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EVALUATION OF THE PROTECTIVE EFFECTS OF REACTIVE SULFIDE ON THE ACUTE TOXICITY OF SILVER TO RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract—Acute 96-h toxicity tests were performed with juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to $AgNO_3$ in either the absence or the presence of 100 nM reactive sulfide to evaluate the protective effect of aqueous sulfides against ionic Ag toxicity. The sulfide was presented in the form of zinc sulfide (ZnS) clusters under oxic conditions. Silver was lost from the water column during the course of the experiment, so mean measured Ag concentrations were used to generate all median lethal concentration (LC50) data. The system was complicated in that Ag_2S precipitated because of the need for large amounts of Ag to obtain lethal effects in the presence of ZnS. Some of the losses of Ag could be explained by complexation with ZnS and formation of solid Ag_2S. Other losses were probably the result of partial adsorption to exposure-chamber walls or to complexation with ligands or functional groups within organic material produced by the fish. The LC50 (95% confidence interval) values generated using measured concentrations for total Ag were 139 (122–162) nM in the absence of sulfide and 377 (340–455) nM in the presence of 100 nM sulfide. The LC50 values generated using measured concentrations from filtered (pore size, 0.45 μm) water samples were 122 (105–145) nM in the absence of sulfide and 225 (192–239) nM in the presence of 100 nM sulfide. These results suggest a stoichiometric protection of sulfides up to a 2:1 ratio of Ag:sulfide. Greater accumulation of Ag at the gills was measured in fish exposed to $AgNO_3$ in the presence of sulfide.

Keywords—Biotic ligand model *Oncorhynchus mykiss* Sulfide Silver toxicity Zinc sulfide clusters

INTRODUCTION

The bioavailability of metal ions within aquatic systems is influenced strongly by various complexing agents [1], including inorganic ligands (e.g., chloride, bicarbonate, thiosulfate), simple organic ligands (e.g., amino acids), and complex polydisperse organic ligands as might be found in humic and fulvic acids. Although acid-volatile sulfide is recognized as an important ameliorative agent in the toxicity of metals associated with anoxic sediments [2,3], its importance as a complexing agent in oxic surface waters has, until recently, been overlooked, largely because reduced sulfur is inherently unstable in the presence of molecular oxygen [4]. However, metastable sulfide has been reported in oxic water systems at concentrations from less than 1 nM up to several hundred nanomolar in rivers [5–7] and a few thousand nanomolar in waters emanating from sewage treatment plants [7,8] and nearly always exceed the environmental concentration of Ag at the same locations (for review, see [9,10]).

Predictive metal toxicity models, such as the biotic ligand model (BLM) [11], attempt to predict the bioavailability of metals in aquatic systems on the basis of metal speciation. For such a model to be effective, all the major ligands likely to affect metal ion bioavailability should be considered. Sulfide is one of the naturally occurring ligands that need to be incorporated into the Ag BLM. Sulfide has a very high binding affinity for Ag(I) (log K = 13.6 for $Ag^+ + HS^- \leftrightharpoons AgHS$) [12], and the formation of stable Ag–sulfide complexes within Ag-contaminated waters is likely to protect aquatic organisms from toxicity caused by Ag^+. Silver sulfide salts added to water are poorly soluble; even when kept in suspension at high concentration, they exert negligible toxicity [13].

To better simulate oxic sulfide–containing waters, we developed an exposure system that used synthetic zinc sulfide (ZnS) clusters [14]. In a previous study, we used this system to evaluate the ameliorative effect of relatively low levels of Cr(II)-reducible sulfide (CRS) on Ag^+ toxicity in *Daphnia magna* [15]. In the presence of environmentally realistic concentrations of reducible sulfide (~25 nM), acute toxicity of Ag, measured as the median lethal concentration (LC50), was substantially reduced. The reduction that occurred suggested a stoichiometric binding of Ag^+ by ZnS.

As a follow-up to the *Daphnia* studies, we used a similar approach to determine if sulfide was similarly protective against acute Ag^+ toxicity to rainbow trout (*Oncorhynchus mykiss*). Data for both fish and invertebrates are required before sulfide can be incorporated into predictive models, such as the BLM. Because the incipient LC50 value for rainbow trout exposed to Ag^+ is more than 10-fold greater than that for *D. magna* (compare Hogstrand et al. [16] with Bianchini et al. [15]), elevated yet environmentally realistic concentrations of sulfide were employed. A solution containing 100 nM sulfide (as ZnS clusters) was used for the tests described here. To determine a 96-h LC50, we exposed the fish to Ag concentrations ranging between 250 and 800 nM.

