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Environmental Toxicology and Chemistry, Vol. 21, No. 6, pp. 1294–1300, 2002
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Printed in the USA
0730-7268/02 $9.00 + .00

EVALUATION OF THE EFFECT OF REACTIVE SULFIDE ON THE ACUTE TOXICITY OF SILVER (I) TO *DAPHNIA MAGNA*. PART 2: TOXICITY RESULTS

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(*Received* 1 *May* 2001; *Accepted* 2 *January* 2002)

Abstract—The protective effect of reactive sulfide against $AgNO_3$ toxicity to *Daphnia magna* neonates was studied. Acute (48-h) toxicity tests were performed in the absence (<5 nM) and presence of low (~25 nM) and high (~250 nM) concentrations of zinc sulfide clusters under oxic conditions. In both the presence and the absence of sulfide, lower mean lethal concentration (LC50) values were observed when measured as opposed to nominal silver concentrations were used in calculations. This reflected the fact that measured total silver concentrations were lower than nominal concentrations due to losses of silver from solution observed during the experiment. High concentration (~250 nM) of sulfide completely protected against toxicity up to the highest silver concentration tested (2 μg/L [19 nM]) with measured silver data. In the presence of environmentally realistic levels of sulfide (~25 nM) in receiving waters, acute silver toxicity was reduced by about 5.5-fold. However, when filtered (0.45 μm) silver concentrations alone were considered, toxicity (48-h LC50) was similar in the absence (0.22 μg/L) and presence (0.28 μg/L) of sulfide. The difference between measured total and filtered silver was attributed to chemisorption of the metal sulfide onto the membrane filter and provides evidence that the toxic fraction of silver is that which is unbound to sulfide. Accumulation of silver was greater in daphnids exposed to silver in the presence of sulfide than in its absence, even though a toxic effect was not observed under these conditions. In this case, silver appears to be incorporated by daphnids rather than merely adsorbed on the surface. Our results point out the need to incorporate sulfide into the acute biotic ligand model and to assess its potentially large role in preventing chronic toxicity.

Keywords—Biotic ligand model *Daphnia magna* Waterborne silver Acute toxicity Sulfide

INTRODUCTION

A very significant recent finding in aquatic geochemistry is that metastable sulfide complexes appear to occur commonly in oxic surface freshwaters at picomolar to nanomolar concentrations [1–5]. This is of great environmental significance since sulfide has a very high binding affinity for metal cations (log K for Ag[I] = 13.6 [6]) and thus represents a hitherto uninvestigated and environmentally important ligand in oxic surface waters. The observed sulfide concentrations are high enough to remove silver from other ligands. They are also high enough to potentially exert a detoxifying effect on silver, which is generally present in these media at concentrations lower than the sulfide [3,5]. Previous studies indicate that chemical ligands have a protective effect on the toxicity of silver to aquatic organisms [7–9], but sulfide as a ligand has not been tested directly.

In light of this, we performed two sets of acute toxicity tests in order to assess the possible protective effect of reactive sulfides against $AgNO_3$ toxicity in *Daphnia magna* neonates. This freshwater crustacean was employed as test species because the most sensitive freshwater organisms appear to be cladocerans and amphipods when silver is added as $AgNO_3$ [10]. Furthermore, one critical area for improvement of the current version [11] of the biotic ligand model for silver is the need for more invertebrate data. At present, the biotic ligand model [11] is calibrated using toxicity data for *Daphnia*, but the actual gill binding constants and most of the water chemistry interaction data come from previous work on fish.

The two acute toxicity tests with zinc sulfide clusters and $AgNO_3$ include one at approximately 250 nM sulfide, representative of waters impacted by sewage treatment plants, and another at approximately 25 nM sulfide, representative of comparatively clean natural waters. Some aspects of silver accumulation in *D. magna* whole body at approximately 25 nM were also considered. We employed zinc sulfide cluster as a suitable reactive sulfide source because of its easy synthesis and stability in pure water at low micromolar concentrations [12,13]. For details regarding the chemical properties of this compound as well as the chemical parameters controlling the toxicity tests reported in the present study, refer to the companion paper describing the chemical system during the toxicity tests [14].

