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EFFECTS OF CHLORIDE, CALCIUM, AND DISSOLVED ORGANIC CARBON ON SILVER TOXICITY: COMPARISON BETWEEN RAINBOW TROUT AND FATHEAD MINNOWS

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Abstract—The effects of independently altering chloride, calcium, and dissolved organic carbon (DOC) on the toxicity of silver (presented as $AgNO_3$) were compared between rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*). The 96-h median lethal concentration (LC50) toxicity tests for both species were performed under the same conditions, within the same containers. In addition, the effect of altering [Cl^-] on silver-induced perturbations to body Na^+ influx and gill silver load was studied. Toxicity tests were conducted in synthetic soft water (50 µM Na^+, 50 µM Cl^-, 50 µM Ca^{2+}, 0.3 mg DOC/L). The [Cl^-], [Ca^{2+}], and [DOC] were adjusted by the addition of NaCl, $CaNO_3$, or humic acid, respectively. On the basis of total silver, increasing [Cl^-] over a range of 50 µM to 1,500 µM resulted in a 4.3-fold increase in the 96-h LC50 values (decrease in toxicity) for rainbow trout, but did not significantly affect the 96-h LC50 values for fathead minnows. Increasing water [Ca^{2+}] (from 50 to 2,000 µM) had only a small influence (1.5-fold increase) on the 96-h LC50 values in either species. However, increasing DOC levels (from 0.3 to 5.8 mg DOC/L) significantly increased the 96-h LC50 values (2.7- to 4.1-fold increases) in both species. If the 96-h LC50 values are calculated on the basis of ionic silver, Ag^+ (utilizing the aquatic geochemical computer program MINEQL+), then, in the case of rainbow trout, toxicity correlates to Ag^+. However, this correlation does not exist for fathead minnows. Increasing [Cl^-] did not affect the degree of perturbation of Na^+ influx during acute exposure (first 4 h) to 8 µg Ag/L in either species, nor did it affect the whole-body silver uptake rates, but it did reduce the gill silver load. These results demonstrate that differences exist in the way in which water chemistry ameliorates silver toxicity between rainbow trout and fathead minnows.

Keywords—Silver toxicity   Water chemistry   Rainbow trout   Fathead minnows   Physiology

INTRODUCTION

Silver, when presented as silver nitrate in laboratory water, is one of the most toxic metals to freshwater fish, with median lethal concentration (LC50) values of between 5 and 60 µg Ag/L [1–9]. However, inorganic complexes of silver, such as silver thiosulfate, silver sulfide, and silver chloride, have been shown to be relatively benign in acute toxicity tests. Both LeBlanc et al. [6] and Hogstrand et al. [8] found that Ag, when presented in the form silver thiosulfate or silver chloride, is less toxic by at least orders of magnitude than silver nitrate for both fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*). Both studies illustrate the importance of speciation in silver toxicity.

Regulations for silver discharge in the United States acknowledge the importance that water chemistry may play in influencing toxicity. U.S. Environmental Protection Agency (U.S. EPA) guidelines, designed to help authorities in the regulation of silver, have designated hardness (principally the calcium content of the water) as the sole factor affecting silver toxicity and have incorporated it into the following equation:

$$\text{maximum total recoverable Ag (µg/L)}$$
$$= \exp[1.72(\ln \text{hardness}) - 6.52] \qquad (1)$$

This equation is derived from interlaboratory studies of $AgNO_3$ toxicity to daphnids, fathead minnows, and rainbow trout [4]. However, the validity of this hardness equation has been re-

cently questioned [8–10]. Galvez and Wood [9] showed that, for juvenile rainbow trout, water chloride had a much greater effect in ameliorating silver toxicity than did water hardness. Analysis of the water geochemistry in the toxicity test by the aquatic geochemical computer program MINEQL+ [11] showed that silver toxicity correlated to Ag^+. Furthermore, Davies et al. [2] found that when hardness was increased from 26 mg/L (as $CaCO_3$) to 350 mg/L, a twofold increase occurred in the LC50 value for juvenile rainbow trout. Based on the above U.S. EPA hardness equation, an increase in hardness of this magnitude should result in a >80 times increase in the LC50 value. Erickson et al. [12] reported a significant protective effect of hardness (2.5-fold increase in LC50) in 30-d-old fathead minnows over the hardness range of 48 to 249 mg/L, but this effect again was much less than that predicted by the hardness equation (17-fold).

Further evidence that water geochemistry influences silver toxicity comes from studies investigating the physiological and biochemical perturbations induced by silver exposure in rainbow trout. In freshwater rainbow trout the toxic mechanism of $AgNO_3$ involves the inhibition of Na^+ and Cl^- uptake at the gills [13,14], and, more specifically, the inhibition of the sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase) enzyme, which is situated on the basolateral membrane of the gill [13]. Bury et al. [15] and McGeer and Wood [16] showed that an increase in water chloride reduces the degree of silver-induced disruption to Na^+ balance. Moreover, an increase in water calcium had no effect on the silver inhibition of Na^+ influx and Na^+/K^+-ATPase activity [15].