The large Ag concentration (compared to the *Daphnia* tests [15]) required to cause mortality in trout changes the chemistry of the system. Here, the system is saturated with respect to $Ag_2S_{(solid)}$ for the concentration ranges of Ag used in the this study. In addition, for the total sulfide and Ag concentrations, AgHS will be the predominant Ag species in solution as long as enough $S(II^-)$ is available to complex the Ag [12]. For the

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other ion concentrations, AgCl predominates after S(II$^-$) sites are saturated. By contrast, $ZnS_{(solid)}$ is not saturated. These conditions can be expressed as follows: First,

$$ZnS_{(solid)} \leftrightharpoons ZnS_{(aqueous)} \quad (1)$$

with stability constants of $10^{-5.6}$ and $10^{-3.4}$ for α and β forms of $ZnS_{(solid)}$, respectively. Given a ZnS concentration of 10^{-7} M, $ZnS_{(solid)}$ should not form. Second,

$$2AgHS \leftrightharpoons Ag_2S_{(solid)} + HS^- + H^+ \quad (2)$$

with a stability constant of

$$K = \frac{[Ag_2S_{(solid)}][HS^-][H^+]}{[AgHS]^2} = 10^8 \quad (3)$$

Rearranging Equation 3 and substituting in $[HS^-] = 10^{-7}$, $[H^+] = 10^{-8}$, and $[Ag_2S_{(solid)}] = 1$, you find that $[AgHS] = 10^{-11.5}$ M and that $[Ag_{(total)}] = 1.5 \cdot [AgHS] = 10^{-11.3}$ M $\cong$ 0.005 nM for saturation with respect to $Ag_2S_{(solid)}$. Thus, all Ag test solutions are saturated with respect to $Ag_2S_{(solid)}$. That is, the maximum concentration of AgHS possible in this system is exceeded, and precipitation of Ag_2S occurs. The same conclusion can be obtained using ZnS rather than HS^- as the source for the sulfide because of the reaction

$$ZnS + H^+ \leftrightharpoons Zn^{2+} + HS^- \quad (4)$$

For AgCl, the speciation is

$$[Ag_{(total)}] = [Ag^+] + [AgHS] + [Ag_2S] + [AgCl] \quad (5)$$

As noted above, all the sulfidic species are consumed to form Ag_2S. Thus, Equation 5 simplifies to

$$[Ag_{(total)}] = 200 \times 10^{-9} + [Ag^+] + [AgCl] \quad (6)$$

where the 200 nM reflects the consumption of 100 nM of HS^- to form $Ag_2S_{(solid)}$. Note also that $[AgCl]/[Ag^+] = K[Cl^-] = 10^{3.08} \cdot 10^{-3.11} \sim 10^0 \sim 1$. Thus, Equation 6 becomes

$$[Ag_{(total)}] = 200 \times 10^{-9} + 2[Ag^+] \quad (7)$$

For $AgCl_{(solid)}$ formation, one can write

$$AgCl_{(solid)} \leftrightharpoons Ag^+ + Cl^- \quad (8)$$

with a solubility product of

$$K = [Ag^+][Cl^-] = 10^{-9.42} \quad (9)$$

Substituting into Equation 9 $[Cl^-] \sim 10^{-3.1}$, $AgCl_{(solid)}$ saturation leads to $[Ag^+] = 10^{-6.32} \sim 490$ nM. For saturation with respect to $AgCl_{(solid)}$, the equation for $Ag_{(total)}$ becomes

$$[Ag_{(total)}] = [Ag_2S_{(solid)}] + [Ag^+] + [AgCl]$$
$$= 200 + 490 + 490 = 1{,}160 \text{ nM Ag} \quad (10)$$

None of the treatment concentrations of Ag were as great as this concentration. Thus, we conclude that all treatments were saturated with respect to $Ag_2S_{(solid)}$ and that no treatment was saturated with respect to $AgCl_{(solid)}$. Furthermore, all treatments consumed the sulfide to form solid Ag_2S, and the Ag remaining in solution partitioned equally between Ag^+ and AgCl.

MATERIALS AND METHODS

Preparation of ZnS clusters

Zinc sulfide clusters were formulated in deoxygenated, dechlorinated Hamilton (ON, Canada) tap water. The composition of Hamilton tap water was as follows: $[Na^+] = 0.6$ mM, $[Cl^-] = 0.7$ mM, $[Ca^{2+}] = 1.0$ mM, $[HCO_3^-] = 1.9$ mM, $[Mg^{2+}] = 0.2$ mM, $[SO_4^{2-}] = 0.25$ mM, $[K^+] = 0.05$ mM, $[NH_3] < 0.005$ mM, pH 7.8–8.0, dissolved organic carbon = 3 mg/L, hardness = 140 mg/L (as $CaCO_3$). The cations were measured by atomic absorption spectroscopy, and the anions were measured by coulometric titration or high-performance liquid chromatography. Hardness was measured by titration. Dechlorination (<0.01 mg/L) was achieved by filtration through a bed of activated carbon. The water was deoxygenated by bubbling with argon and maintained thereafter under an argon blanket.