MATERIALS AND METHODS

D. magna *maintenance*

Several colonies of adult gravid *D. magna* were obtained from Aquatic Research Organisms (ARO strain, lot 090600 DM, Hampton, NH, USA). According to the ARO data sheet, the brood origination was U.S. Environmental Protection Agency (Cincinnati, OH, USA) and the daphnids had been

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reared in a freshwater static renewal system with water saturated in dissolved oxygen, pH 7.5, hardness approximately 150 mg $CaCO_3$/L, and 25°C. *D. magna* were fed phytoplankton and a slurry of yeast, cerophyll, and trout chow. On arrival at our laboratory, the *D. magna* colonies were gradually acclimated to synthetic water in an incubator. To ensure standardized conditions for water chemistry, synthetic water used for all tests was prepared as a single batch employing 1,000 L of reverse osmosis–purified water in a food-grade polyethylene tank. This water was designed to resemble Lake Ontario (Canada) water and was slightly lower in hardness (115 vs 150 mg $CaCO_3$/L) and higher in pH (8.2 vs 7.5) than the culture water used by the supplier. The synthetic water was reconstituted to the following composition: 1.0 mM $CaCO_3$, 0.15 mM $MgSO_4$, and 0.6 mM NaCl. It was bubbled with pure CO_2 for 24 h to ensure that $CaCO_3$ went into solution and then was bubbled with air for 48 h to ensure removal of excess CO_2 and atmospheric equilibration. Water was then left to stand for at least two weeks prior to use. During the acclimation period, *D. magna* were fed a slurry of yeast, cerophyll, and trout chow, and water was not aerated but renewed daily. Temperature was maintained at 22 to 23°C for the first 24 h of the acclimation period and was then fixed at 20°C. Light regime was fixed at a 16:8-h light:dark photoperiod.

After *D. magna* acclimation, reproduction rate was measured to ensure it met established criteria for a healthy population (15–20 neonates per adult every 3–4 d). Provided that the reproductive rate was satisfactory, we proceeded with the particular colony. To collect neonates, adults were confined in a fine-screen mesh net suspended in a 1-L glass aquarium containing synthetic water at 20°C. During this time, feeding was withheld. Neonates passed through the mesh and into the aquarium of synthetic water and were collected within 6 to 24 h of hatch for toxicological studies.

Acute toxicity tests

Acute (48-h) toxicity tests were performed in borosilicate glass beakers containing 250 ml of synthetic water, pre-equilibrated to 20°C. This high volume-to-daphnia ratio was employed to minimize accumulation of dissolved organic carbon during the tests and to maximize the volume-to-wall surface ratio. All glassware used was new and was acid washed in 1% HNO_3 (trace metal grade, Merck Chemicals, Darmstadt, Germany) and rinsed thoroughly with synthetic water prior to use. Zinc sulfide clusters (2–4 μM) were prepared and measured as described in the partner publication [14].

Acute $AgNO_3$ toxicity was tested at two different sulfide concentrations (mean ± standard deviation): 250 ± 10 nM and 22 ± 0.4 nM, referred to hereafter as approximately 250-nM and approximately 25-nM tests, respectively. Unlabeled $AgNO_3$ (SigmaUltra, Sigma Chemical, St. Louis, MO, USA) containing a proportion of radioactive ^{110m}Ag (RISOE National Laboratory, Roskilde, Denmark) to facilitate analyses of silver concentrations in the test solutions and organisms was added into the test solutions 3 h prior to introduction of *D. magna* neonates. The final specific activity of radiolabeled silver in all test solutions was 0.72 μCi/μg total silver. Silver concentrations tested depended on sulfide concentration in the test solutions. In the absence of sulfide, nominal concentrations were 0 (control), 0.05, 0.1, 0.2, 0.5, 1, and 2 μg/L (0, 0.46, 0.93, 1.9, 4.6, 9.3, and 19 nM) in both toxicity tests. In added-sulfide tests, nominal silver concentrations were 0 (control), 0.2, 0.5, 1, 2, 5, 10, and 20 μg/L (0, 1.9, 4.6, 9.3, 19, 46, 93, and 185 nM) at approximately 250 nM sulfide and 0 (control), 1, 2, 3, 4.5, 6, 8, and 10 μg/L (0, 9.3, 19, 28, 42, 56, 74, and 93 nM) at approximately 25 nM sulfide. Final silver concentrations were obtained from $AgNO_3$ stock solutions (10 and 1 mg/L) acidified with 1% HNO_3. Total silver concentrations in stock solutions as well as in some treatments (>2 μg/L in the presence of ~250 nM sulfide) were checked by graphite furnace atomic absorption spectrometry (AA-1275 with GTA-9 atomizer, Varian, Toronto, ON, Canada). However, because of analytical difficulties faced during silver measurements by graphite furnace atomic absorption spectrometry in samples containing sulfide, only results from experiments where silver concentrations in the test solutions were measured by the radiotracer technique have been shown and used for calculations. These analytical problems are discussed in detail in the partner publication [14].

Silver (total and filtered) and sulfide concentrations were followed over the 48-h experiment. The ^{110m}Ag radioactivity in filtered (Acrodisc 0.45-μM polyethersulfone in-line filters, Pall Gelman Laboratory, Ann Arbor, MI, USA) and nonfiltered water samples (2 ml) was determined using a gamma counter (Minaxi gamma Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada). Reactive sulfide was quantified by formation and spectrophotometric measurement of methylene blue sulfide according to the method of Cline [15]. As an additional check on water quality, total organic carbon was monitored throughout the experiment performed at approximately 25 nM sulfide. Analytical techniques employed and results of the chemical analyses are described in detail in the partner publication [14].

