Silver accumulation in Daphnia magna in the presence of reactive sulfide

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Abstract

Previously, we demonstrated a higher silver body burden when Daphnia magna were exposed to silver in the presence of environmentally relevant concentrations (25 nM) of reactive sulfide, but the explanation was unclear. In the present study, D. magna were exposed to AgNO₃ (0.93 μg Ag/L = 8.6 nM as a mixture of cold Ag and ¹¹⁰⁸Ag) in synthetic water in either the presence or absence of 25 nM sulfide as zinc sulfide clusters. After 1-h exposure, daphnids were transferred to clean water for up to 5-h depuration. At different times of Ag exposure and depuration, daphnids were randomly sampled for whole body silver burden. Also, after 1 h, daphnids were sampled for silver accumulation in “gills” (small organs on the thoracic appendages), digestive tract, and carcass. Other groups were exposed to the same silver and sulfide concentrations for 1 h and then sampled for whole-body autoradiography. Silver body burden was about two-fold higher in the presence of sulfide. A two-fold increase in silver burden in “gills” and digestive tract, but not in carcass, was also observed in the presence of sulfide. Absolute differences due to sulfide were greatest in digestive tract and explained most of the difference in whole body burden. Transfer to clean water caused a significant drop in silver concentration in whole body and all compartments to similar levels in the two groups after 5-h depuration. These results indicate that the higher silver body burden observed in the presence of sulfide is mainly due to sulfide-bound silver in the digestive tract of the daphnids. This conclusion is supported by autoradiography, which showed a high concentration of silver in the digestive tract of daphnids exposed to Ag/sulfide.

1. Introduction

Several different geochemical or biological modeling approaches have been developed in an attempt to predict acute silver toxicity in aquatic invertebrate and vertebrate species (Wood et al., 1999). A mod-
eling approach that takes into account the geochemistry of the biological ligand has been developed to predict silver toxicity to freshwater fish, and has been extended to daphnids (Janes and Playle, 1995; Paquin et al., 1999; Bury et al., 2002). According to the "biotic ligand model" (BLM), the gill is considered a negatively charged ligand (the biotic ligand) to which Ag+ can bind. Toxic effects are considered a function of the degree of saturation of sites of toxicity on the biotic ligand by Ag+. The gill modeling approach presents some advantages over simply considering speciation of silver in the water column (Wood et al., 1999; Wood, 2001). For example, current versions of the BLM take into account the competition between other cations and Ag+ for sites of toxicity on the gills as well as the influence of different waterborne complexing agents on silver speciation and availability (Paquin et al., 1999; McGeer et al., 2000).

The importance of complexing agents to the bioavailability and toxicity of metals in general is widely accepted (Campbell, 1995). In the aquatic environment, these substances may include inorganic ligands (e.g. chloride, bicarbonate, thioulate), simple organic ligands (e.g. amino acids, EDTA) and complex polydisperse organic ligands such as humic and fulvic acids (Bianchini and Bowles, 2002). Generally, it is considered that these ligands will decrease bioavailability of metals by decreasing free metal ion concentrations (Morel, 1983).

The occurrence of metastable sulfide (Cutter and Oatts, 1987; Luther and Tsamakis, 1989; Adams and Kramer, 1999) and thiols (Tang et al., 2000; al-Farawati and van den Berg, 2001) in a variety of fully oxygenated marine and freshwater systems has been reported in the literature. These findings have sparked interest in the potential for reduced sulfur to influence the bioavailability and toxicity of metals (Kramer et al., 2002).

The most sensitive freshwater organisms to waterborne silver appear to be cladocerans and the generally less sensitive amphipods with values of acute lethal concentration for 50% of the individuals tested (LC50) as low as 0.3 μg/L of total silver when silver is added as AgNO3 in the absence of food (Ratte, 1999; Bianchini et al., 2002; Bury et al., 2002). For this reason, the present version of the silver BLM has been calibrated using toxicity data for Daphnia (Paquin et al., 1999). Previously, (Bianchini et al., 2002), we demonstrated an important protective effect of reactive sulfide against AgNO3 toxicity to Daphnia magna neonates. However, we also found that silver accumulation was greater in daphnids exposed to silver in the presence of sulfide than in its absence, even though toxicity was prevented by sulfide. Therefore, the aim of the present study was to explain why there is a greater silver accumulation without accompanying toxicity in daphnids exposed to waterborne silver in the presence of environmentally realistic levels of reactive sulfide.