The preparation of ZnS clusters is described by Bowles et al. [14]. Briefly, Zn(II) nitrate (10 μM) was added to sodium sulfide (10 μM) in 40 L in a 50-L polyethylene bottle (Nalgene, Rochester, NY, USA). The anoxic solution was stirred for 4 h before expelling the argon blanket and allowing the solution to equilibrate by passive diffusion with the laboratory atmosphere. The solution was aged for 6.5 d to ensure the absence of unreacted sulfide and to allow complete cluster development [14]. Before use, the concentration of reactive sulfide within the solution was quantified by the spectrophotometric measurement of methylene blue sulfide (MBS; see below). This allowed us to calculate appropriate dilution factors for the subsequent toxicity experiment. Typical values of [S(II)$^-$] ranged between 3 and 5 μM; this represented 30 to 50% yields of ZnS.

Determination of reactive sulfide

The CRS was determined using the method described by Bowles et al. [17]. Duplicate 30-ml aliquots were taken from each tank at each sampling time during the toxicity experiment. Acidic $CrCl_2$ (5 ml of 6 M HCl, 5 ml of 1 M $CrCl_2$ in 0.5 M HCl) was added to each sample, and the samples were purged for 30 min into a trapping solution (15 ml of 0.05 M NaOH). Sulfide in the trapping solutions was determined by use of a modified MBS method [18]. The trapping solutions were acidified with 500 μl of MBS reagent (*N*,*N*-dimethyl-*p*-phenylenediamine oxalate; 8 mM) and $FeCl_3$ (8 mM) in 50% HCl (vol/vol). Sulfide was then determined by comparing the colorimetric response of MBS (670 nm) to a calibration curve of known MBS standards.

Determination of total organic carbon

Total organic carbon (TOC) was determined using a Dohrmann DC-180 ultraviolet-persulfate TOC analyzer (Tekmar-Dohrmann, Mason, OH, USA) with an infrared carbon dioxide detector. One 10-ml aliquot was taken from each tank at the beginning of each day just after water renewal and again at the end of the day before the next renewal. The samples were acidified (2% HNO_3) to remove inorganic carbon and purged with He to remove carbon dioxide. Triplicate 0.5-ml samples were injected into the analyzer.

Determination of Ag concentrations

The Ag concentration in each water sample was determined by radiometric measurement using spiked ^{110m}Ag (RISØE, Roskilde, Denmark). This method was used because many water-Ag determinations of high accuracy were required throughout the duration of the toxicity tests and because analytical difficulties are encountered using graphite furnace analysis of Ag in the presence of sulfide clusters [19]. Enough $^{110m}AgNO_3$ was added to 100 mM $AgNO_3$ solutions to provide working stock solutions with specific activities ranging between 0.4 and 1.0 μCi/μg Ag. The specific activity of the working stock solution was verified by measurement of ^{110m}Ag radioactivity in a gam-

ma counter (MINAXI Auto-gamma 5000 series; Canberra-Packard, Meridan, CT, USA) and measurement of the total Ag concentration by graphite furnace atomic absorption spectroscopy (Varian AA-1275 with GTA-9 atomizer; Palo Alto, CA, USA) using operating conditions recommended by the manufacturer and a multielement standard (Inorganic Ventures, Lakewood, NJ, USA). The working stock solution was used to prepare experimental exposure solutions (see below). Quadruplicate samples were used for Ag determination in a gamma counter with precautions on energy-window selection [20]. Duplicate, unfiltered water samples were used to estimate total Ag concentration ($[Ag_{total}]$). To distinguish between solid-phase and dissolved-phase Ag, a second duplicate set of water samples was filtered ($[Ag_{filtrate}]$) through acrodisc 0.45-μm polyethersulfone in-line filters (Gelman, Ann Arbor, MI, USA) with polypropylene syringes.

Acute toxicity tests

Juvenile rainbow trout (weight, <1.0 g) were obtained from Humber Springs Hatchery (Orangeville, ON, Canada) and maintained at 14 ± 1°C within a 240-L tank supplied with dechlorinated Hamilton tap water at a rate of 2.5 L/min. The water in the tank was vigorously aerated with air from a compressed air supply in our animal care facility. The fish were acclimated for at least two weeks before use. The trout were fed commercial trout pellets (Silver Cup for Salmon Fry, Nelson & Sons, Murray, UT, USA) during acclimation. The fish were not fed during the 24-h period before each test to reduce the production of fecal waste during the test.