After 3 h pre-equilibration, 10 *D. magna* neonates were then placed in each beaker and maintained without aeration. Triplicates for each silver concentration, with and without sulfide, were provided. Measurements were made to ensure that water remained sufficiently oxygenated (P_{O_2} = 164.2 ± 1.9 mmHg; $n = 10$) and the water pH was constant (8.23 ± 0.014; $n = 10$) over 24 h of experiment. *Daphnia magna* were not fed during tests. Temperature was maintained at 20°C, and light regime was fixed at 16:8-h light:dark photoperiod.

After 24 h, *D. magna* neonates were transferred to a new set of test solutions prepared 3 h prior to transfer as previously described. For the second 24 h of exposure, living *D. magna* were removed from the original beakers and transferred using a fine-screen mesh net (~250 nM sulfide experiment) or held (< 5 s) in plastic pipettes (~25 nM sulfide experiment) during solution changeover. At 24 and 48 h (~250 nM sulfide experiment) or 6, 24, and 48 h (~25 nM sulfide experiment) of exposure, mortality (%) in each experimental beaker was recorded. The death criteria adopted were a change to milky coloration and lack of movement even after mild stimulation. The 48-h LC50 values and the respective 95% confidence intervals were estimated on the basis of cumulative mortality data using probit analysis [16]. These values were estimated on the basis of both nominal and mean measured total and filtered silver concentrations over the respective period of test, and the results were compared.

Free Ag^+ ion concentrations were also calculated from the measured silver concentration for 48-h LC50 results in the absence and presence of approximately 25 and approximately 250 nM sulfide. Free Ag^+ were calculated using the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) critical stability constant (log β) for $AgCl^0$ of 3.31 [17] (I, ionic strength = 0) and corrected to an I = 0.077 of

the reactor solution of $10^{3.23}$ using the Güntelberg expression for Ag^+ and Cl^- and assuming the activity coefficient of $AgCl^0$ = 1. The conditional stability constant (log K' = 8.9) for the silver–zinc sulfide complex (Ag–SZn) was measured in the same matrix as used for the toxicity tests and needs no correction for ionic strength. The Ag–SZn conditional stability constant was assumed to be constant for 25- and 250-nM sulfide concentrations. Total organic carbon was not considered as a site for silver binding since the measured concentrations were very low (generally <0.1 mg/L [14]).

A 48-h LC50 test was also conducted under the same experimental conditions to check on possible toxicity due to zinc from zinc sulfide clusters to *D. magna* neonates. Zinc test solutions were prepared from a $ZnSO_4$ (Anachemia Canada, Montreal, QC, Canada; analytical grade) stock solution. Final zinc concentrations ranged from 0 to 1.7 mg/L (0–26 μM).

Silver accumulation studies

In a third test, total silver accumulation in whole body was assessed in living *D. magna* after 48 h exposure in the absence and presence of sulfide (~25 nM). In the absence of sulfide, nominal silver concentrations tested were equal to those employed in the toxicity tests described previously: 0 (control), 0.05, 0.1, 0.2, 0.5, 1, and 2 μg/L (0, 0.46, 0.93, 1.9, 4.6, 9.3, and 19 nM). In added-sulfide tests (~25 nM), they were 0 (control), 0.2, 0.5, 1, 1.5, 2, 3, and 5 μg/L (0, 1.9, 4.6, 9.3, 13.9, 19, 28, and 46 nM). Silver accumulation in living and dead *D. magna* over 1 h exposure to a single silver concentration (0.93 ± 0.02 μg/L) in the absence and presence (~25 nM) of sulfide was also measured. To perform this last experiment, daphnids were killed by brief exposure to subzero temperature. In both experiments, test solutions were prepared following the same procedure adopted in the acute toxicity test described previously. Experimental conditions were maintained as described for the toxicity tests. Measurements of total silver as well as sulfide in water samples were also performed as described for the toxicity tests.

After silver exposure, living daphnids from the 48 h test were collected using plastic pipettes, dried on filter paper (Whatman No. 1, Scarborough, ME, USA) without a previous wash procedure, weighed using an electronic microscale (Mettler UMT2, Mettler-Toledo, Greifensee, Switzerland; 0.001-mg accuracy), and transferred to plastic vials. Those exposed for 1 h to silver received a 15-s wash in a concentrated (1 mg silver/L) $AgNO_3$ solution to displace loosely bound ^{110m}Ag before drying on filter paper, weighing, and transfer to plastic vials. The ^{110m}Ag radioactivity in daphnids was then measured as described for water samples. Total silver concentration in *D. magna* was expressed in μg silver/g wet weight. All data were expressed as mean ± standard error. Significant differences ($p < 0.05$) in silver accumulation values in the absence and presence of sulfide (~25 nM) were assessed by analysis of variance in combination with the least significant difference test using the software Statistica® (Statsoft, Tulsa, OK, USA).