2. Materials and methods

2.1. Daphnid maintenance

Colonies of adult gravid D. magna (ARO strain, lot #090600 DM) were obtained from Aquatic Research Organisms (ARO, Hampton, NH, USA). The brood origination of these colonies was United States Environmental Protection Agency (Cincinnati, OH, USA), and the daphnids had been reared in a freshwater static renewal system with water saturated in dissolved oxygen, pH 7.5, hardness ~150 mg CaCO3/L, and 25°C. D. magna were fed phytoplankton and YCT (a slurry of yeast, cerophyll, and trout chow). Upon 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 1000 L of reverse osmosis purified water in a food-grade polyethylene tank. This water was reconstructed to the following composition (final pH 8.23): 1.0 mM CaCO3, 0.15 mM MgSO4, and 0.6 mM NaCl. It was bubbled with pure CO2 for 24 h to ensure that CaCO3 went into solution and then was bubbled with air for 48 h to ensure removal of excess CO2 and atmospheric equilibration. Water was then left to stand for at least 2 weeks prior to use. During the acclimation period, D. magna were fed algae (Ankistrodesmus convolutus; 1.82 x 109 cells/L = 33 mg dry weight/L) and YCT (18.5 mg dry weight/L). Water was not aerated, but renewed daily. Temperature was maintained at 22–23°C for the first 24 h of the acclimation period and was then fixed at 20°C. Photoperiod was fixed at 16L:8D.

After acclimation, reproduction rate of the adult daphnids was measured to ensure it met previously established criteria (Bianchini et al., 2002) for a healthy
population (15–20 neonates per adult every 3–4 days). Provided that the reproductive rate was satisfactory we proceeded with the particular colony. Adults were collected for experiments using plastic pipettes.

2.2. Silver accumulation

Adult daphnids were acutely exposed to a single concentration (nominal = 1 μg Ag/L; total measured = 0.93 μg Ag/L = 8.66 nM) of silver (AgNO₃) as a mixture of “cold” Ag (Sigma/Iltra, Sigma Co., St. Louis, MO) and radioactive ¹¹⁰ᵐAg (RISO National Laboratory, Roskilde, Denmark). The final measured specific activity of radiolabeled silver was 2.7 × 10⁴ Bq/μg total silver.

Acute silver exposure was performed in borosilicate glass beakers (five beakers per experimental condition) containing 500 mL of synthetic water, pre-equilibrated to 20°C, and no more than 14 daphnids in each. Experiments were done in the absence or in the presence of 25 nM sulfide as zinc sulfide complexes. Zinc sulfide clusters were prepared and measured as previously described (Bowles et al., 2002).

It is important to note that the silver concentration employed in this study corresponds to the upper level of the 95% confidence interval (0.62–0.95 μg Ag/L) of the 6-h-LC₅₀ value (0.75 μg Ag/L) calculated based on measured total silver and reported for neonates D. magna under the same experimental conditions and in the absence (<5 nM) of sulfide. Thus, with this silver concentration we would expect a measurable silver body burden with a minimal mortality within the 1-h period of exposure. In the presence of sulfide (25 nM), the 6-h-LC₅₀ value reported is 2.85 (2.60–3.12) μg Ag/L. Thus, the concentration of zinc sulfide clusters employed here (25 nM) was selected considering its protective effect against acute silver toxicity as previously determined (Bianchini et al., 2002).

The levels of Zn attained in the test system are far below the toxic threshold for D. magna (Bianchini et al., 2002). Furthermore, this concentration of sulfide is environmentally relevant since metastable sulfide complexes appear to occur commonly in oxic surface freshwaters at picomolar to nanomolar concentrations (Bianchini and Bowles, 2002).