Acute, static-renewal, 96-h toxicity tests were performed in acid-washed, 80-L polyethylene tubs (Newell Rubbermaid, Wooster, OH, USA). Each tub contained 40 L of test solution at a temperature of 14 ± 1°C. Previous experience with sulfide–Ag systems highlighted the need to maintain large ratios of water volume to wall surface during static-renewal tests to avoid substantial losses of sulfide and Ag to the walls of the exposure chambers [15,19]. We also kept biomass loading low by using small fish (weight, <1 g) to reduce losses of sulfide and Ag to the surface of the fish and to organic matter produced by the fish [21]. Furthermore, we replaced 95% of the water at each renewal to reduce the accumulation of organic matter over the 96-h test. Control solutions that contained no Ag were used in each test. The fish were not fed during the course of the tests. Each test chamber was aerated with an air-stone during the tests. Water samples were taken at regular intervals for the analysis of Ag, sulfide, or TOC. All water samples were taken from approximately 2 cm below the water surface, near the center of the tank, after a 15-min settling period during which aeration was stopped. All test chambers were monitored hourly for mortality (except for 8 h during the night). Mortality was indicated by an absence of opercular movement. Dead fish were removed, and those fish that had died less than 1 hour before being removed were rinsed in deionized water and then dissected to isolate gills, liver, and carcass for Ag analyses. Two tests were performed. The first test was performed without the addition of sulfide, and the second included sulfide (nominally 100 nM).

Nonsulfide test

Ten animals were allocated impartially to each of seven exposure tanks; the average biomass loading was 30.8 mg/L. Seven nominal concentrations of Ag were used: 0 (control), 65, 93, 130, 185, 260, and 371 nM as $AgNO_3$. At the start of each test and after each 24-h interval, pre- and postrenewal water samples were analyzed for Ag.

Sulfide test

Zinc sulfide solutions were prepared in Hamilton tap water 3.5 h before the start of the test and 3.5 h before each 24-h renewal by adding the appropriate volume of the ZnS stock solution to tap water followed by thorough mixing. Test solutions were individually prepared in each test chamber by introducing 40 L of sulfide solution and an appropriate volume of spiked $AgNO_3$. The test solutions were allowed to equilibrate for 3 h with aeration before the trout were added [15,19]. The maximum possible release of Zn^{2+} from the clusters was calculated to be well below the lowest toxicity thresholds for Zn^{2+} to trout [22,23]. Ten animals were allocated impartially to each of seven exposure tanks; the biomass loading was 21.8 mg/L. Seven nominal concentrations of Ag were used: 0 (control), 250, 316, 400, 500, 630, and 790 nM as $AgNO_3$. At the start of the test and after each 24-h interval, pre- and postrenewal water samples were taken for analysis of Ag, sulfide, and TOC. At 8-h intervals, additional water samples were taken for Ag and sulfide analysis.

Calculations and data analysis

The Spearman-Karber statistic [24] and Probit analysis [25] were used to generate estimates of the LC50. The regressions were generated using mortality data over the 96-h duration of each test and the average concentration for each exposure tank during each test. For the test without sulfide, this included eight measured concentrations (pre- and postrenewal) for each exposure tank, and for the test with 100 nM sulfide, this included 16 measured concentrations (every 8 h, including pre- and postrenewal) for each exposure tank. Probit regressions were used to generate mortality curves with 95% confidence interval (95% CI) between the 1% lethal concentration and the 99% lethal concentration values for each test. An analysis of variance with Tukey's post-hoc test was used to determine if significant ($\alpha = 0.05$) differences existed among TOC concentrations over subsequent days. Student's *t* tests for unpaired data were used to test for significant differences ($\alpha = 0.05$) in the concentrations of accumulated Ag in gills, liver, and carcass between tests in the presence and absence of sulfide. The data for gill-Ag accumulation and carcass-Ag accumulation were transformed (log) to obtain normally distributed data for the Student's *t* tests.

RESULTS

Sulfide, Ag, and TOC concentrations

In the test without sulfide, Ag losses (± standard error [SE]) during each 24-h period averaged 9.4 ± 1.6% in unfiltered samples and 23.0 ± 2.3% in filtered samples. In the test with 100 nM sulfide, substantial losses of both CRS and Ag occurred over each 24-h period (Fig. 1). The average daily loss (± SE) of sulfide in each 24-h period was 53.6 ± 2.1%. The average daily losses (± SE) of Ag in unfiltered and filtered samples were 46.7 ± 2.2% and 58.6 ± 3.6% respectively. On a molar basis, the losses of Ag from the water column were greater than the losses of sulfide. The average daily loss of sulfide (± SE) was 54.7 ± 4.8 nM. The average daily loss (± SE) of Ag ranged from 146 ± 18 nM in the 250 nM tank to 349 ± 38 nM in the 790 nM tank in unfiltered samples and from 126 ± 5 nM in the 250 nM tank to 268 ± 36 nM in the 790 nM tank. For all exposure concentrations, total Ag at the