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Table 1. Measured water ion concentrations, pH, dissolved oxygen (DO_2), and dissolved organic carbon (DOC) levels. Values are mean $\pm$ SD[a]

Predicted Cl:Ca:DOC	Cl^- (μM)	Ca^{2+} (μM)	Na^+ (μM)	K^+ (μM)	Mg^{2+} (μM)	DO_2[b]	pH	DOC[c] (mg/L)
Chloride								
50:50:0.3	67.8 $\pm$ 15.8 $n = 40$	47.6 $\pm$ 9.3 $n = 42$	83.9 $\pm$ 8.5 $n = 47$	10.9 $\pm$ 2.5 $n = 48$	4.7 $\pm$ 5.6 $n = 48$	91.2 $\pm$ 1.1 $n = 6$	6.2 $\pm$ 0.03 $n = 6$	NM
250:50:0.3	240.7 $\pm$ 34.2 $n = 40$	51.7 $\pm$ 3 $n = 42$	257.2 $\pm$ 11.6 $n = 47$	11.2 $\pm$ 2.4 $n = 47$	3.8 $\pm$ 3.2 $n = 48$	90.3 $\pm$ 3.8 $n = 6$	6.3 $\pm$ 0.04 $n = 6$	NM
800:50:0.3	782.2 $\pm$ 68.2 $n = 34$	65.2 $\pm$ 22.7 $n = 48$	868 $\pm$ 120.1 $n = 47$	12.7 $\pm$ 3.2 $n = 48$	14.1 $\pm$ 3.3 $n = 46$	94.9 $\pm$ 1.1 $n = 6$	6.6 $\pm$ 0.04 $n = 6$	NM
1500:50:0.3	1426 $\pm$ 139 $n = 33$	91.2 $\pm$ 71.6 $n = 64$	1590 $\pm$ 155 $n = 63$	14 $\pm$ 2.9 $n = 64$	3.6 $\pm$ 3.8 $n = 64$	92.1 $\pm$ 4.7 $n = 6$	6.7 $\pm$ 0.2 $n = 6$	NM
Calcium								
50:500:0.3	46.3 $\pm$ 10 $n = 35$	499 $\pm$ 27.8 $n = 48$	75.2 $\pm$ 10.6 $n = 48$	11.7 $\pm$ 2.5 $n = 48$	12.2 $\pm$ 7.9 $n = 48$	94.9 $\pm$ 2.5 $n = 6$	6.6 $\pm$ 0.07 $n = 6$	NM
50:2000:0.3	58.9 $\pm$ 26.5 $n = 28$	2316 $\pm$ 252 $n = 45$	85.7 $\pm$ 21.1 $n = 45$	12 $\pm$ 2 $n = 44$	12 $\pm$ 4 $n = 44$	79.2 $\pm$ 14.3 $n = 6$	6.5 $\pm$ 0.03 $n = 6$	NM
DOC								
50:50:1	74.3 $\pm$ 16.6 $n = 42$	32 $\pm$ 13.1 $n = 54$	110.6 (137) $n = 46$	15.2 $\pm$ 2.6 $n = 54$	3 $\pm$ 2.7 $n = 54$	NM	6.7 $\pm$ 0.07 $n = 6$	1.63 $\pm$ 0.14 $n = 4$
50:50:5	95.2 $\pm$ 19.4 $n = 42$	34.5 $\pm$ 22.4 $n = 64$	150.6 $\pm$ 21.6 $n = 62$	14.6 $\pm$ 1.6 $n = 64$	3.5 $\pm$ 3.7 $n = 64$	NM	6.7 $\pm$ 0.08 $n = 6$	5.82 $\pm$ 0.12 $n = 4$

[a] n = number of samples measured; NM = not measured.
[b] The DO_2 concentration is presented as a percent of air-saturated water.
[c] The DOC levels for reverse osmotic dechlorinated Hamilton tap water (controls) are 0.3 mg/L [15].

Analysis of the water geochemistry by computer programs such as MINEQL+ [11] shows a good correlation between the water ionic silver (Ag^+) concentration and the silver-induced physiological disruptions in rainbow trout [15,16].

In contrast to the profound protective effects that chloride has on silver toxicity to rainbow trout [9,15,16], Brooke et al. [17] and Karen et al. [18] reported that chloride has only a very minor protective influence on silver toxicity to 4-d-old fathead minnows. Erickson et al. [12] reported a modest increase in silver toxicity to 30-d-old fathead minnows when water chloride was increased 2.5-fold. Consequently, the first purpose of this study was to ascertain the effect of chloride on silver toxicity to fathead minnows and rainbow trout by conducting toxicity tests under the same conditions. The second purpose of this study was to compare the effect of water chloride on silver-induced perturbations to Na^+ balance and silver accumulation in the gills and body of both rainbow trout and fathead minnows. In addition, the relative influences of calcium and dissolved organic carbon (DOC) on silver toxicity were assessed. Calcium is included because of its importance in the U.S. EPA hardness equation (see above). Dissolved organic carbon is prevalent in freshwater systems and has been shown to reduce heavy metal toxicity [19–21]. Specifically, Erickson et al. [12] found that elevating DOC from 1.5 mg/L to 10.5 mg/L increased the silver LC50 values for 30-d-old fathead minnows by more than fourfold. Moreover, DOC has also been shown to reduce the accumulation of silver on the gills [15,22], as well as prevent silver-induced physiological perturbation to Na^+ balance in rainbow trout [15].

