SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 21, No. 6, pp. 1286–1293, 2002

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 1: DESCRIPTION OF THE CHEMICAL SYSTEM

KARL C. BOWLES,† ADALTO BIANCHINI,‡ COLIN J. BRAUNER,§ JAMES R. KRAMER,*† and CHRIS M. WOOD‖
†School of Geography and Geology, McMaster University, Hamilton, Ontario L8S 4K1, Canada
‡Departamento de Ciências Fisiológicas, Fundação Universidade Federal do Rio Grande, Rua Eng. Alfredo Huch 475, Rio Grande, RS 96.201-900, Brazil
§Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, California 921282-4614, USA
‖Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

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

Abstract—Experiments were designed to assess the potential protective effect of the presence of sulfide against the acute (48-h) toxicity of silver(I) to *Daphnia magna*. Tests were conducted in borosilicate glass beakers (250 ml) in moderately hard synthetic water. Toxicity solutions were replaced after 24 h by static renewal method. This paper describes the chemical system, and the acute toxicity results are presented in a companion paper. Sulfide was below detection limit (<5 nM) in controls with no sulfide added. Sulfide, added as zinc sulfide clusters at approximately 35- or approximately 350-nM concentration, dropped in concentration to approximately 25 and 250 nM, respectively, over the 24-h period of measurements. Silver also decreased in concentration during the experiment (up to 59%), and the rate of loss was greater in the absence of sulfide compared with the presence of sulfide. A filtration experiment indicated a 1:1 binding ratio of silver to sulfide and a conditional stability constant for the Ag(I)–zinc sulfide complex of log $K' = 8.9$. The losses of sulfide and silver during the experiments highlighted the need for regular monitoring of the important chemical components of the system, even during short (48-h) toxicity tests.

Keywords—Zinc sulfide clusters Silver Daphnids Toxicity

INTRODUCTION

Current understanding suggests that silver toxicity to aquatic organisms is strongly affected by binding of the metal to strong ligands [1,2]. Recently, it has been confirmed that reactive sulfide is present in a wide range of oxygenated natural fresh and saline waters [3–7]. This overcomes earlier thinking that sulfide is not stable in oxygenated waters and suggests that sulfide needs to be reevaluated as an important ligand for trace metals in aquatic systems. Reactive sulfide has been quantified at concentrations from low nanomolar to a few hundred nanomolar in river waters and publicly owned treatment works outfalls [3,7]. These concentrations are greater than the concentrations of silver(I) found in the same waters [7]. Since sulfide is known to bind silver(I) strongly, it is expected that sulfide will be the dominant ligand affecting the speciation of silver(I) in freshwaters. Currently, the photographic and other industries are regulated on their silver emissions to aquatic systems without taking into account the effect of sulfide as a ligand. It is, therefore, extremely important for both environmental and economic reasons to elucidate the effect of the binding with sulfide on the toxicity of silver(I).

Unfortunately, the nature of the reactive sulfide in natural waters has not yet been rigorously characterized. Some authors have shown that sulfide is stabilized in natural waters by the presence of soft metals, predominantly zinc, iron, and copper with traces of other metals, such as silver [3]. One possibility is that the sulfide exists as inorganic metal sulfide cluster molecules [3,8]. Another possibility is that sulfide is stabilized by metals associated with natural organic matter [9]. Other authors have suggested that thiols are also important forms of reduced sulfur in natural waters [10]. The relative merits of these arguments are complex and not suitable for full discussion here. From a toxicological perspective, it is important that a candidate form of reactive sulfide be chosen that is inherently nontoxic to the test organisms and represents, in principle, the sulfide found in the environment.

We chose zinc sulfide clusters as being a suitable candidate for the reactive sulfide. These clusters probably consist, in solution, of a mixture of polynuclear species with base units of Zn_3S_3 and $Zn_4S_6^{4-}$ [8] and can be easily synthesized in pure water at low micromolar concentrations [8,11]. The aim was to react silver(I) with these clusters in solution at concentrations of sulfide appropriate to natural environmental levels and to determine the acute toxicity of the solution to *Daphnia magna*. This species was chosen since daphnids have been shown to be among the most sensitive of aquatic organisms to silver(I) [12]. Because of the unusual nature of the sulfide ligand and the extremely low concentrations of ligand and metal used in this study, the chemical parameters controlling the toxicity tests were thoroughly evaluated in tests without daphnia present and also during the toxicity tests themselves. This paper describes the chemical system for two sets of acute toxicity tests with zinc sulfide clusters and silver(I): one at approximately 250 nM sulfide and one at approximately 25 nM sulfide. These concentrations are representative of the mid- to high range of sulfide concentrations measured in natural waters [3]. The first test (~250 nM sulfide) was conducted largely as a preliminary study to the low sulfide study and is reported in less detail. A companion paper details the toxicological results of the experiments [13].

* To whom correspondence may be addressed (kramer@mcmaster.ca).

METHODS

All glassware, plastic ware, and filtration equipment was acid cleaned in 1 or 10% nitric acid. Exceptions to this were materials used only for radiometric counting of ^{110m}Ag, which is not prone to contamination because of low background levels, and vials for methylene blue sulfide (MBS) determination, which were used as purchased. Reagents were ACS grade unless otherwise specified.

Preparation of zinc sulfide clusters

Preparation of the zinc sulfide clusters is described in full in Bowles et al. [11]. Briefly, zinc(II) nitrate (final concn. 10 nM) was added to sodium sulfide (10 nM) in oxygen-free water (500–800 ml) under an argon blanket. The anoxic solution was stirred for 2 h before exposing it for equilibration with oxygen in the laboratory atmosphere. Cluster solutions were aged for >4 d prior to use in toxicity tests to ensure the absence of any unreacted sulfide and also the complete development of the clusters [11]. Reactive sulfide in the cluster solutions was measured daily to ensure that the clusters were stable and to allow calculation of appropriate dilution factors for the toxicity experiments.

