hardness did not. Erickson et al. [2] also observed differences in copper toxicity for <24-h-old FHM under different Ca:Mg ratios at hardnesses of 146 and 246 mg/L as $CaCO_3$ but not at a hardness of 106 mg/L. However, differences in our study may be due to other factors (e.g., alkalinity, pH, and Na concentration) besides the Ca:Mg ratio, as the ionic composition of the waters were different between the two studies (Table 2).

Based on the competitive exclusion of the cupric ion (Cu^{2+}) at Na^+ channels in the fish gill (the mode of action for Cu [13]), we believe Ca^{2+} is a better inhibitor of copper toxicity than Mg^{2+} is for RBT and <24-h FHM in hard water. The effect of different Ca:Mg ratios (expressed as calcium concentration) significantly affected copper toxicity for RBT in hard waters (Fig. 1a) ($p = 0.0007$). Studies conducted by Welsh et al. [4] suggest that the Ca:Mg ratio is also an important regulator of copper toxicity to salmonids in softer waters (hardness 40 and 90 mg/L). The sensitivity of copper to <24-h-old FHM was also influenced by higher Ca:Mg ratios in hard waters (>146 mg/L), but this effect was not evident in softer waters [2]. From other experiments conducted in our laboratory with RBT (unpublished data), other factors (e.g., alkalinity and dissolved organic carbon) may become more important in mitigating copper toxicity in softer waters. Magnesium appears to be a better inhibitor of copper for *D. magna* (in hard water) (Fig. 1b) ($p = 0.014$), while both Ca and Mg were equally competitive inhibitors of copper for *C. dubia, Gammarus* sp., and 28-d-old FHM. The difference in response to copper under different Ca:Mg ratios in hard waters may be due to decreased gill permeability at higher Ca concentrations [14] but not by greater Mg concentrations [15]. While this explains why RBT and <24-h-old FHM are more sensitive to copper in 1:1 Ca:Mg waters compared to 4:0 Ca:Mg waters, it does not explain why 28-d-old FHM were not influenced by different Ca:Mg ratios. Nor does it explain why the invertebrates were either unaffected or more sensitive at higher Ca:Mg ratios.

Although the absolute toxicity of copper can change for certain species because of differences in Ca:Mg ratios, the real question is, How does this impact the ambient water quality criteria (AWQC) for copper? The acute AWQC or criterion maximum concentration is calculated as half the final acute value, which is derived from the four lowest genus mean acute values [16]. For copper, the four most sensitive genera are the Cladocerans (*Ceriodaphnia* and *Daphnia*), Northern squawfish (*Ptychocheilus;* common name has been changed to Northern pikeminnow), and amphipod (*Gammarus*) [8]. The copper toxicity for *C. dubia* and *Gammarus* sp. was not significantly affected by changes in Ca:Mg ratios. Furthermore, *D. magna* were affected by differences in Ca:Mg ratio (at least in hard waters), but they are more sensitive to copper at higher Ca:Mg ratios. Since many of the *Daphnia* studies were conducted in natural waters that tend to have a higher Ca:Mg ratio [4], any difference in toxicity due to changes in Ca:Mg ratio may be accounted for in the AWQC derivation. However, because organism sensitivity to copper under different Ca:Mg ratios may change because of hardness, studies should also be conducted over a range of water hardnesses to determine potential effects. Based on our studies, it appears that for copper the potential confounding effects of Ca:Mg ratios to the AWQC are minimal.

Our studies suggest that the copper sensitivity for some, but not all, organisms is influenced by differences in the Ca:Mg ratio. However, until sufficient studies have been conducted to fully evaluate this relationship for multiple species in waters of varying hardnesses, we also recommend that calcium and magnesium concentrations be matched with known Ca:Mg ratios in waters of interest when preparing laboratory waters for certain testing purposes (e.g., whole effluent toxicity testing and water-effect ratios) [4]. These relationships should also be evaluated for other metals, as the Ca:Mg ratio has been shown to affect the toxicity of cadmium [5,17] and nickel [18].

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