MATERIALS AND METHODS

Toxicity tests

Juvenile rainbow trout, weight 2.2 $\pm$ 1.2 g, were obtained from Humber Spring Hatchery, Orangeville, Ontario, Canada and fathead minnows, weight 0.23 $\pm$ 0.28 g, were obtained from Aquatic Biosystems, Fort Collins, Colorado, USA. Fish were kept in dechlorinated Hamilton (ON, Canada) tap water

([Na^+], 0.5 mM; [Cl^-], 0.7 mM; [Ca^{2+}], 1 mM; [Mg^{2+}], 0.2 mM; [K^+], 0.05 mM; pH 7.8–8.0; 17°C) for 2 weeks before acclimation to test water. Fish were gradually acclimated to synthetic soft water, generated by reverse osmosis (RO, Andersen, Dundas, ON, Canada) of Hamilton dechlorinated tap water, over a period of 2 weeks. During this period the ratio of soft water to hard water that the fish received was increased. The water temperature was maintained at 17°C. After 2 weeks, the fish received RO water with the addition of NaCl and $CaNO_3$ to attain a final soft water composition of 50 μM Na^+, 50 μM Cl^-, and 50 μM Ca^{2+} (K^+ and Mg^{2+} levels and pH of the soft water are reported in Table 1; titratable alkalinity to pH 4 was 8.25 mg $CaCO_3$/L). Fish were maintained in this synthetic water for at least 2 weeks before testing, during which time both species were fed a commercial ration (Martin Mills, Tavistock, ON, Canada) at 1.5% of their body weight daily.

All toxicity tests were performed in a static-renewal system and followed American Society for Testing and Materials guidelines [23]. To enable direct comparison between the toxicity of silver to rainbow trout and fathead minnows, tests were conducted in the same container in a temperature-controlled room at 17°C, a temperature that both species could experience in the natural environment, with a 16-h light and 8-h dark light regime. Test media, consisting of RO water and the appropriate salts (NaCl or $CaNO_3$, respectively, to obtain the desired Cl^- or Ca^{2+} concentrations) were stored in 200-L containers in the experimental room with constant aeration for 24 h before use. For acclimation of fish to the various test waters, 60 rainbow trout and 60 fathead minnows were placed in separate 72-L tanks containing 50 L of the aforementioned medium for 48 h. After 24 h, 50% of the test water was exchanged with fresh media and the tanks were well aerated throughout the acclimation period.

Experimental tanks were designed so that two nylon mesh chambers (approximate size 15 $\times$ 15 $\times$ 40 cm) were suspended in 20 L of test medium. During the 96-h experiment, the test

solutions were continuously aerated to maintain high dissolved oxygen levels (Table 1) and to enable thorough mixing of the eluents. For each test, at least six (maximum of nine) concentrations over the range of 3.2 to 40 μg AgNO₃/L were used to elucidate a 96-h LC50 value. Each experimental tank was assigned a silver concentration and then randomly distributed in the temperature-controlled room. Clean techniques ensured that the controls never experienced silver. Ten rainbow trout and 10 fathead minnows were placed in separate chambers within the same tank, and the total weight of the fish to water volume ratio never exceeded 2 g/L.

Silver was added from a stock of 1 M $AgNO_3$ (in 0.1 M HNO_3) that was stored in the dark. The water silver concentrations were monitored frequently (every 3–4 h over the first 48 h and periodically thereafter) and the concentrations were maintained by the addition of an appropriate volume of the stock $AgNO_3$ solution. The silver concentration to which the fish were exposed was calculated as a time-weighted average of the total silver measured throughout the experimental period. Each day 80% of the medium from each tank was replaced with fresh medium supplemented with an appropriate volume of the 1 M $AgNO_3$ stock so as to maintain the desired silver levels. Two 10-ml water samples were taken before and after the medium change. The first water sample was used for analysis of water ions, total ammonia, and total silver levels (see below), whereas the second water sample was filtered through 0.45-μm filters (Millipore, Bedford, MA, USA) for measurement of dissolved silver levels.

Water analysis

Aliquots from the 10-ml daily water sample were taken for Cl^- and total ammonia analysis, and the remainder of the sample was acidified with 0.05% by volume of trace metal grade concentrated HNO_3 for analysis of ions and silver. Water Na^+, Ca^{2+}, K^+, and Mg^{2+} concentrations were measured by flame atomic absorption (Varian AA 1275, Varian Ltd., Mississauga, ON, Canada). Water silver levels were measured by graphite furnace atomic absorption spectrophotometry (Varian AA 1275 fitted with a GTA-95 atomizer). Water Cl^- levels were measured by the colorimetric mercuric thiocyanate method [24] and water ammonia by the salicylate–hypochlorite method [25]. Water pH (GK2401C electrode) and dissolved oxygen levels (E5046 electrode) were measured using Radiometer equipment (Radiometer Instruments, Copenhagen, Denmark). Dissolved organic carbon levels were determined by a Rosemount Analytical DC-180 automated total organic carbon analyzer (Folio Instruments, Kitchener, ON, Canada).