Determination of reactive sulfide

Reactive sulfide was quantified by formation and spectrophotometric measurement of MBS. Not all sulfide bound to silver(I) is measurable by the MBS method since it is only sparingly soluble in the reagent under our analytical conditions [11]. Therefore, the MBS value is not equivalent to total reactive sulfide when silver(I) is present at an appreciable proportion of the sulfide concentration. The MBS method was chosen, however, because of its relative simplicity and its sensitivity, which allows determination of sulfide at low nanomolar concentrations.

Methylene blue sulfide (MBS) measurements were conducted according to the method of Cline [14]. Briefly, 20 ml of sample were pipetted by plastic-tipped micropipette into an unused 40-ml borosilicate glass vial. Mixed diamine reagent (MDR) was added (2 ml) and the vial quickly stoppered. The solution was briefly shaken by hand to mix and allowed to react for >30 min in the dark. The absorbance of the methylene blue was measured at 670 nm using a Cary 50 spectrophotometer (Varian, Palo Alto, CA, USA) with a 10-cm pathlength quartz cell.

The MDR was prepared by carefully mixing the following two solutions: 2.25 g of *N,N*-dimethyl-*p*-phenylenediamine oxalate ($[C_8N_2H_{12}]_2{\cdot}C_2O_4H_2$, Baker, Phillipsburg, NJ, USA) dissolved in 660 ml of concentrated H_2SO_4 and 340 ml of water, and 5.4 g of ferric chloride ($FeCl_3{\cdot}6H_2O$, BDH, Toronto, ON, Canada) dissolved in 100 ml of concentrated HCl and diluted to 200 ml with water. The reagent was stored in a 1-L brown polyethylene (high-density polyethylene) bottle (Nalgene, Rochester, NY, USA). Note that the mixing of water and strong acids results in considerable generation of heat, and appropriate use of safety equipment is required.

We found that light-scattering particles were frequently present in the test solutions containing daphnids, possibly because of shedding of exoskeletons or fecal material, which could interfere with the spectrophotometric measurement. When measuring the MBS with the spectrophotometer, we allowed any particles to settle to the bottom of the cell, as evidenced by taking sequential readings until a plateau was reached. Controls with solutions containing *D. magna* neonates but with no added sulfide confirmed that with appropriate consideration a detection limit of <5 nM could be achieved. Note that filtration of the solutions to remove particulates is not appropriate (see the following discussion and [10]).

Determination of silver concentrations

Measurement of spiked ^{110m}Ag (RISØE, Roskilde, Denmark) was used to determine total silver concentrations in the approximately 250-nM sulfide tests, where nominal Ag was ≤9.3 nM (1 μg/L) and in all samples in the approximately 25-nM sulfide test. The ^{110m}Ag was added to the working cold silver stock solution so that the final specific activity after dilution was 0.72 μCi/μg silver. The ^{110m}Ag radioactivity in water samples (2 ml) was determined using a gamma counter (Minaxi Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada) according to the guidelines of Hansen et al. [15].

Graphite furnace atomic absorption spectrometry (GFAAS) was used in the approximately 250-nM sulfide tests to determine silver concentrations where nominal Ag was >9.3 nM (1 μg/L). A 10-μl aliquot from a 2-ml subsample (acidified with 0.5% HNO_3 and stored in a polypropylene vial) was placed onto the furnace of the GFAAS (Varian AA-1275 with GTA-9 atomizer, Varian, Walnut Creek, CA, USA) by an autosampler. Measurement was made according to standard operating conditions as documented by the manufacturer with no matrix modifier. Because of adsorption of sulfide clusters onto vessel walls, GFAAS is not appropriate for the determination of silver in solutions containing sulfide unless certain precautions are undertaken. The problems and possible solutions are described in the Results section of this paper.

Filtration: Size separation versus surface adsorption

Filtration of zinc sulfide clusters has been shown to be problematic because of losses of clusters that bind to filtration membranes [11]. In many cases, the loss may be quantitative. For this reason, filtration was not appropriate as a means of size discrimination for the test solutions in this study. We did use filtration, however, to assess the proportion of the silver(I) that was bound to the sulfide clusters. This assumes that all silver(I) bound to the clusters is expected to be lost on the membrane, whereas silver bound to other dissolved ligands (e.g., $AgCl^0_{(aq)}$) should pass the membrane. It is also assumed that no silver is bound to particles, other than sulfide clusters, which exceed the cutoff of the filtration membrane. This assumption is approximate, however, and would be applicable only to the relatively simple system of zinc sulfide clusters and silver studied here.

Acrodisc 0.45-μm polyethersulfone in-line filters (Gelman, Ann Arbor, MI, USA) were used with polypropylene syringes to filter solutions in the approximately 25-nM sulfide study for determination of MBS and silver. Measured MBS in filtered samples was always below the detection limit (5 nM). This confirms quantitative losses of the sulfide clusters to the filters.

Determination of organic carbon

Samples for total organic carbon analyses (10 ml) were stored in acid-cleaned borosilicate vials. Measurement was conducted on a Dohrmann organic carbon analyzer after removing inorganic carbon by acidification with one drop of concentrated nitric acid and purging for >5 min with a stream of nitrogen gas.

Experimental design

Synthetic hard water (~250 ml) was measured, in triplicate, into borosilicate glass beakers for each silver concentration, with and without sulfide. The hard water was synthesized by reconstituting reverse-osmosis water to the following composition (final pH = 8.23): 1.0 mM $CaCO_3$, 0.15 mM $MgSO_4$, and 0.6 mM NaCl. Water 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. The water was not bubbled during tests.

Zinc sulfide clusters (2–4 μM MBS) were diluted into the synthetic hard water to give an appropriate concentration. As detailed in the Introduction, two sets of tests were performed in the absence (<5 nM) and in the presence of low (~25 nM) and high (~250 nM) concentrations of Zn-sulfide clusters.