RESULTS

Acute toxicity tests

Control survival in the approximately 250-nM sulfide experiment was 73 and 83% in the absence and in the presence of sulfide, respectively. In the approximately 25-nM sulfide experiment, it corresponded to 100 and 97% in the absence and in the presence of sulfide, respectively. The lower survival in the controls in the approximately 250-nM sulfide experiment can be attributed, at least in part, to the use of a fine-screen mesh net to remove and transfer daphnids from the original beakers during solution changeover as opposed to the use of a plastic pipette in the approximately 25-nM sulfide experiment.

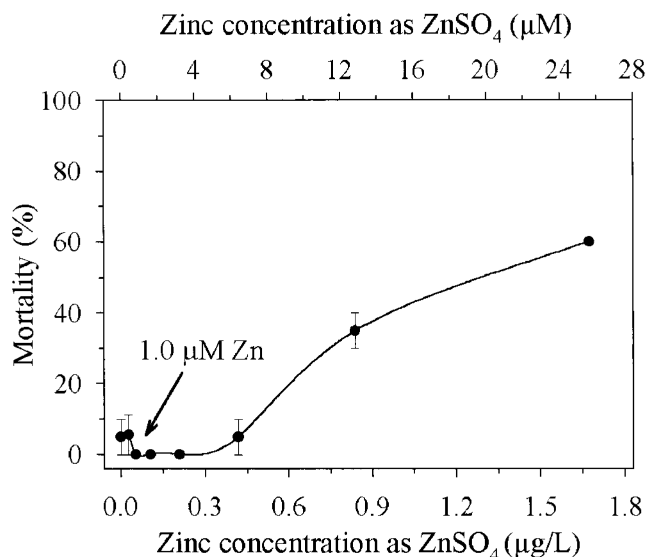

Fig. 1. Mortality of *Daphnia magna* neonates after 48-h exposure to zinc as $ZnSO_4$ in synthetic hard water. The zinc concentration indicated by the arrow corresponds to the maximum zinc concentration that could be encountered in the toxicity tests using zinc sulfide clusters. Data are means ± one standard error (n = 3).

On the basis of nominal zinc concentrations, 48-h mean lethal concentration (LC50, 95% confidence interval [CI]) value for *D. magna* neonates was estimated as 21.8 (17.1–31.9) μM zinc (Fig. 1). This concentration is far greater than the maximum possible zinc concentration in the zinc sulfide cluster experiment, that is, 1.0 μM Zn^{2+} in the approximately 250-nM zinc sulfide treatment.

The LC50 values significantly changed according to the index of silver concentration considered, that is, nominal or mean measured total or measured filtered concentration (Table 1). The LC50 values also progressively decreased with time (6, 24, and 48 h). In the absence or in the presence of sulfide (~25 nM), 48-h LC50 values estimated using mean measured total concentrations corresponded to about 70% of those simultaneously estimated based on nominal concentrations (Table 1). This difference was due to the progressive loss of silver from the test solution during the 24-h static period [14]. In turn, a slight further decrease was observed in 48-h LC50 when measured filtered, as opposed to measured total, concentrations were employed, at least in the absence of sulfide. However, in the presence of sulfide, the situation was dramatically different.

Mortality of *D. magna* neonates exposed to silver was dramatically reduced by the presence of sulfide in the experimental medium (Figs. 2 and 3). In the absence (<5 nM) of sulfide, 48-h LC50 values estimated were similar (0.18 and 0.26 μg/L) in the two sets of toxicity tests performed when measured total silver concentrations were considered (Table 1). In the presence of high concentration of sulfide (~250 nM), mortality of *D. magna* neonates was about 15% and not significantly different from that observed in the control condition of the respective toxicity test, even at the highest silver con-

Table 1. Acute silver toxicity (added as $AgNO_3$) to neonates of *Daphnia magna* in the absence (<5 nM) and in the presence (~250 and ~25 nM) of reactive sulfide (zinc sulfide clusters). The mean lethal concentration (LC50) values and respective 95% confidence intervals were estimated after different times of exposure based on both nominal and measured (total and filtered, 0.45-μm) silver concentrations. MBS = methylene blue sulfide; CI = confidence intervals; ND = no data; NT = no toxicity observed