Flux measurements

Juvenile rainbow trout, weight 4.22 ± 1.89 g, were obtained from Humber Spring Hatchery, and fathead minnows, weight 2.71 ± 0.75 g, were obtained from Spring Valley trout farm, Petersburg, Ontario, Canada. Fish were kept in dechlorinated Hamilton tap water (for composition see above) for at least 2 weeks before conducting experiments. Fish where fed 1% of their body weight per day during this period.

Whole-body Na^+ and silver influxes were performed in 100 ml of test medium in light-shielded plastic bags. About 24-h before commencement of the experiment, 100 ml of synthetic soft water containing 0.01 μCi ¹¹⁰ᵐAgNO₃/L (94.3 MBq, Amersham International, Courtaboeuf, France) was placed in the plastic bags to saturate silver binding sites on the plastic. Fluxes were performed in synthetic soft water supplemented

with 0.5 mM CaNO₃ and 0.25 mM NaNO₃, and adjusted to pH 7.6 with 1 M KOH. The $[Cl^-]$ was adjusted to 50 μM in the control series and 50, 250, or 800 μM in the experimental series by the addition of KCl. To each container 2 μCi ²⁴Na⁺ (nuclear reactor at McMaster University, Hamilton, ON, Canada) was added in the form of a trace amount of sodium carbonate, which had negligible effect on water total Na^+ level. In addition, in all experimental series, 1 μCi ¹¹⁰ᵐAg/L was added in the form of $AgNO_3$ (equivalent to 8 μg total Ag/L, as measured by graphite furnace atomic absorption spectrophotometry, see above). Individual rainbow trout or fathead minnows were then added to the plastic bags. The flux period lasted for 4 h, during which time the medium was continuously aerated. The first 5-ml water samples were taken 15 min after the fish had been added to the plastic bag; this delay allowed for mixing of the eluent. Further water samples were taken at 4 h. All water samples were immediately acidified with 0.05% HNO_3 and Na^+ and silver concentrations were measured as described above (see *Water analysis*). After 4 h the fish were euthanized by an overdose of MSS-222 (Syndel Pharmaceuticals, Vancouver, BC, Canada) and then washed in 150 mg NaSO₃/L for 1 min, 250 μg AgNO₃/L for 1 min, dechlorinated Hamilton tap water for an additional 1 min, and then blotted dry. This wash regime removed loosely bound ¹¹⁰ᵐAg and ²⁴Na⁺ from the surface of the fish. The gills were dissected from the body and counted separately. Radioactivities of the water, body, and gills were measured on a gamma counter (Packard Instruments, Downers Grove, IL, USA).

The radioactivity in the samples emitted by ²⁴Na⁺ was calculated from the initial total counts per minute (cpm) (composed of both ²⁴Na⁺ and ¹¹⁰ᵐAg cpm) minus the cpm after 10 half-lives (150 h) of ²⁴Na⁺ (representing the proportion attributable to ¹¹⁰ᵐAg alone). This value was then corrected for the decay of the ²⁴Na⁺ isotope during counting.

Gill loads (of Ag) and whole-body influx rates of sodium and silver were calculated from the appearance of radioactivity in the tissues as follows:

$$\text{whole-body influx} = q/(SA \cdot t \cdot wt) \tag{2}$$

where q represents the cpm for gill or whole body, t is the time (h), wt the wet weight of the sample, and SA is the specific activity of the water calculated from

$$SA = [(cpm_i/[ion]_i) + (cpm_f/[ion]_f)]/2 \tag{3}$$

where cpm_i represents the initial cpm per milliliter in the water, cpm_f represents the final cpm per milliliter in the water, and $[ion]_i$ and $[ion]_f$ represent the initial and final sodium or silver concentrations of the water, respectively. The division by time was omitted for calculation of gill silver load.