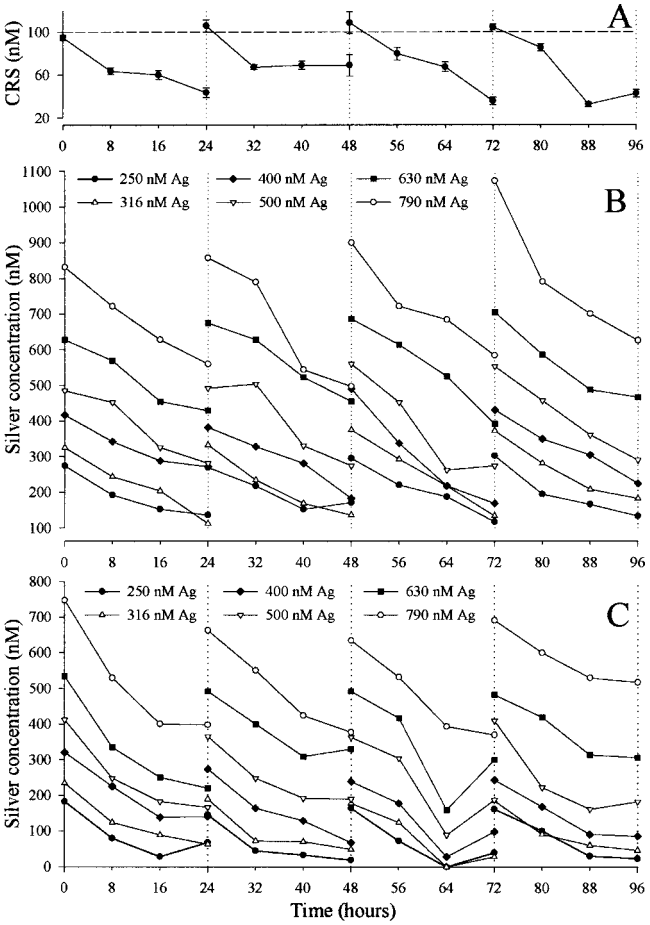

Fig. 1. Results of water sampling during the sulfide-added toxicity test. (**A**) Total chromium reducible sulfide (CRS) over 96 h. The results for all exposure tanks were pooled to provide mean concentrations (control tank excluded). Error bars represent standard error. (**B**) Total silver concentration within water sampled 2 cm below the water surface in the middle of each tank. Individual data sets are labeled according to the predicted nominal concentration of Ag. (**C**) Silver concentration in water samples after filtering through 0.45-μm polyethersulfone filters to remove solid-phase silver. Individual data sets are labeled according to the predicted nominal concentration of Ag (unfiltered). All data points for silver concentration represent means of duplicate samples.

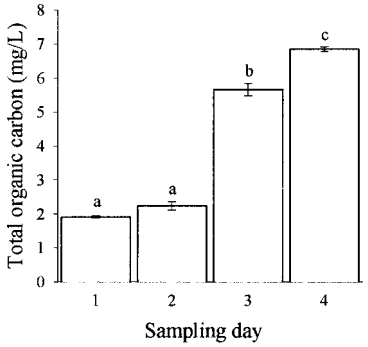

Fig. 2. Average concentrations of total organic matter in exposure tanks (control tank excluded) at the beginning of the test (day 1) with 100 nM sulfide and after each renewal in exposure tanks. Error bars represent standard error. Different lowercase letters above bars indicate statistical differences between days ($p < 0.05$, analysis of variance).

beginning of each renewal increased with each successive day, indicating an accumulation of Ag with successive renewals (Fig. 1B). Filtration of the water samples abolished this trend (Fig. 1C).

The postrenewal TOC measurements indicated an increase in TOC over successive days (Fig. 2). Prerenewal measurements suggested less TOC at the end of each 24-h period than at the beginning, likely reflecting a settling-out of organic matter. Organic matter was visible in all exposure tanks as long threads of mucus. Some of these threads, especially at the higher concentrations (630 and 790 nM tanks) were black, suggesting the presence of Ag_2S.

Acute toxicity

Control survival was 100% for each test. Using measured [Ag_{total}] data, the LC50 value in the test without sulfide was 139 nM using Probit analysis. Filtering water samples in the test without sulfide had only a minor effect on the magnitude of the resultant LC50 value (Table 1 and Fig. 3). Using measured [$Ag_{filtrate}$] data, the LC50 value was 122 nM, representing a 12.5% reduction in the LC50, which resulted in only a minor shift to the left for the mortality curve (Fig. 3). Similar results were obtained using Spearman-Karber regression. Table 1 and Figure 3 also provide numerical toxicity data (expressed as μg/L).