AgNO₃ and zinc sulfide clusters were added into the test solution 3 h prior to introduction of D. magna. Final silver concentration was obtained from an AgNO₃ stock solution (1 mg/L) acidified with 1% HNO₃. Total silver concentration in the stock solution was verified by graphite furnace atomic absorption spectrometry (GF-AAS; Varian AA-1275 with GTA-9 atomizer, Palo Alto, CA, USA). The final concentration of zinc sulfide clusters was obtained from a stock solution (2 μM measured as methylene blue sulfide). Total silver and sulfide concentrations were measured in the experimental media. The ¹¹⁰ᵐAg radioactivity in water samples (2 mL) was measured using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra-Packard, Toronto, Ont., Canada), considering the precautions on window selection, to avoid possible complications from ¹⁰⁹Cd contamination, as outlined in Hansen et al. (2002). Reactive sulfide was quantified by formation and spectrophotometric measurement of methylene blue sulfide according to the method of Cline (1969).

After the 3 h pre-equilibration, 14 daphnids were placed in each of five beakers (70 daphnids in each experimental condition) and maintained without aeration. After 1-h silver exposure, daphnids were transferred to clean synthetic water using plastic pipettes and maintained under the same experimental conditions for up to 5 h. Daphnids were not fed during tests.

At different times of Ag exposure (0, 5, 15, 30, and 60 min) and depuration (5, 15, 30, 60, 80, 130, and 300 min), one daphnid from each beaker was randomly sampled for whole body silver burden measurement. Daphnids were collected using plastic pipettes, washed for 15 s in a concentrated (1 mg silver/L) AgNO₃ solution to displace loosely bound ¹¹⁰ᵐAg, blotted dry on filter paper (Whatman no. 1; Clifton, NJ, USA), weighed using an electronic microscale (Mettler UMT2; 0.001 mg accuracy; Mettler-Toledo, Columbus, OH, USA), and transferred to plastic vials. The ¹¹⁰ᵐAg radioactivity in daphnids was then measured using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra-Packard, Mississauga, Ont., Canada), with the precautions on window selection outlined by Hansen et al. (2002). Total silver concentration in D. magna was expressed in μg silver/g wet weight. Wet weight was corrected considering the percentage of water trapped into the daphnid exoskeleton, as determined by Stobbart et al. (1977).

Also, after 1-h exposure and after 1-h exposure followed by 5-h depuration, one daphnid from each beaker was collected and washed as described above.
They were dissected for measurement of silver accumulation in "gills" (small organs with the thoracic appendages (Kikuchi, 1983)), digestive tract, and carcass. Tissue samples were dissected under stereoscopic microscope, weighed using an electronic microscale (Mettler UMT2; 0.001 mg accuracy; Mettler-Toledo, Columbus, OH, USA), and transferred to plastic vials. The $^{110m}$Ag radioactivity in tissue samples was measured as described for whole body samples.

2.3 Whole-body autoradiography

Other groups of daphnids were exposed to the same silver and sulfide concentrations employed in the silver accumulation studies. However, the final specific activity of radiolabeled silver was $2.7 \times 10^5$ Bq/µg total silver. After 1-h exposure, daphnids were then collected using plastic pipettes, washed for 15 s in a concentrated (1 mg silver/L) AgNO₃ solution to displace loosely bound $^{110m}$Ag, blotted dry on filter paper (Whatman no 1; Clifton, NJ, USA), and prepared for whole-body autoradiography (Ullberg et al., 1982; Inza et al., 2001). The daphnids were embedded in a carboxymethylcellulose gel and frozen in a slurry of hexane and dry ice. Two 10-µm-thick sections were obtained every 100 µm with a specially designed cryomicrotome (Leica CM3600, Leica, Nussloch, Germany), at $-20^\circ$C. The 15 pairs of sections obtained from each group were freeze-dried and applied on regular X-ray film (Kodak X-omat) for 2 days at $-20^\circ$C. After exposure, films were processed as recommended by the manufacturer. Representative sections were also exposed on phosphor screens for 24 h, scanned digitally with a Cyclone Phosphor Imager, and the radioactivity distribution in the sections was visualized and quantified using the software Optiquant ver. 3.1 (Packard Instrument Co., IL, USA). Results obtained from phosphor screens are expressed as digital light units per mm² of section surface (DLU/mm²).