Calculations and statistics

The LC50 values were calculated either by probit analysis (SPSS 6 computer package, Chicago, IL, USA) or by the methods of Litchfield and Wilcoxon [26] and are reported with the 95% confidence limit. Values are considered different when the CLs did not overlap. Water geochemical analysis was performed using the MINEQL+ computer program [11], with the addition of the conditional equilibrium constants for Ag-DOC and H-DOC taken from Janes and Playle [22]. A one-way analysis of variance followed by a least significant difference (LSD) test (STATISTICA, StatSoft, Tulsa, OK, USA) was used
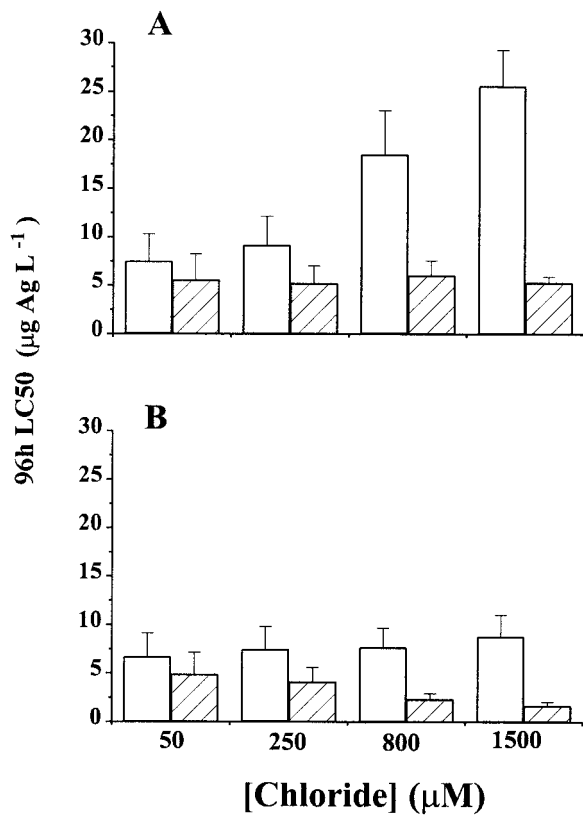
Fig. 1. The 96-h median lethal concentration (LC50) values + 95% confidence limits for rainbow trout (**A**) and fathead minnows (**B**) at various chloride concentrations. The open bars represent total silver values (μg Ag/L) and the hatched bars represent ionic silver values (μg Ag⁺/L) calculated by MINEQL+ [11].

to test differences (at $p < 0.05$) among treatments in Na⁺ influx rates and gill silver loads.

RESULTS

Measured water [Cl⁻] and [Ca²⁺] were in the ranges of 95 to 114% and 99.8 to 115.8 %, respectively, of the predicted values. Measured DOC concentrations were slightly higher than predicted; a predicted value of 1 mg DOC/L corresponded to a measured value of 1.63 mg DOC/L, and 5 mg DOC/L

corresponded to 5.82 mg DOC/L (Table 1). Water ammonia values averaged 12.74 ± 10.1 μM total ammonia ($n = 294$) and measured dissolved silver values (the silver concentration after filtration through a 0.45-μm filter) were between 81 and 94% of the measured total silver values for Cl⁻ and Ca²⁺ and 71 to 75 % of the measured total silver concentration in the DOC experiment.

Influence of chloride

For rainbow trout, an increase in water [Cl⁻] resulted in an increase (decrease in toxicity) in the 96 h total silver LC50 values. A 30 times increase in [Cl⁻] increased the LC50 values by 4.3 times (Fig. 1 and Table 2). In contrast, for fathead minnows, the 96-h total silver LC50 values remained virtually constant (1.3-fold increase, not significant) over a water [Cl⁻] range of 50 to 1,500 μM (Fig. 1 and Table 2). Thus, at higher [Cl⁻] levels (800 μM, 1,500 μM) total silver was significantly more toxic to fathead minnows than to rainbow trout. For rainbow trout, the 96-h LC50 value based on ionic silver (Ag⁺, as calculated from MINEQL+) remained constant, at about 5 μg/L. However, for fathead minnows, the 96-h ionic silver LC50 values decreased significantly as [Cl⁻] increased (Fig. 1 and Table 2).

Influence of calcium

In both species, increasing [Ca²⁺] over the range of 50 to 2,000 μM resulted in only a very small change in the 96-h LC50 (1.5-fold increase, not significant), for both total silver and ionic silver (Fig. 2 and Table 2).

Influence of DOC

For both species, an increase in the water DOC levels from 0.3 mg/L to 5.8 mg/L resulted in a substantial increase in the 96-h total silver LC50 values (Fig. 3 and Table 2). At 5.8 mg DOC/L a 4.1 times increase occurred in the 96-h total silver LC50 value for rainbow trout (7.5 to 27.7 μg/L) and a 2.7 times increase occurred for fathead minnows (6.7 to 18 μg/L). At this highest DOC level (5.8 mg/L) the 96-h LC50, expressed as total silver, was significantly greater (less toxic) in rainbow trout than in fathead minnows. For rainbow trout, different DOC concentrations did not significantly affect (based on geochemical model calculations [11]) the 96-h ionic silver LC50 values, which remained close to 5 μg/L. However,

Table 2. Summary of 96-h median lethal concentration values ± 95% confidence limits