Table 1. Nominal and measured Ag concentrations and median lethal concentration (LC50) values for 96-h acute toxicity tests with and without 100 nM sulfide[a]

Test	Zinc sulfide cluster concn.	Nominal [Ag] (nM)	Mean measured [Ag_{total} ± SE] (nM)	Mean measured [$Ag_{filtrate}$ ± SE] (nM)	LC50 (95% CI)	
					Unfiltered samples	Filtered samples
1	No sulfide	65	63 ± 2.8	51 ± 3.7	Spearman-Karber	Spearman-Karber
		93	96 ± 3.1	80 ± 4.6	136 (120–155) nM	117 (101–135) nM
		130	126 ± 5.3	103 ± 7.2	14.7 (12.9–16.8) μg/L	12.6 (10.9–14.5) μg/L
		185	177 ± 4.6	162 ± 10.3	Probit	Probit
		260	260 ± 8.0	220 ± 11.3	139 (122–163) nM	122 (105–145) nM
		371	368 ± 6.9	291 ± 31.0	15.0 (13.2–17.5) μg/L	13.1 (11.3–15.7) μg/L
2	100 nM	250	199 ± 14.8	83 ± 20.8	Spearman-Karber	Spearman-Karber
		316	239 ± 21.0	100 ± 23.4	397 (343–460) nM	231 (186–286) nM
		400	313 ± 22.5	162 ± 28.6	42.8 (37.0–49.6) μg/L	24.9 (20.1–30.9) μg/L
		500	397 ± 26.8	245 ± 34.4	Probit	Probit
		630	551 ± 24.7	360 ± 38.0	377 (340–455) nM	225 (192–293) nM
		790	719 ± 37.8	522 ± 42.7	40.7 (36.7–49.1) μg/L	24.3 (20.7–31.6) μg/L

[a] CI = confidence interval; SE = standard error.

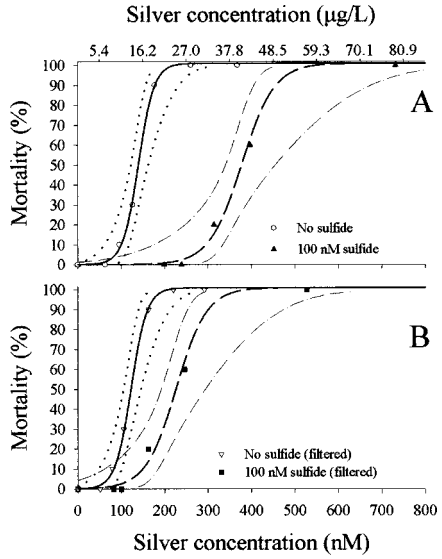

Fig. 3. Mortality curves (with 95% confidence intervals) for juvenile rainbow trout exposed to waterborne $AgNO_3$ in either the absence (open circles and triangles, solid lines) or presence (closed triangles and squares, dashed lines) of 100 nM sulfide. (**A**) Plots represent the total silver concentrations (i.e., [Ag_{total}]). (**B**) Plots represent the concentration of silver in samples after filtering through 0.45-μm polyethersulfone filters (i.e., [$Ag_{filtrate}$]). All plots were generated using Probit regression (SPSS, Chicago, IL, USA).

Addition of 100 nM ZnS had a pronounced effect on the LC50 and established a wide discrepancy between LC50 values generated using Ag concentrations measured in filtered or unfiltered water samples (Table 1 and Fig. 3). Using measured [Ag_{total}] data, the LC50 value (using Probit analysis) in the presence of 100 nM sulfide was 377 nM. This was 2.7-fold higher (i.e., less toxic) than that estimated in the absence of sulfide. Using measured [$Ag_{filtrate}$] data, the LC50 value was 225 nM. Therefore, filtration of the samples resulted in an apparent 40% reduction in the LC50 value, as indicated by the large shift to the left for the mortality curve of dissolved Ag in the presence of sulfide (Fig. 3B) relative to the mortality curve of total Ag in the presence of sulfide (Fig. 3A). These data indicate that at the LC50 concentration, 152 nmol Ag/L were unable to pass through the filter. However, the LC50 for filtered samples was still 1.7-fold greater than that estimated in the absence of sulfide, indicating the presence of nonbioreactive Ag within the filtrates. Spearman-Karber analysis provided a similar outcome (Table 1).