		Nominal		Measured			
				Total		Filtered	
Nominal MBS (nM)	Time of exposure (h)	LC50 μg/L (nM)	95% CI μg/L (nM)	LC50 μg/L (nM)	95% CI μg/L (nM)	LC50 μg/L (nM)	95% CI μg/L (nM)
<5	24	0.89 (8.2)	0.73–1.08 (6.8–10.0)	0.52 (4.8)	0.44–0.64 (4.1–5.9)	ND	ND
	48	0.26 (2.4)	0.17–0.37 (1.6–3.4)	0.18 (1.7)	0.12–0.25 (1.1–2.3)	ND	ND
~250	24	NT	NT	NT	NT	ND	ND
	48	NT	NT	NT	NT	ND	ND
	6	0.95 (8.8)	0.79–1.21 (7.3–11)	0.75 (6.9)	0.62–0.95 (5.7–8.8)	0.54 (5.0)	0.46–0.67 (4.3–6.2)
<5	24	0.51 (4.7)	0.44–0.59 (4.1–5.5)	0.33 (3.1)	0.29–0.39 (2.7–3.6)	0.25 (2.3)	0.23–0.28 (2.1–2.6)
	48	0.40 (3.7)	0.33–0.46 (3.1–4.3)	0.26 (2.4)	0.23–0.30 (2.1–2.8)	0.22 (2.0)	0.19–0.25 (1.8–2.3)
	6	3.29 (30.5)	3.02–3.59 (28.0–33.2)	2.85 (26.4)	2.60–3.12 (24.1–28.9)	1.06 (9.8)	0.94–1.19 (8.7–11.0)
~25	24	2.69 (24.9)	2.43–2.97 (22.5–27.5)	1.90 (17.6)	1.71–2.13 (15.8–19.7)	0.45 (4.2)	0.39–0.55 (3.6–5.1)
	48	2.07 (19.2)	1.79–2.37 (16.6–21.9)	1.47 (13.6)	1.26–1.70 (11.7–15.7)	0.28 (2.6)	0.24–0.32 (2.2–3.0)

centration (nominal = 2 μg/L) evaluated (Fig. 2). In the presence of low concentration of sulfide (~25 nM), the 48-h LC50 was about 5.5-fold higher than that estimated in the absence of sulfide (Table 1 and Fig. 3A). However, when measured filtered silver concentrations rather than measured total concentrations were considered, the toxicity curves became virtually identical (Fig. 3B), and 48-h LC50 values estimated in the absence of sulfide were very similar to those obtained in the presence of sulfide (~25 nM). They corresponded to 0.22 and 0.28 μg/L, respectively (Table 1 and Fig. 3B).

The fraction of mononuclear species depends only on the formation constant and ligand concentration. The tests without reactive sulfide are quite simple, resulting in $[Ag^+] = [AgCl^0]$ within 1%. The speciation calculation is more variable when sulfide is present because of the uncertainty in the reactive sulfide (initial = 35 nM; mean measured over 48 h = 22 nM)

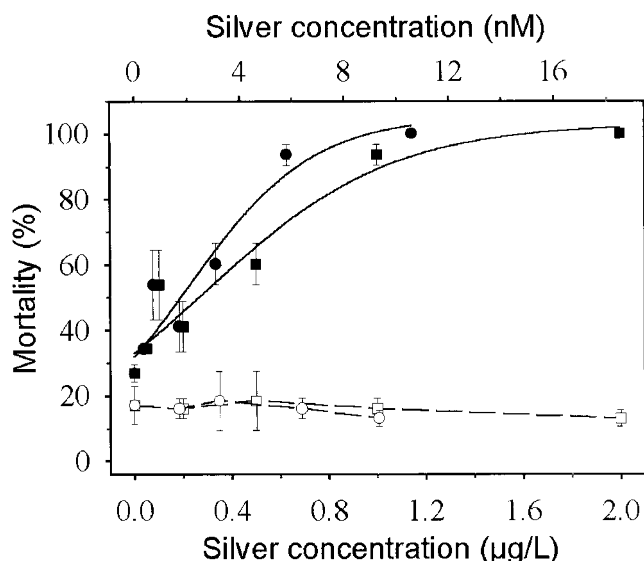

Fig. 2. Mortality of *Daphnia magna* neonates after 48-h exposure to silver as $AgNO_3$ in the absence (<5 nM; solid lines and closed symbols) and in the presence (~250 nM; dashed lines and open symbols) of zinc sulfide clusters in synthetic hard water. Squares correspond to nominal concentrations of silver, while circles correspond to measured total concentrations of silver. Data are means ± one standard error (n = 3).

and the variation in the conditional stability constant (log K') for the silver sulfide (Ag–SZn) from 8.7 to 9.2 with a mean of 8.9 [14]. The calculated mean and range of Ag–SZn for 35 nM reactive sulfide is 93% (86–97%) and for 22 nM reactive sulfide is 90% (85–95%). The calculated free Ag^+ ion for the 48-h LC50 (respective 95% CI) value based on measured total silver (Table 1) gives 0.13 (0.12–0.15) μg/L when sulfide is absent. Similarly, the calculated free Ag^+ ion from the 48-h LC50 value (Table 1), the mean log K' (8.9) for Ag–SZn, and mean measured sulfide concentration over 48 h (22 nM) is 0.07 μg/L with a range of 0.02 to 0.13 μg/L when the variation in 48-h LC50, log K' for Ag–SZn, and sulfide concentration is considered. Although an overlap exists for free Ag^+ ion in the absence and in the presence of sulfide, the mean concentration of free Ag^+ ion in the presence of sulfide (22 nM) is 46% lower than in the absence of sulfide. This difference can be attributed to the assumptions inherent in the methodology to obtain the Ag–SZn conditional stability constant as well as the variation in the results for sulfide concentration and the log K' value.