Water chemistry Cl (μM):Ca (μM):DOC[a] (mg/L)	Fathead minnows		Rainbow trout	
	Total silver (μg Ag/L)	Ionic silver[b] (μg Ag⁺/L)	Total silver (μg Ag/L)	Ionic silver[b] (μg Ag⁺/L)
Chloride treatment				
50:50:0.3	6.7 ± 2.5	4.9 ± 2.3	7.5 ± 2.8	5.6 ± 2.7
250:50:0.3	7.5 ± 2.4	4.1 ± 1.5	9.2 ± 3.0	5.2 ± 1.8
800:50:0.3	7.7 ± 2.0	2.3 ± 0.6	18.5 ± 4.5	6.1 ± 1.5
1500:50:0.3	8.8 ± 2.3	1.7 ± 0.4	25.6 ± 3.7	5.3 ± 0.6
Calcium treatment				
50:500:0.3	7.8 ± 2.4	6.1 ± 2.1	9.9 ± 4.8	8.0 ± 3.3
50:2000:0.3	9.9 ± 2.6	8.4 ± 1.9	10.5 ± 2.3	8.9 ± 1.6
DOC treatment				
50:50:1.6	13.3 ± 5.5	6.2 ± 1.2	18.4 ± 2.7	10.6 ± 2.1
50:50:5.8	18.0 ± 3.2	0.4 ± 0.3	27.7 ± 2.8	5.1 ± 2.9

[a] DOC = dissolved organic carbon.
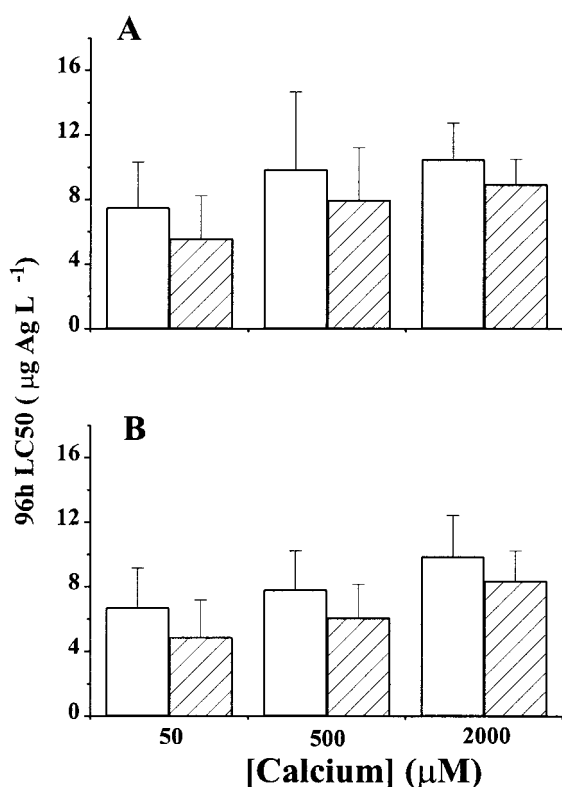[b] Ionic silver was calculated by the geochemical speciation program MINEQL+ [11].

Fig. 2. The 96-h median lethal concentration (LC50) values + 95% confidence limits for rainbow trout (**A**) and fathead minnows (**B**) at various calcium concentrations. The open bars represent total silver values (μg Ag/L) and the hatched bars represent ionic silver values (μg Ag$^+$/L) calculated by MINEQL+ [11].

Fig. 3. The 96-h median lethal concentration (LC50) values + 95% confidence limits for rainbow trout (**A**) and fathead minnows (**B**) at various dissolved organic carbon (DOC) concentrations. The open bars represent total silver values (μg Ag/L) and the hatched bars represent ionic silver values (μg Ag$^+$/L) calculated by MINEQL+ [11].

for fathead minnows, the highest DOC level (5.8 mg/L) resulted in a decrease in the 96-h ionic silver LC50 values (Fig. 3 and Table 2).

Flux measurements

The total silver concentration for the flux experiments was set at 8 μg AgNO$_3$/L. Altering the chloride concentration to 50, 250, or 800 μM corresponded to changes in the [Ag$^+$] of 6.2, 4.5, and 2.4 μg AgNO$_3$/L, respectively (calculated from MINEQL+ [11]). The whole-body Na$^+$ influx rate was about 50% higher in control rainbow trout compared to control fathead minnows. Acute exposure to silver resulted in a significant (30–40%) reduction in the whole-body Na$^+$ influx rate in both species (Table 3); the inhibition was independent of the water [Cl$^-$]. Rainbow trout accumulated more silver in their gills and exhibited higher body silver uptake rates during the 4-h exposure period than did fathead minnows (approximately twofold differences; Table 4). Increasing [Cl$^-$] did not affect the body silver uptake rate in either species, but did tend to lower the gill silver load in both. This effect was statistically significant only at 800 μM [Cl$^-$] for rainbow trout (65% reduction); the 50% reduction in gill silver burden for fathead minnows at this [Cl$^-$] was not significant (Table 4).