Silver accumulation

At the time of death, fish exposed to $AgNO_3$ in the presence of 100 nM ZnS accumulated much greater gill-Ag burdens than those fish exposed to $AgNO_3$ in the absence of 100 nM sulfide (Fig. 4). The gills of fish exposed to $AgNO_3$ in the absence of sulfide accumulated 148 nmol Ag/g. In contrast, the gills of fish exposed to 100 nM sulfide accumulated an average of 1,219 nmol Ag/g, or 8.2-fold that of the fish exposed in the absence of sulfide. Neither the liver nor the carcass accumulated substantial quantities of Ag in either treatment, although the carcass of fish exposed to 100 nM sulfide also accumulated 8.8-fold the amount of Ag compared to those fish exposed in the absence of sulfide (Fig. 4).

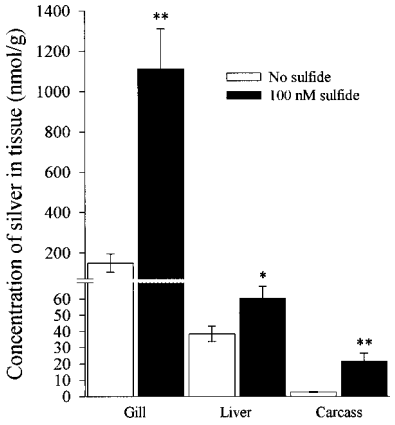

Fig. 4. Concentration of silver bound to fish tissues at the time of death. Tissue included in the no-sulfide data were from fish exposed to either 260 and 371 nM Ag as $AgNO_3$ ($n = 20$) for up to 96 h, and tissues included in the 100 nM sulfide data were from fish exposed to between 400 and 800 nM Ag as $AgNO_3$ ($n = 27$) for up to 96 h. Error bars represent standard error. Note that the *y*-axis is split into two ranges. Statistical differences were detected between treatments at $p < 0.01$ (*) and $p < 0.001$ (**) in unpaired Student's *t* tests.

DISCUSSION

Until now, the importance of sulfide as an ameliorative ligand in Ag^+ toxicity to fish has, to our knowledge, been untested. The present study demonstrates that environmentally realistic concentrations of waterborne reactive sulfide (in the form of ZnS clusters) offer substantial protection in juvenile rainbow trout from the acute toxicity associated with ionic Ag^+. When 100 nM ZnS clusters were present, LC50 values as much as 2.7-fold greater than incipient LC50 values in the absence of sulfide were observed. The protective effect of sulfide likely is a general phenomenon that is relevant to a wide range of species.

The acute tests in the absence of sulfide were performed under conditions similar to those of acute toxicity tests previously carried out within our laboratory [16]. Although the 96-h LC50 value reported here (and expressed as total measured Ag) is slightly higher than that reported by Hogstrand et al. [16], a generous overlap exists between the respective 95% CIs. This result confirms the high toxicity of Ag^+ when presented as $AgNO_3$ in noncomplex aquatic media (for review, see [26,27]). Water chemistry influences Ag toxicity, and several anionic ligands, including chloride, dissolved organic carbon, and thiosulfate, can ameliorate the acute toxicity of Ag to rainbow trout by competing with the gill for Ag binding [13,16,28–31]. The results presented here demonstrate that reactive sulfide should be added to this list and may be the most important protective ligand in field situations, because it is effective at such low concentrations.

During the test with 100 nM ZnS clusters, substantial losses of both sulfide and Ag occurred. Much of these losses were an anticipated consequence of the use of ZnS clusters. In similar tests with *D. magna*, Bowles et al. [19] reported 24 to 32% losses of sulfide in preparations using approximately 250 nM sulfide and losses of greater than 50% in preparations using approximately 25 nM sulfide. The losses in this study are similarly attributed to adsorption to the walls of the exposure tanks as well as to formation of particulate Ag_2S or insoluble Ag_2S/Zn^{2+} species [32]. Bowles et al. [19] also reported losses of Ag of as much as 68%, which parallel the losses of Ag reported in the present study. Such losses highlight the ne-

cessity of regular monitoring and the use of measured rather than nominal Ag concentration data when using ZnS clusters. The use of a radio-isotopic measurement technique greatly facilitates this approach.

Interpreting the changes in total [Ag(I)] and filtered [Ag(I)] throughout the ZnS treatments is difficult, because the kinetics of Ag complexation with sulfide species is largely unknown. The addition of Ag^+ to ZnS clusters results in an immediate reaction of Ag^+ with S atoms on the surface of the clusters, which in turn results in the formation of {Zn-S-Ag-S-Zn} clusters. Although these larger clusters are soluble, they may not be represented in the filtrates of the initial samples, because they retain at least some of the chemisorptive properties of ZnS clusters and may adsorb to the filters [14]. With increased time, Ag eventually displaces Zn from the clusters to form insoluble Ag_2S or variably soluble and colloidal Ag_2S/Zn^{2+} species. How many of these species will be excluded from the filtrates on a size basis is unknown, as is to what extent their chemisorptive properties have changed. It is clear from the present investigation, however, that substantial amounts of nonbioreactive Ag pass through the filters.