Silver(I) nitrate containing a proportion of radioactive ^{110m}Ag was spiked into the test solutions at $t = -3$ h. The addition of ^{110m}Ag was chosen to give at least 160 counts/min in subsamples (2 ml) of the lowest-silver-concentration treatments. Neonate (<24 h old) *D. magna* were introduced into the test solutions by disposable pipettes at $t = 0$ h. At $t = 21$ h, a new set of test solutions was made for each treatment by spiking 750 ml of synthetic hard water with zinc sulfide clusters and silver nitrate. At $t = 24$ h, the daphnia were removed from the original beakers by pipettes, and the water was replaced with the fresh solution (250 ml per beaker). The daphnids were held briefly in the pipettes while the water was replaced and then pipetted back into the fresh solution for the second 24 h of exposure. Only living daphnids were transferred into fresh solutions. Beakers that exhibited 100% mortality after 24 h were not used in the second 24 h. Maintenance of the daphnids is described in the partner publication [13].

An experiment was conducted in the absence of organisms to assess the losses of silver and zinc sulfide to borosilicate glass beakers and polyethylene beakers over 48 h in order to assess the suitability of each as the test material. In these tests, silver(I) (2 and 10 μg/L; 19 and 93 nM) was added to two glass beakers and two polyethylene beakers. Similarly, zinc sulfide clusters (~30 nM) were added to separate glass and polyethylene beakers. The losses of silver and sulfide over 48 h in the glass beakers were comparable (73 and 85%, respectively). In polyethylene beakers, the loss of silver(I) was considerably less than the loss of sulfide (37 and 80%, respectively). Borosilicate glass was, therefore, chosen for all further experiments to allow for better comparison of acute toxicity between systems with and without sulfide present.

In other preliminary tests (data not shown), we showed that it was not possible to saturate reactive sites on the beakers with sulfide in order to reduce adsorptive losses. This can be explained since the sulfide cluster, once adsorbed, forms a substrate for further chemisorption. This property has been reported at higher concentrations by researchers aiming to create thin films of metal sulfides by successive application of the metal and sulfide to a surface such as silicon [16,17]. These experiments suggested that renewal of the test water was necessary in order to maintain sulfide and silver(I) concentrations at roughly appropriate concentrations. After several preliminary tests, we decided that the water would be replaced once (at 24 h) during the 48-h acute toxicity tests and that regular monitoring of the silver and MBS was essential to allow estimation of mean concentrations for calculation of silver mean lethal concentration (LC50) to *D. magna* neonates.

As an additional check on water quality, natural organic matter was monitored (as total organic carbon) throughout the experiment conducted with approximately 25 nM sulfide. This monitoring is important since natural organic matter has been shown to affect the toxicity of silver(I) [18]. At $t = 0$, total organic carbon was always <0.1 mg/L. At the end of the 24-h periods, the total organic carbon ranged from <0.1 to 0.3 mg/L. The results confirm that introduction of natural organic matter (e.g., from daphnid shedding) was not a significant perturbation of the system.

RESULTS

Experiment A: Approximately 250 nM sulfide

Sulfide concentrations. Spot checks were conducted to confirm that sulfide was not present in the beakers to which no sulfide was added. Measured MBS was below the limit of detection (<5 nM) in the tested beakers.

The mean initial sulfide (MBS) was measured at $t = -3$ h in all beakers with added sulfide. The concentrations (± standard deviation) were 240 ± 3.3 nM ($n = 13$) on day 1 and 293 ± 6.8 nM ($n = 7$) on day 2 of the experiment. Sulfide was lost from solution over each 24-h period of the experiment because of adsorption on the beaker walls. The loss in the silver-free beakers was 32 and 24% on day 1 and day 2, respectively. The rate of sulfide loss from solution was approximately linear, and the mean MBS (± range) for day 1 was 203 ± 20 nM ($n = 2$) and for day 2 was 250 ± 10 nM ($n = 2$). In the beakers with silver present, the yield of the MBS reaction is less than 100% (see *Methods*) and results in an apparent loss of sulfide from the solution. Apparent losses ranged from 24 to 80% as silver concentration increased. In all cases, some sulfide was measurable at the conclusion of the experiment.

Silver concentrations. Losses of silver concentrations over 24 h in the sulfide-free controls ranged from 25 to 66% and were generally related to the initial silver concentration (Table 1). The mean silver concentrations listed in Table 1 for 24 h were calculated from the $t = 0$ and $t = 24$-h measurements. Similarly, the mean silver concentrations for 48 h were calculated from the combined $t = 0$ and 24-h measurements from both days. These values were used in the calculations of 24- and 48-h LC50 for *D. magna* neonates [13].

In the sulfide-containing treatments, analytical problems occurred at silver concentrations >10 nM (Fig. 1A). These samples were measured with GFAAS as opposed to the other samples, which were analyzed by ^{110m}Ag gamma counting. The lower values obtained with GFAAS were attributed to losses of silver sulfide onto the walls of the sample vials before the measurements could be made. This hypothesis was confirmed by measurements with ^{110m}Ag gamma counting, which showed that the silver on the walls and in solution could be measured separately (Fig. 1B). ^{110m}Ag gamma counting was not affected by this problem since the entire vial could be placed in the counter. In the beakers that were measured by ^{110m}Ag gamma counting, the losses from solution were similar those in the sulfide-free treatments (42–65%).