Silver accumulation studies

After 48 h exposure to silver, whole-body accumulation of total silver in living daphnids was significantly different in the presence (~25 nM) and in the absence (<5 nM) of sulfide. The range where silver concentrations overlapped (nominal = 0.2–0.5 μg/L) was small because of high mortality of daphnids beyond a concentration of 0.5 μg/L in the absence of sulfide. Nevertheless, in this range, it appears that much more accumulation of silver occurred in the presence than in the absence of sulfide (Fig. 4A).

As in the preceding test, silver accumulation in living *D. magna* over 1 h exposure to approximately 1 μg silver/L was about 10-fold higher in the presence of approximately 25 nM sulfide than in the absence (<5 nM) of sulfide, even when cold displacement was used to remove any loosely bound radioactive silver. On the other hand, silver body burden in *D. magna* killed by exposure to low temperature prior to silver exposure was very low and unaffected by the presence of sulfide (~25 nM) in the test solution (Fig. 4B).

DISCUSSION

In the present study, acute toxicity tests were performed in order to assess any possible protective effect of reactive sulfide

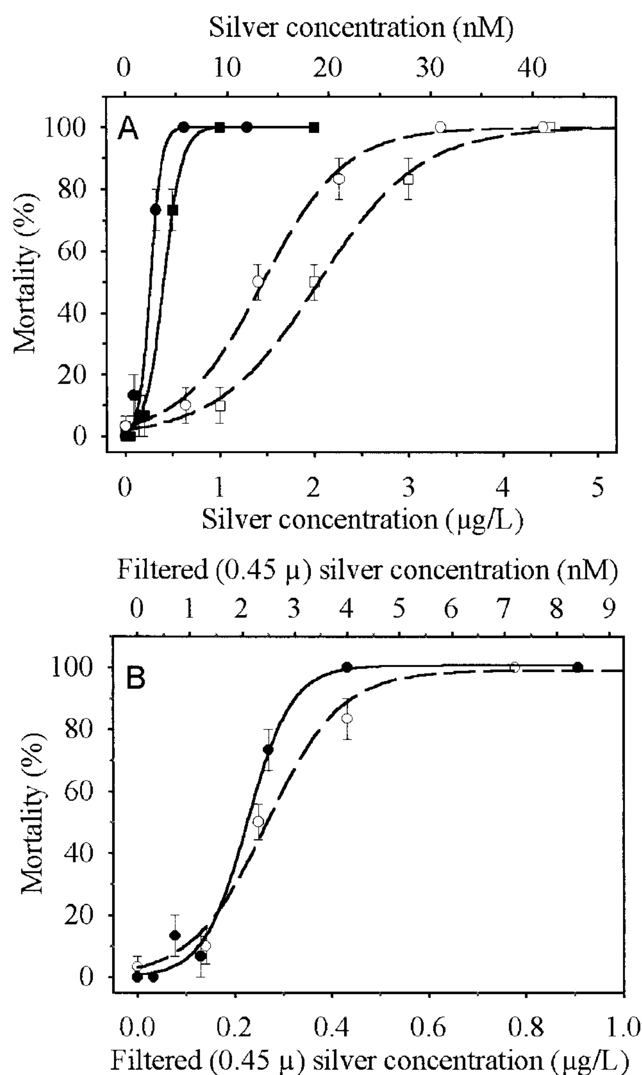

Fig. 3. Mortality of *Daphnia magna* neonates after 48-h exposure to silver as $AgNO_3$ in the absence (<5 nM; solid lines and closed symbols) and in the presence (~25 nM; dashed lines and open circles) of zinc sulfide clusters in synthetic hard water. (**A**) Squares (■) correspond to nominal concentrations of silver, while circles correspond to measured total concentrations of silver. (**B**) Open circles (○) correspond to measured filtered (0.45-μm) concentrations of silver in the presence of zinc sulfide clusters. Closed circles (●) represent filtered concentrations in the absence of zinc sulfide clusters. Data are means ± one standard error (n = 3).