The mortality curves presented in Figure 3 illustrate (at least partially) the complexities noted above. The use of measured [$Ag_{filtrate}$] data for the regression has the effect of shifting the LC50 (mortality curve) to the left. In the test without sulfide, this shift is minor (12.5%) and reflects the scarcity of competing ligands within the system. The conventional view is that the Ag remaining within the filtrate represents the toxic fraction, because Ag bound to the ZnS clusters or organic matter (see below) is excluded either on the basis of particle size or as a consequence of chemisorption to the filter membranes [14,19]. Bianchini et al. [15] made this assessment when interpreting *Daphnia* toxicity data in the presence of approximately 35 nM sulfide, because the acute toxicity values generated using measured [$Ag_{filtrate}$] data produced similar LC50 values irrespective of the presence of sulfide. The interpretation that only Ag^+ will pass through a 0.45-μm filter is not tenable considering the data in the present study. Although substantially left-shifted, the LC50 value generated using [$Ag_{filtrate}$] data for the 100 nM sulfide test is still approximately 100 nM higher than the mortality curve for the test without sulfide, suggesting that approximately 100 nM nontoxic Ag must still be within the filtrates. In the absence of Ag^+, ZnS clusters are expected to chemisorb 100% to filter membranes [14]. However, as indicated above, some of the Ag–sulfide/Ag–ZnS moieties that form with addition of Ag^+ appear to be less prone to chemisorption [32] and may pass through the filters. Additionally, the nontoxic fraction of the filtrates could include filterable $AgCl_n$ species, some of which appear to be nontoxic in acute tests [16,28]. Furthermore, Ag adsorbed to colloidal organic particles less than 0.4 μm in size likely are able to pass through a 0.45-μm filter [33]. The origin of organic matter within the system was the fish.

The presence of fish within the system undoubtedly affected the profile of water-Ag concentrations. In preliminary trials, it became clear that the presence of fish accelerated the rate of loss of sulfide and Ag from solution. However, the losses of Ag to gills, liver, and carcass are negligible compared to the total losses. For example, fish exposed to 500 nM Ag (average exposure time before sampling, 82 h) accumulated a total of 402 nmol of Ag, which represented approximately 1% of the Ag that was lost. In all other exposure concentrations, the proportion of Ag accumulated by the fish was even less. Adsorption to, or absorption by, the fish therefore cannot explain the excessive losses of Ag. A more plausible explanation is that the lost Ag adsorbed to insoluble organic matter or complexed with dissolved organic matter produced by the fish that subsequently precipitated out of solution. Silver also has a high affinity for reactive sites in organic matter (log K ~9.0–11.3) [8,34]. This interpretation is consistent with the visible and measurable accumulation of organic matter (i.e., TOC) within the exposure tanks and with the occurrence of blackened strings of mucus within tanks with high concentrations of Ag.

An explanation for the high levels of organic matter in the tests in the presence of sulfide can be found in the fact that fish exposed to Ag in the presence of sulfide accumulated much higher gill-Ag burdens than fish exposed to Ag in the absence of sulfide. The Ag was probably in a nonacutely toxic form, as indicated by the elevated LC50 values, but likely caused respiratory stress in the fish, resulting in a much greater production of organic matter in the form of mucus [35,36].

The substantial accumulations of Ag at the gill are intriguing. In their studies with *Daphnia*, Bianchini et al. [15] also noted whole-body accumulations of Ag that were 10-fold greater in animals exposed to Ag in the presence of sulfide compared to those animals exposed to Ag in the absence of sulfide. Simple surface binding to the exoskeleton of the animals was eliminated as an explanation in those studies. In the present study, surface binding may have been the primary contribution to the carcass accumulations, considering that the differences between accumulations of Ag in the liver were relatively small. However, the high gill accumulations are conspicuous, and they call into question the use of body or tissue burdens as an indicator of biological risk [37] for Ag.

CONCLUSION

Predictive models, such as the BLM [11,38], should become more reliable by incorporating sulfide as a competitive ligand, because sulfide exerts a substantial ameliorative effect on acute Ag toxicity in rainbow trout. The high gill-Ag burden reported in the present study may require scrutiny in further studies. Predictive models, such as the BLM, rely on the quantification of gill burden as an indicator of toxicity and may be challenged by the data presented here. Also, although sulfide has been shown to have an ameliorative effect on acute toxicity in both trout and *Daphnia* [15], the high gill-Ag burdens (in trout) and high body-Ag burdens (in *Daphnia*) may be associated with respiratory or other stresses. If so, they may have detrimental effects in longer-term chronic bioassays. Clearly, the next step is to evaluate whether reactive waterborne sulfide offers long-term protection against chronic Ag toxicity to aquatic organisms.

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