Experiment B: Approximately 25 nM

Sulfide concentrations. Measured MBS in the controls with no added sulfide was below the analytical detection limit (<5 nM) in all but one sample (6 nM). Therefore, sulfide contamination was not problematic during the experiment. This also

Table 1. Measured mean silver concentrations over 24 and 48 h, with (~250 nM) and without (<5 nM) zinc sulfide. Figures in italics are results of graphite furnace atomic absorption spectrometry analyses and are not reliable (see *Discussion* and Fig. 1). Ag loss = 100 × (Initial Ag − Final Ag)/Initial Ag. MBS = methylene blue sulfide; SD = standard deviation; ND = no data

	Nominal		0–24 h		0–48 h	
MBS (nM)	Ag (μg/L)	Ag (nM)	Mean Ag (nM [±SD])	Ag loss (%)	Mean Ag (nM [±SD])	Ag loss (%)
<5	0.05	0.46	0.33 ± 0.07	50	0.36 ± 0.09	25
	0.1	0.93	0.65 ± 0.06	50	0.71 ± 0.07	34
	0.2	1.9	1.6 ± 0.2	50	1.7 ± 0.2	36
	0.5	4.6	3.0 ± 0.5	56	3.1 ± 0.6	55
	1.0	9.3	5.6 ± 0.3	58	5.8 ± 0.3	54
	2.0	19	*10.6 ± 0.5*	*66*	ND	ND
~250	0.2	1.9	1.7 ± 0.1	52	1.7 ± 0.2	45
	0.5	4.6	3.2 ± 0.2	55	3.2 ± 0.5	49
	1.0	9.3	6.0 ± 0.5	58	6.4 ± 1.6	42
	2.0	19	*11 ± 1*	*65*	*9.3 ± 1.4*	*43*
	5.0	46	*9.4 ± 2.3*	*56*	*12 ± 7*	*20*
	10	93	*15 ± 3*	*63*	*19 ± 9*	*−18*
	20	185	*28 ± 3*	*68*	ND	ND

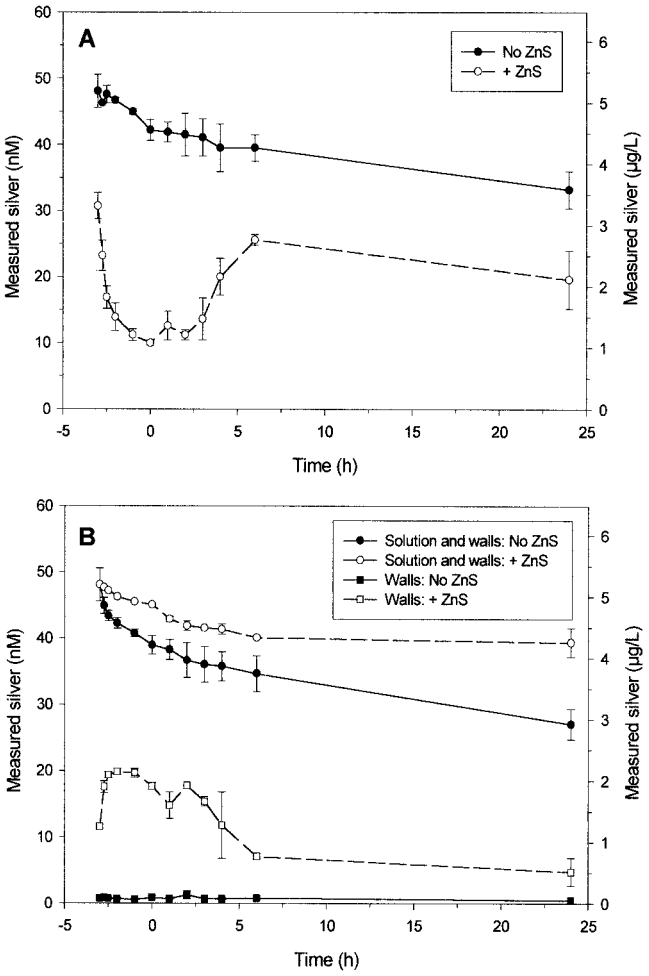

Fig. 1. Comparison of silver concentrations during a toxicity test measured by different techniques (46 nM nominal silver, 240 nM initial sulfide). (**A**) Graphite furnace atomic absorption spectrometry; (**B**) ^{110m}Ag gamma counting. Walls refers to walls of sample vial, not walls of the test beakers. Error bars represent standard deviation of triplicate measurements.

confirmed that material shed by the neonate daphnids did not affect the MBS spectrophotometric measurement.

In the sulfide-added beakers, the initial sulfide concentration was 35 ± 3 nM (n = 8) on day 1 and 32 ± 2 nM (n = 6) on day 2. The MBS losses over day 1 are shown in Figure 2 for the beakers with 0 nM Ag and 9.3 nM Ag (1 μg/L). As expected, the concentration of measured MBS in the presence of silver is lower than in the absence of silver. Note that the difference between the two values at any given time after 3 h is approximately 10 nM, which is close to the added silver concentration. This implies that the binding of silver to sulfide occurs at a 1:1 ratio if none of the sulfide bound to silver was available for reaction with MDR. The mean MBS concentration over 48 h in the absence of silver was 22 ± 0.5 nM (mean ± range, n = 2).

Total silver concentrations. Total measured silver concentrations are summarized in Table 2. As with sulfide, silver was lost from solution over time. The concentrations of silver used in the experiments with and without sulfide were substantially different because of the differences in the LC50 for silver in

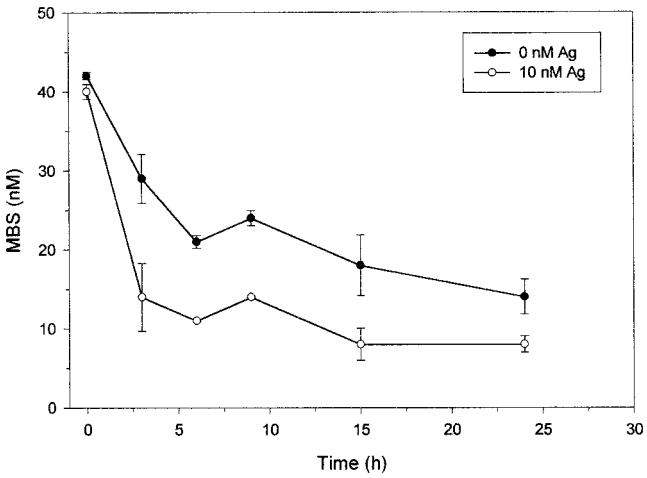

Fig. 2. Loss of reactive sulfide (methylene blue sulfide [MBS]) from synthetic hard-water solution over 24 h. Initial sulfide = 35 nM. Legend denotes nominal initial silver concentration. Error bars represent range of duplicate measurements.