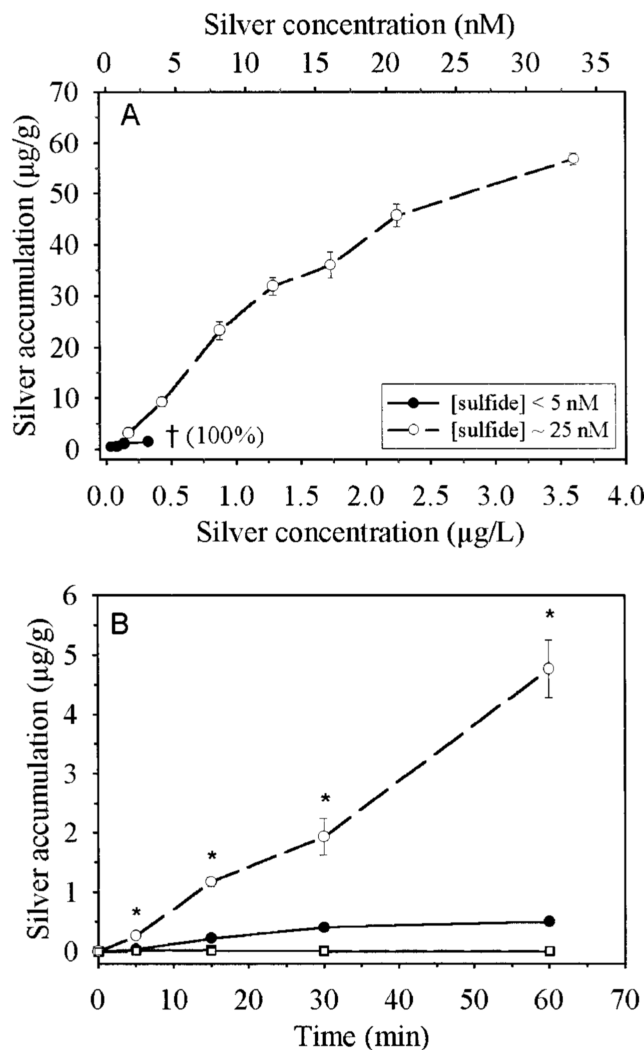

Fig. 4. Whole-body silver accumulation in *Daphnia magna* neonates in the absence (<5 nM; solid lines and closed symbols) and in the presence (~25 nM; dashed lines and open symbols) of zinc sulfide clusters in synthetic hard water. (**A**) Accumulation in living neonates after 48-h exposure to silver in the absence and in the presence of sulfide. In the absence of sulfide, no survivors were observed beyond a concentration of 0.5 μg silver/L. Data are means ± one standard error (n = 5–9). (**B**) Accumulation in living (circles) and in dead (cold-killed, squares) neonates over 1-h exposure to 0.93 ± 0.02 μg silver/L in the absence and in the presence of sulfide. Note the overlap in the dead *D. magna* data set for the two treatments. Data are means ± one standard error (n = 4). The asterisk (*) indicates significant differences ($p < 0.05$) between mean accumulation values for living *D. magna* in the absence and the presence of sulfide after the same time of exposure.

against ionic silver toxicity in *D. magna* neonates. The 48-h LC50 values estimated in absence of sulfide in the two sets of toxicity tests performed are in agreement with the previous toxicity studies that showed that daphnids are among the most sensitive freshwater organisms to acute silver toxicity when silver is added as $AgNO_3$ [10]. However, the 48-h LC50 values, expressed as total measured silver, that were estimated in the present study (0.18 and 0.26 μg/L) were lower than those (0.5–1.0 μg/L [18–21]) previously reported for *D. magna*. These differences could be attributed, at least in part, to differences in the chemistry of the water used in the toxicity tests. In fact, it has been demonstrated that increased alkalinity [20] and dissolved organic carbon [20,21] significantly decreased acute toxicity of $AgNO_3$ to *D. magna*. In the present study, alkalinity (100 mg/L as $CaCO_3$) was generally lower than that employed by other authors, and dissolved organic carbon levels were very low (generally <0.1 mg/L [14]) since we used synthetic water, and daphnids were not fed during the toxicity tests. Furthermore, in the present study, measured concentrations of silver were employed, which was not the case in some of the earlier studies. Our findings clearly point out the need for monitoring silver concentrations during toxicity tests because of the progressive loss of silver from the test solutions.

Toxicity data obtained in the present study clearly demonstrate that reactive sulfide effectively protected *D. magna* against acute silver toxicity. As demonstrated in the companion paper [14], zinc sulfide clusters proved to be feasible as a form of sulfide to use in these toxicity tests. A log K' value for the silver–zinc sulfide equilibrium was calculated as 8.9. This val-

ue needs to be confirmed by rigorous analytical tests since it is conditional to the ionic medium of these toxicity tests. Toxicity results obtained and described in the present paper show that zinc from zinc sulfide clusters is not toxic to *D. magna* neonates at the concentrations employed. This statement is based on the fact that the 48-h LC50 estimated for zinc (21.8 μM) is far greater than any concentration *D. magna* neonates encountered in the zinc sulfide cluster experiments, that is, 1.0 μM zinc in the approximately 250-nM zinc sulfide treatment. The protective effect of reactive sulfide against silver toxicity was observed in concentrations of sulfide representative of a range of natural waters. In fish, it has also been demonstrated that the presence of ligands that bind Ag(I) (e.g., Cl^-, natural organic matter, $S_2O_3^{2-}$) or compete with Ag(I) for toxic sites on gills (e.g., Na^+, Ca^{2+}) profoundly influences acute $AgNO_3$ toxicity [7,22–25].