DISCUSSION

Conducting toxicity tests on rainbow trout and fathead minnows under the same conditions, and within the same container, allows direct comparison of the effects of freshwater chemistry on silver toxicity between the species. Aquatic environments can vary dramatically in their ionic composition, but variation
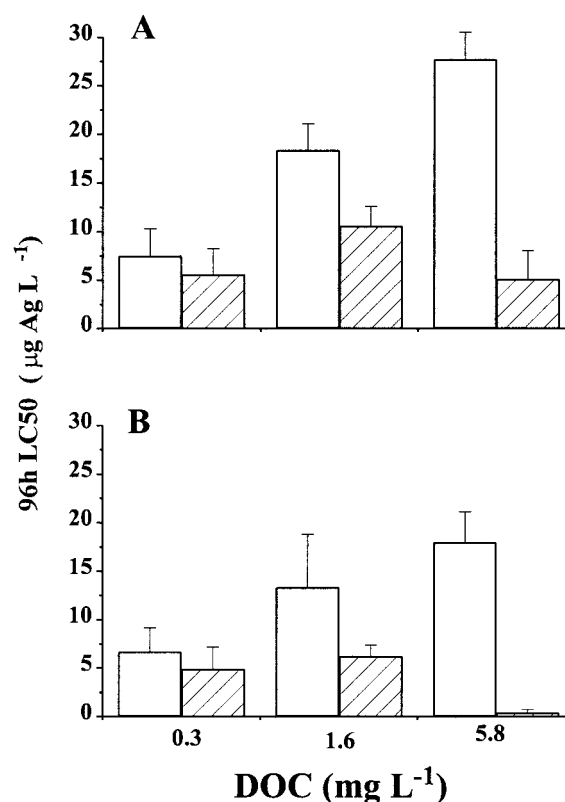
in the concentration of a single ion, as was the case in this study, is rare in the natural environment. Results from this study show that toxicity of silver to rainbow trout decreases, with respect to total silver levels, when water chloride or DOC levels are altered, but altering calcium levels does not significantly affect the 96-h LC50 total silver values. Toxicity of silver to rainbow trout correlates to the water [Ag$^+$], supporting the notion that it is the ionic forms of heavy metals that are toxic [27]. Increasing DOC concentration reduces the toxicity of silver to fathead minnows, but chloride and calcium have only a minor effect on the 96-h total silver LC50 values. No clear relationship exists between toxicity and Ag$^+$ for fathead

Table 3. Whole-body Na$^+$ influx rates (μmol/kg/h) for fathead minnows and rainbow trout in control conditions (50 μm Cl$^-$, 500 μm Ca^{2+}, 250 μm Na$^+$) and with the addition of 8 μg Ag/L (as AgNO$_3$) at different water chloride concentrations

	Fathead minnows (μmol/kg/h)	Rainbow trout (μmol/kg/h)
Control	246.5 ± 19.7	360.7 ± 29.5
Chloride concentration		
50	195.9 ± 21.7	269.0 ± 14.7[a]
250	149.7 ± 14.5[a]	236.9 ± 32.6[a]
800	169.7 ± 24.8[a]	221.9 ± 21.9[a]

[a] Significantly different from control values for the appropriate species ($p < 0.05$, one-way analysis of variance followed by a least significant difference test). Each value represents the mean ± SEM for seven or eight fish.

Table 4. Gill silver load (µg/kg gill) and body silver uptake (nmol/kg/h) for fathead minnows and rainbow trout exposed to 8 µg Ag/L (as AgNO$_3$) labeled with ^{110m}Ag at different water chloride concentrations

Cl$^-$ concentration (µM)	Fathead minnows		Rainbow trout	
	Gills (µg/kg)	Body (nmol/kg/h)	Gills (µg/kg)	Body (nmol/kg/h)
50	329 ± 71	13.1 ± 1.5	521 ± 69	25.3 ± 2.1
250	235 ± 69	11.3 ± 3.0	562 ± 136	35.9 ± 9.0
800	164 ± 37	12.2 ± 2.1	192 ± 33[a]	22.8 ± 4.7

[a] Different from the other values in the same column ($p < 0.05$, one-way analysis of variance followed by a least significant difference test). All other values are not significantly different from each other. Each value represents the mean ± SEM for seven or eight fish.

minnows. This dichotomy cannot be explained by any obvious difference between the species in the silver-induced disturbance to whole-body Na$^+$ influx or accumulation of silver in the gills or body over a 4-h acute exposure to 8 µg Ag/L (as AgNO$_3$); trends in all were very similar in the two species (Tables 3 and 4). However, we noticed that fathead minnow mortalities generally occurred sooner than those of rainbow trout at the same silver concentration in these tests, which may indicate a difference in the mode of toxic action or pathway for silver entry.

Chloride

Chloride, when present in excess so that it forms AgCl$_n$ complexes, has been shown to significantly reduce silver toxicity to fathead minnows [6] and rainbow trout [8]. In the present study, the chloride levels were chosen so that no cerargyrite formed and that only the aqueous Ag$^+$, and the aqueous silver chloride species AgCl and AgCl$_2^-$, levels varied. Chloride strongly protected rainbow trout against silver toxicity when the latter was expressed as total silver. Similarly, Galvez and Wood [9] found that chloride increased the median lethal time values for rainbow trout at a fixed silver concentration of 100 µg Ag/L. Hogstrand and Wood [10] summarized additional data indicating that chloride protects against silver toxicity to rainbow trout. In all studies silver toxicity correlated to Ag$^+$. The strong relationship between Ag$^+$ and toxicity was also observed in physiological studies on silver-induced disturbances. Both Bury et al. [15] and McGeer and Wood [16] showed that perturbations to Na$^+$ balance in rainbow trout induced by silver exposure correlate to water [Ag$^+$].