Table 2. Measured silver concentrations in solution over 6, 24, and 48 h in the absence and presence of sulfide. Ag loss = 100 × (Initial Ag − Final Ag)/Initial Ag. SD = standard deviation; ND = no data

	Nominal		0–6 h[a]		0–24 h[b]		0–48 h[c]	
MBS (nM)	Ag (μg/L)	Ag (nM)	Mean Ag (nM [±SD])	Ag loss (%)	Mean Ag (nM [±SD])	Ag loss (%)	Mean Ag (nM [±SD])	Ag loss (%)
<5	0.05	0.46	0.40 ± 0.05	5.3	0.35 ± 0.08	32	0.35 ± 0.10	25
	0.1	0.93	0.92 ± 0.07	3.2	0.83 ± 0.10	27	0.77 ± 0.11	40
	0.2	1.9	1.5 ± 0.2	3.9	1.3 ± 0.25	32	1.3 ± 0.26	35
	0.5	4.6	3.6 ± 0.1	8.7	3.1 ± 0.15	40	3.0 ± 0.39	40
	1	9.3	7.2 ± 0.6	11	5.8 ± 0.9	50	ND	ND
	2	19	15 ± 2	10	12 ± 2	59	ND	ND
~25	1	9.3	7.8 ± 0.7	24	6.2 ± 1.4	49	5.9 ± 2	54
	2	19	16 ± 2	18	13 ± 4	39	13 ± 5	40
	3	28	24 ± 0.8	19	20 ± 1	37	21 ± 2	32
	4.5	42	37 ± 2	16	31 ± 4	37	31 ± 4	35
	6	56	50 ± 4	14	41 ± 5	35	ND	ND
	8	74	65 ± 3	17	53 ± 4	39	ND	ND
	10	93	84 ± 5	12	70 ± 8	33	ND	ND

[a] Mean of measurements at 0 and 6 h on day 1 only.
[b] Mean of measurements at 0, 6, 9, 15, and 24 h on day 1 only.
[c] Mean of measurements at 0, 6, 9, 15, and 24 h on each of day 1 and day 2.

the presence and absence of sulfide [13]. In previous work, however, we had greater overlap of silver concentrations and can use these data to compare the rate of loss of silver with or without sulfide present. Regressions for the rate of loss of silver over a 24-h period are listed in Table 3. The regression slopes are higher in the absence of sulfide, which indicates that sulfide helps to prevent the adsorption of silver to the vessel walls. It is interesting to note that in the absence of sulfide, the percentage loss of silver over time increases with increasing silver concentration, whereas this trend is reversed in the presence of sulfide (Table 2, 24-h data). This is further evidence for the need to closely monitor the actual concentrations of chemical species in solution during toxicity tests.

Filtered silver concentrations. In the absence of sulfide, filtered silver accounted for 69 to 93% of the total measured silver (Table 4). At higher total silver concentrations, the proportion of filterable silver was lower, possibly suggesting a small amount of precipitation of AgCl. Computer modeling (MINEQL+ Ver 4.0), however, suggested that precipitation of AgCl did not occur. Chemical parameters of the modeling procedure were entered on the basis of solution chemistry described in the experimental design section.

Table 3. Slopes of regressions: measured silver versus time over 24 h, with and without sulfide (~25 nM methylene blue sulfide). Data from previous work (see *Discussion*). ND = no data

Nominal Ag (nM)	Sulfide <5 nM		~25 nM sulfide	
	Slope (pM/h)	r^2	Slope (pM/h)	r^2
0.46	5.4	0.9844	ND	ND
0.93	9.8	0.9596	ND	ND
1.9	24	0.9539	16	0.9766
4.6	75	0.9293	45	0.9931
9.3	190	0.9335	140	0.9946
14	ND	ND	200	0.9855
19	430	0.9847	270	0.9733
28	ND	ND	440	0.9872
46	ND	ND	730	0.9851
93	ND	ND	1,450	0.9852

In the presence of sulfide, the proportion of filterable silver (17–62%) was much less than in the absence of sulfide (Table 4). Filtered silver concentrations increased as total silver concentrations increased, which is consistent with silver having exceeded the stoichiometric binding ratio with the sulfide clusters. Bowles et al. [11] present evidence that the removal of zinc sulfide clusters on filters is caused by chemisorption rather than simply size exclusion of particles. Since silver is complexed by the sulfide, the filtered silver concentrations can be used as a measure of the extent to which the silver has been complexed by the sulfide.

Figure 3 shows the relationship between filtered silver and total silver concentrations. Silver concentrations in the absence of sulfide were linearly correlated with total silver (slope = 0.784, $r^2 = 0.940$, $n = 40$). Silver concentrations in the presence of sulfide showed a biphasic distribution with filtered silver concentrations close to zero until the concentration of sulfide (~22 nM) was exceeded. The linear regression for the silver data points in excess of the sulfide concentration (slope = 0.840, $r^2 = 0.929$, $n = 20$) was similar to the regression for silver without sulfide present. The filtered silver concentrations, with sulfide present, on day 1 at $t = 0$ were proportionately higher than at all other times (see Fig. 3, open triangles). This suggests that the silver sulfide moiety that initially forms may be smaller or less prone to chemisorption than clusters after further stabilization. When these points were removed from the data set, the linear regression (slope = 0.781, $r^2 = 0.943$, $n = 13$) was virtually identical with the regression in the absence of sulfide. The x intercept of this regression was 24 nM, which corresponds well with the mean measured sulfide value (22 nM), clearly indicating 1:1 binding between silver and sulfide.