For example [26,27], silver is discharged from the photographic industry complexed with thiosulfate, which has a high affinity for Ag(I) (log *K* 8.8–13.6 [28]). After passing through publicly owned treatment works, thiosulfate is likely to be reduced to sulfide, which has a higher affinity for silver (log *K* 13.6–17.7 [6]). The log *K*′ value (8.9) used in the present study is a conditional value obtained in the presence of Zn(II) [14]. Natural waters also contain high levels of Cl^- (log *K* 3.3–5.5 [28]) and natural organic matter (log *K* 9.0–9.2 [28]), which can also bind Ag(I). At acute levels, all these bound forms of silver are much less toxic than $AgNO_3$ in fish [7,21,22,24,29,30].

The present study shows that in *D. magna* neonates, silver bound to sulfide present in the water under oxic conditions also appears to be much less toxic than $AgNO_3$. The toxic fraction of silver is possibly that fraction filterable through a 0.45-μm membrane filter since 48-h LC50 values calculated from filtered measured silver concentrations were similar in the absence (<5 nM) and in the presence (~25 nM) of sulfide. As described in the companion paper, the nonfilterable silver is believed to be associated with sulfide that binds by chemisorption to the filter membrane, and its separation is not related to size discrimination [14]. The silver that passes the membrane is assumed, on the basis of the same principle, to be that which is unbound to sulfide. This filterable fraction likely consists of free Ag^+ ion and $AgCl^0_{(aq)}$ complex. Either or both of these may be the moiety causing acute toxicity, though the free ion is usually considered the more likely candidate [7,10,11,21–25,28–30]. The similarity of free Ag^+ ion and $AgCl^0_{(aq)}$ concentrations in the tests, with and without sulfide present, is in agreement with the accumulation study that suggests that the sulfide-bound silver is not acutely toxic to *D. magna* neonates. The results from the whole-body silver accumulation experiments indicate that silver is in some way incorporated by the daphnids and not simply adsorbed on their exoskeleton. However, we cannot at present eliminate the possibility that silver may also have been ingested by the daphnids. The experiments also demonstrate that silver accumulation is much higher in *D. magna* exposed to silver in the presence of sulfide (~25 nM) and that this accumulation has no acute toxic effect.

Traditionally, $AgNO_3$ has been the form of silver used in acute toxicity tests, chosen because it dissociates freely in solution to yield large amounts of the free ion Ag^+ [31]. In general, free cationic metals are the most toxic because of their interaction with anionic sites on the gill [32,33], and thus the use of $AgNO_3$ represents a worst-case scenario. Taking into account that in the present study $AgNO_3$ was used as silver source, our results strongly suggest that naturally occurring sulfide will influence acute toxicity and probably also chronic toxicity in the most sensitive freshwater organisms. Indeed, this may be the reason why waterborne chronic silver toxicity has never been demonstrated in natural field situations. The regulatory implications of this finding are immense because virtually all laboratory toxicity studies carried out to date have been inadvertently conducted in the absence of metastable sulfides because of water purification processes (chlorination and subsequent dechlorination or distillation or reverse-osmosis treatment) used in the preparation of the test solutions. Even in the presence of freshwater sulfide levels as low as 1 nM, sulfide could complex far more silver than would otherwise be chronically toxic.

Thus, the results from the toxicological studies presented here indicate the need to incorporate sulfide into the present version of the acute biotic ligand model [11]. An improvement of this model for silver will result from the incorporation of sulfide with a stoichiometry of 1:1 since the chemical studies reported in the companion paper indicated a 1:1 binding ratio of silver to sulfide [14]. Furthermore, the present study indicates the need to assess the potentially large role of sulfide in preventing chronic toxicity. Clearly, extrapolation of laboratory-based chronic silver toxicity studies in the absence of metastable sulfides to natural waters with metastable sulfides may well be unnecessarily overprotective and costly.

Acknowledgement—The authors thank N. Ogden, M. Ernste (both McMaster University), and T. Smith (Kodak Canada). Funding was provided via research grants from Kodak Canada and the Natural Sciences and Engineering Research Council (NSERC) of Canada Collaborative Research and Development Program. A. Bianchini is a research fellow (203457/86-2) of the Conselho Nacional de Desenvolvimento Ciêntífico e Tecnológico of Brazil. C.M. Wood is supported by the Canada Research Chair Program.

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