The 96-h total silver LC50 values for fathead minnows did not vary over a chloride range of 50 to 1,500 µM. The result is similar to those of [17] and [18], which showed a very minor protective effect of chloride on silver toxicity to 4-d-old fathead minnows. In contrast, Erickson et al. [12] found that the addition of sodium chloride actually increased silver toxicity to 30-d-old fathead minnows. Nevertheless, the overall conclusion is that chloride does not protect fathead minnows against silver toxicity as it does in rainbow trout. Moreover, the 96-h Ag$^+$ LC50 values declined with increasing chloride concentrations. This suggests that other Ag complexes, such as AgCl^0 and AgCl$_2^-$, contribute to toxicity in fathead minnows. Indeed, Erickson et al. [12], based on their toxicity data in fathead minnows, proposed that AgCl^0 may contribute to toxicity because it may be more readily transported by this species across membranes to the site of action. Copper toxicity to fathead minnows has been found to be modified by altering

water chemistry [19–21,28]. The 96-h total copper LC50 values increase as water alkalinity and pH levels are changed. However, when these values are expressed as ionic copper, the 96-h LC50 values decrease, suggesting that species of copper other than Cu^{2+} are toxic to fathead minnows [17].

Assuming that the site for silver toxicity to freshwater fish is the gill [10,13,14], then the difference in the toxic response of rainbow trout and fathead minnows when water chloride concentrations are altered suggests that the gills of these two species respond differently to AgCl complexes. Physiological studies show that silver inhibits Na$^+$ influx in rainbow trout (Table 2; [13–16]), as well as in fathead minnows (Table 3). Chloride did not protect against perturbations to Na$^+$ balance induced by acute exposure to 8 µg/L of silver (AgNO$_3$) in either species. In the case of rainbow trout, this contrasts to the results from two previous studies [15,16], and is probably a consequence of the higher silver concentration (8 µg AgNO$_3$/L compared to 3.2 µg AgNO$_3$/L) and the shorter exposure times (4 h compared to 6 and 48 h) used in the present study. The reduction in gill silver levels in rainbow trout, the site of silver toxicity in this species [13–16], may explain why chloride protects against silver toxicity, but the same phenomenon occurred in fathead minnows without the accompanying protection. Silver toxicity to freshwater rainbow trout correlates to Ag$^+$ [9,10,13,15,16], but this scenario does not appear to be apparent for fathead minnows [12,17,18], because other silver complexes may be toxic. The physiological reasons for the disparity between the two species are not clear and require further investigation.

Calcium

Increasing water calcium concentrations from 50 to 2,000 µM had only a slight, nonsignificant, effect on 96-h total silver LC50 values for both rainbow trout and fathead minnows (Fig. 2). This small protective effect may be a consequence of the stabilizing effect that Ca^{2+} has on the permeability of the fish gill epithelium [29]. This will reduce the loss of ions via the paracellular pathway that occurs on exposure to silver [30]. These results corroborate those of Galvez and Wood [9] and also add to the debate raised by a number of authors [8–10,15,16] concerning the validity of the U.S. EPA hardness equation, designed to help authorities in the regulation of silver discharge. The reported data are in contrast with those of Erickson et al. [12] who reported a modest protective effect of hardness on silver toxicity. However, it is evident that for such an equation to be reliable, other parameters, such as chloride, DOC, sulfide, and thiosulfate, which influence silver toxicity to a greater extent, must be incorporated.

Dissolved organic carbon

On the basis of total silver, DOC strongly protects both rainbow trout and fathead minnows against silver toxicity. This finding is in close agreement with the data of Erickson et al. [12] on 30-d-old fathead minnows and Karen et al. [18] for 4-d-old fathead minnows. The protective effect of DOC has also been demonstrated for copper toxicity [19,20], as well as for a mixture of copper and cadmium [21]. Protection results from DOC forming silver complexes, which reduces the available metal concentration in the water. Indeed, DOC has been shown to reduce silver gill accumulation in rainbow trout [15,22]. However, the reduction in the 96-h ionic silver LC50 values for fathead minnows at a DOC concentration of 5.8 mg/L suggests that a proportion of these DOC–Ag complexes

may contribute to toxicity. A very similar pattern was observed by Erickson et al. [19] for the effects of DOC on copper toxicity to fathead minnows. They suggested that the DOC–copper complexes are not absorbed by the fish, but that the interaction of these complexes at the gill surface results in a fraction of the metal becoming available.

In conclusion, on the basis of total silver, DOC protects both rainbow trout and fathead minnows against silver toxicity, water chloride protects only rainbow trout, and water calcium has only a negligible effect. For rainbow trout, silver toxicity correlates to Ag^+, but this is not the case for fathead minnows, at least at different water chloride concentrations. The difference between the LC50 values of the two species over the water chloride range and at the highest DOC level tested suggests that rainbow trout and fathead minnows react differently to inorganic–Ag and organic–Ag complexes. These differences warrant further investigation.

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