Filtered and total silver concentrations were used to estimate a conditional binding constant (K') and capacity (L_{total}). In this assessment, unbound silver ([Ag′]) is assumed to be that concentration of silver that passes the filter. The bound silver ([AgL]) is that amount retained on the filter. We assume 1:1 metal to ligand binding so that

Table 4. Measured filtered silver concentrations over the periods 6, 24, and 48 h in the presence and absence of sulfide. MBS = methylene blue sulfide; SD = standard deviation

	Nominal		6 h[a]		24 h[b]		48 h[c]	
MBS (nM)	Ag (μg/L)	Ag (nM)	Filtered Ag (nM [±SD])	% AgT[d] (%)	Filtered Ag (nM [±SD])	% AgT[d] (%)	Filtered Ag (nM [±SD])	% AgT (%)
	0.05	0.46	0.33	83	0.31	87	0.30	86
	0.1	0.93	0.82	89	0.74	90	0.71	93
	0.2	1.9	1.3	85	1.2	89	1.2	92
	0.5	4.6	2.8	77	2.3	76	2.5	82
	1.0	9.3	5.2	71	4.0	69	ND	ND
<5	2.0	19	12	75	8.4	71	ND	ND
	1	9.3	2.3 ± 0.4	29	1.5 ± 0.4	24	1.3 ± 0.4	21
	2	19	4.5 ± 0.7	30	2.7 ± 0.7	21	2.3 ± 0.8	17
	3	28	8.6 ± 0.7	36	4.8 ± 0.7	24	4.0 ± 0.8	19
	4.5	42	15 ± 2	41	8.6 ± 2	28	7.2 ± 2	23
	6	56	24 ± 2	47	15 ± 2	35	ND ±	ND
	8	74	37 ± 0.6	56	24 ± 0.7	48	ND ±	ND
~25	10	93	52 ± 0.8	62	36 ± 2	51	ND ±	ND

[a] Mean of measurements at 0 and 6 h on day 1 only.
[b] Mean of measurements at 0, 6, 9, 15, and 24 h on day 1 only.
[c] Mean of measurements at 0, 6, 15, and 24 h on day 1 and day 2.
[d] AgT = total measured silver.

$$[Ag_{total}] = [Ag'] + [AgL] \qquad [L_{total}] = [L] + [AgL]$$

$$K' = [Ag'][L]/[AgL]$$

Combining the two equations and rearranging gives

$$[Ag'L] = \frac{[Ag']L_{total}}{[Ag'] + K'} \quad (1)$$

The function in Equation 1 was fitted to [Ag′] (filtered) and [AgL] ([Ag_{total}] − [Ag′]) to obtain values for L_{total} and K' using a nonlinear least-squares fitting procedure. Figure 4 shows the plot of data for bound Ag ([ML]) versus free Ag ([M′]) and the best-fit curve (Eqn. 1). The time = 0 h data from both day 1 and day 2 were omitted since reaction with the clusters appeared incomplete at that time (see the previous discussion). The results obtained were log K' = 8.9 (95% confidence range: 8.7–9.2) and L_{total} = 32 nM (26–39 nM).

The calculated binding capacity (32 nM) is higher than the mean measured sulfide (22 nM). Therefore, it may be inferred that the binding is approximately 1.5:1 (silver:sulfide), although this contradicts the previously reported evidence, which indicated a 1:1 binding ratio. It is possible, however, that this method also factors in the component of sulfide adsorbed to the walls of the vessel but that is still available to bind silver. This seems reasonable since the initial sulfide concentrations (35 and 32 nM on days 1 and 2, respectively) were very close to the calculated capacity.

The calculated conditional stability constant (log K' = 8.9) is considerably lower than the thermodynamic log K reported for the equilibrium: $AgHS/Ag^+ \cdot HS^-$ by Martell and Smith (log K = 13.6) [19]. It is likely that the presence of the zinc(II) as a competing metal significantly affects the equilibrium of silver(I) with the sulfide. Literature data are not available for binding to metal sulfide clusters, but it is possible to make an estimate of the contribution of zinc to the calculated condi-

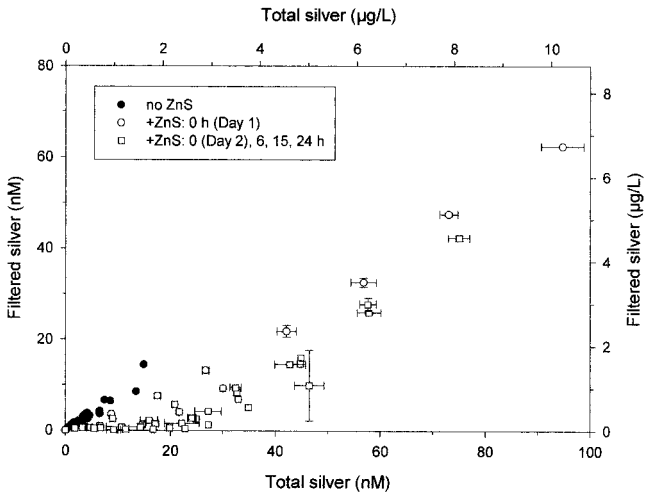

Fig. 3. Relationship between measured filtered and total silver in beakers with and without added sulfide (as zinc sulfide clusters). Data are a composite from day 1 and day 2 of experiment. *X* axis error bars represent standard deviation of triplicate measurements. *Y* axis error bars represent range of duplicate measurements.

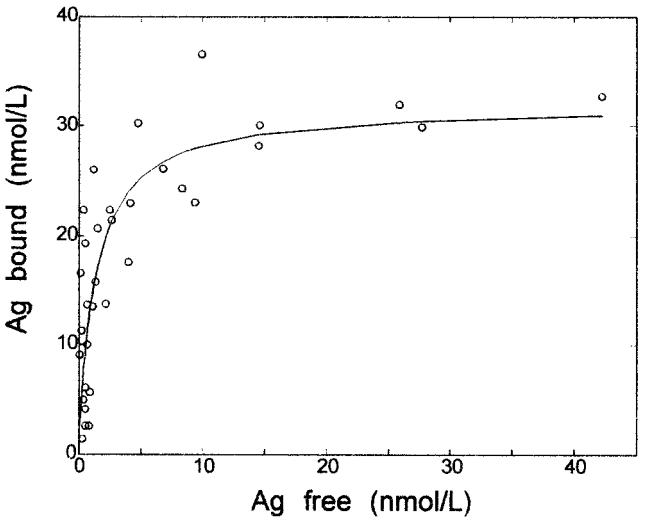

Fig. 4. Nonlinear least-squares fitting of the data derived from filtered and total silver measurements. Ag free = filtered silver concentration; Ag bound = (total silver concentration − filtered silver concentration).

tional constant if the following displacement reaction is substituted for Equation 1:

$$Ag^+ + ZnS^0 + H^+ \Leftrightarrow AgSH + Zn^{2+}$$

$$K_{(2)} = [AgSH][Zn^{2+}]/[Ag^+][ZnS^0][H^+] \quad (2)$$

Equation 2 can be broken down to the following equilibria with reported log *K* values from the National Institute of Standards and Technology (Gaithersburg, MD, USA) Critical database [19]:

$$Ag^+ + HS^- \Leftrightarrow AgSH$$

$$K_{(3)} = [AgSH]/[Ag^+][HS^-] = 10^{13.6} \quad (3)$$

$$Zn^{2+} + HS^- \Leftrightarrow ZnS^0 + H^+$$

$$K_{(4)} = [ZnS^0][H^+]/[Zn^{2+}][HS^-] = 10^{5.0} \quad (4)$$

These can be rearranged to give equilibrium (2), where

$$K_{(2)} = K_{(3)}/K_{(4)} = 10^{8.6} \quad \text{or} \quad \text{Log } K_{(2)} = 8.6$$

The stability constant for Equation 2 is dependent on the concentrations of Zn^{2+} and H^+ in solution. Substituting in values of pH = 8.23 and [Zn] = 22 nM gives an apparent conditional log $K'_{(2)}$ = 9.2. This value is similar to the experimental conditional constant (log K' = 8.9). Despite this, the conditional stability constant reported here should be treated with caution until appropriately thorough analytical studies are performed to confirm or refute this result. Furthermore, the use of filtration data to separate species must be considered appropriate for this experimental setup only.

DISCUSSION

Zinc sulfide proved to be feasible as a form of sulfide to use in the toxicity tests. Losses of sulfide during the experiment were substantial, however, which is in agreement with the findings of other work that reported zinc sulfide losses to a variety of materials, including borosilicate glass, at similar concentration ranges [11]. Losses of silver during the toxicity experiments were also substantial. For example, in a test solution containing nominally 19 nM silver and 25 nM zinc sulfide, the silver concentration was measured to drop by 39% over 24 h (Table 2). Based on the data reported in the companion paper [13], accumulation of silver by the daphnids accounted for <5% of the measured silver loss from solution. Since the daphnids are exposed to silver over the complete duration of the experiment, it is possible that the greatest mortality will occur in the initial few hours of each 24-h period when the silver concentrations are highest. This may be particularly important in the presence of sulfide since filtered silver concentrations, which may be the bioactive fraction, may drop to below the LC50 values despite the total silver remaining comparatively high. Despite these concerns, we decided to change the water only once during the 48-h toxicity tests in order to minimize stress to the daphnids [13].

When using GFAAS, analytical difficulties also occurred because of the adsorption of the clusters to container walls after sampling. We found that the simplest solution was to use a radiometric counting method with labeled silver in which the entire sample vial could be counted. Methods in which an aliquot is removed for measurement may suffer from significant analytical artifacts. In order to use such methods, it will be necessary to destroy the metal sulfide clusters before analysis. Studies of the analysis of silver and copper in natural (sulfide-containing) waters suggest that acidification of samples is not sufficient to attain quantitative recoveries and that an oxidative step is required [20,21]. Suitable oxidative steps may involve ultraviolet light treatment [20] or oxidation with peroxide or persulfate [21]. It should be noted that the loss of sulfide onto vessel walls is highly dependent on solution chemistry (e.g., pH and ionic strength, form of sulfide) [11]. Therefore, the extent of loss of sulfide and associated metals (e.g., silver) from natural samples cannot be predicted using the data from this toxicity experiment.

An additional analytical difficulty results from the low solubility of silver sulfide under the conditions of the reaction with the MDR. This results in low sulfide recoveries in the presence of silver and a degradation of precision, depending on the length of time the silver sulfide is allowed to react with the MDR. It is possible that allowing a longer reaction time will recover more of the sulfide. Some researchers have reported the use of CrII as a reagent to allow measurement of total sulfide by reducing the bound metal (e.g., silver or copper) [3,22]. This approach has promise, but caution is required since this method will also reduce a number of other sulfur species to sulfide, such as polysulfides, elemental sulfur, sulfite, and thiosulfate, some of which may occur in the toxicity test solution.

It is important for the toxicity tests that the added silver(I) has time to equilibrate with the sulfide clusters. Other workers have shown that silver will displace zinc from the sulfide clusters in <1 min [3]. This suggests that the amount of time between addition of the silver to the sulfide and exposure of the daphnids should not be critical. Our filtered data suggest, however, that 3 h after the addition of the silver to the sulfide (t = 0 in our experiments), conformational changes may still be occurring that result in a change in the degree to which the clusters adhere to the filters (Fig. 3). Also, we found that the degree to which the clusters adhered to the walls of the sample vials used for silver analysis was changing up to 9 h (t = 6 h) after silver addition (Fig. 1). Therefore, it may be appropriate to allow the silver sulfide clusters to stabilize for a longer period before addition of the daphnia to the test waters.

Despite adsorptive losses of the sulfide and silver, it was possible to monitor the concentrations throughout the experiment to allow calculation of mean concentrations to use in LC50 determinations. If appropriate care is taken, zinc sulfide clusters are a suitable form of sulfide for use in toxicity tests. These conclusions are born out in the acute toxicity results that are discussed by Bianchini et al. [13].

Acknowledgement—Thanks to N. Ogden, M.J. Ernste, D.S. Smith (McMaster University), and J.W. Gorsuch (Eastman Kodak). Funding was obtained from Kodak Canada and the Natural Sciences and Engineering Research Council of Canada Collaborative Research and Development Program. A. Bianchini is a research fellow from the Brazilian CNPq (203457/86-2). C.M. Wood is supported by the Canada Research Chair Program.

REFERENCES

1. Bury NR, McGeer JC, Wood CM. 1999. Effects of altering freshwater chemistry on physiological responses of rainbow trout to silver exposure. *Environ Toxicol Chem* 18:49–55.
2. Bury NR, Galvez F, Wood CM. 1999. Effects of cadmium, chloride and dissolved organic carbon on silver toxicity: Comparison between rainbow trout and fathead minnows. *Environ Toxicol Chem* 18:56–62.
3. Rozan TF, Lassman ME, Ridge DP, Luther GW III. 2000. Evidence for multinuclear Fe, Cu and Zn molecular sulfide clusters in oxic river waters. *Nature* 406:879–882.
4. Theberge SM, Luther GW III, Farrenkopf AM. 1997. On the

existence of free and metal complexed sulfide in the Arabian Sea and its OMZ. *Deep Sea Res* 44:1381–1390.
5. Kramer JR, Adams NWH, Manolopoulos H, Collins PV. 1999. Silver at an old mining camp, Cobalt, Ontario, Canada. *Environ Toxicol Chem* 18:23–29.
6. Cutter GA, Oatts TJ. 1987. Determination of dissolved sulfide and sedimentary sulfur speciation using gas chromatography-photoionization detection. *Anal Chem* 59:717–721.
7. Adams NWH, Kramer JR. 1999. Silver speciation in wastewater effluent, surface waters and pore waters. *Environ Toxicol Chem* 18:2667–2673.
8. Luther GW III, Theberge SM, Rickard DT. 1999. Evidence for aqueous clusters as intermediates during zinc sulfide formation. *Geochim Cosmochim Acta* 63:3159–3169.
9. Manolopoulos H. 2001. Metal sulfides in oxidizing freshwater systems. PhD thesis. McMaster University, Hamilton, ON, Canada.
10. Tang D, Santschi PH. 2000. Sensitive determination of dissolved sulfide in estuarine water by solid-phase extraction and high-performance liquid chromatography of methylene blue. *J Chromatogr A* 883:305–309.
11. Bowles KC, Bell RA, Ernste MJ, Kramer JR, Manolopoulos H, Ogden N. 2002. Synthesis and characterization of metal sulfide clusters for toxicological studies. *Environ Toxicol Chem* 21:693–699.
12. Ratte HT. 1999. Bioaccumulation and toxicity of silver compounds: A review. *Environ Toxicol Chem* 18:89–108.
13. Bianchini A, Bowles KC, Brauner CJ, Gorsuch JW, Kramer JR, Wood CM. 2002. Evaluation of the effect of reactive sulfide on the acute toxicity of silver(I) to *Daphnia magna*. Part II: toxicity results. *Environ Toxicol Chem* (this issue).
14. Cline JD. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol Oceanogr* 14:454–458.
15. Hansen HJM, Grosell M, Jacobsen U, Jørgensen JC, Hogstrand C, Wood CM. 2002. Precautions in the use of ^{110m}Ag, containing transmutation produced ^{109}Cd, as a tracer of silver metabolism in ecotoxicology. *Environ Toxicol Chem* 21:1004–1008.
16. Valkonen MP, Lindroos S, Resch R, Leskala M, Friedbacher G, Grasserbauer M. 1998. Growth of zinc sulfide thin films on (100)Si with the successive ionic layer adsorption and reaction method studied by atomic force microscopy. *Appl Surface Sci* 136:131–136.
17. Lindroos S, Charreire Y, Bonnin D, Leskela M. 1998. Growth and characterization of zinc sulfide thin films deposited by successive ionic layer adsorption and reaction (SILAR) method using complexed zinc ions as the cation precursor. *Mater Res Bull* 33: 453–459.
18. Bury NR, Galvez FG, Wood CM. 1999. Effects of chloride, calcium, and dissolved organic carbon on silver toxicity: Comparison between rainbow trout and fathead minnows. *Environ Toxicol Chem* 18:56–62.
19. Martell AE, Smith RM. 1997. Critically selected stability constants of metal complexes database. NIST Standard Reference Database 46, Ver 4.0. National Institute of Standards and Technology, Gaithersburg, MD, USA.
20. Herrin RT, Andren AW, Shafer MM, Armstrong DE. 2000. Silver complexation in surface water and effluent. *Proceedings,* 11th Annual International Conference on Heavy Metals in the Environment. Contribution 1185. University of Michigan, School of Public Health, Ann Arbor, MI, USA, August 6–10.
21. Simpson SL, Apte SC, Batley GE. 1998. Sample storage artifacts affecting the measurement of dissolved copper in sulfidic waters. *Anal Chem* 70:4202–4205.
22. Radford-Knoery J, Cutter GA. 1993. Determination of carbonyl sulfide and hydrogen sulfide species in natural waters using specialized collection procedures and gas chromatography with flame photometric detection. *Anal Chem* 65:976–982.