2.4 Data presentation and statistical evaluation

All values were expressed as mean ± one standard error of the mean (S.E.M.). Differences in the accumulation of silver in the whole body and in specific tissues (digestive tract, "gills", and carcass) in the presence and absence of sulfide were assessed by analysis of variance followed by the Tukey’s test, after the 1-h period of exposure to AgNO₃ and the 5-h period of depuration. All data were previously tested for normality and homogeneity of variances. The kinetics of whole body silver depuration in both the presence and absence of sulfide was assessed by regression analysis ($[Ag] = [Ag]_0 - bt$, where $[Ag] = $ silver burden in µg Ag/g at a given time "t" of depuration; $[Ag]_0 = $ silver burden in µg Ag/g at the end of 1-h exposure to AgNO₃; $b = $ slope of the regression; $t = $ time of depuration in clean water expressed in minutes). In all statistical analyses, the significance level adopted was 95% ($\alpha = 0.05$).

3. Results

Mean total silver concentration in the experiments was 0.93 ± 0.02 µg Ag/L, close to the nominal value of 1.0 µg Ag/L. There were no mortalities during these 1-h silver exposure and 5-h depuration experiments. Silver was quickly accumulated in both the absence and the presence of reactive sulfide. However, silver body burden was about two-fold higher in the presence of sulfide after 1 h of exposure to silver in the presence of 25 nM of reactive sulfide: A significant drop in silver body burden was observed during the 5-h depuration period in daphnids exposed to silver in both the absence and the presence of sulfide. However, on a relative basis, it was significantly (P < 0.05) faster in the presence ([Ag; µg/g] = 0.8528 − 0.00263t; r² = 0.95) than in the absence of reactive sulfide ([Ag; µg/g] = 0.4722 − 0.00116t; r² = 0.84). The half-life of accumulated silver in the daphnid whole body was of 2.7 h in the presence of reactive sulfide instead of 3.4 h in its absence. Despite the fact that data fitted to a linear regression, virtually no silver was depurated from daphnids exposed to silver in the absence of sulfide over the first 80 min of depuration in clean water. Furthermore, the whole body silver burden was similar in both groups from about 80 min of depuration (Fig. 1).

After the 1-h exposure period, approximately two-fold greater silver burdens in "gills" and digestive tract were observed in the presence of reactive sulfide (Fig. 2), with most in the digestive tract. Considering the corrected mean body wet weight (3.11 mg) of daphnids employed in the present study, and because the digestive tract represented 12.2% of the body mass, this compartment accounted for 47.9% of the whole body silver burden in daphnids exposed for 1 h to sil-
Fig. 1. Whole body silver accumulation in Daphnia magna exposed to 0.93 µg total Ag/L (8.6 nM), as AgNO₃, in the absence and in the presence of reactive sulfide (25 nM), for 1 h and then transferred to clean water for up to 5 h. Data are mean ± 1 S.E.M. (n = 5). The vertical dotted line denotes the end of the silver exposure period and the start of the depuration period. (*) Indicates significantly different mean (P < 0.05) between treatments at the same experimental time.

Fig. 2. Silver accumulation in tissues of Daphnia magna exposed to 0.93 µg total Ag/L (8.6 nM), as AgNO₃, in the absence and in the presence of reactive sulfide (25 nM) for 1 h and then transferred to clean water for 5 h. Data are mean ± 1 S.E.M. (n = 5). Different letters in each period (exposure or exposure + depuration) indicate means significantly different (P < 0.05). (*) Indicates significantly different mean (P < 0.05) between the two periods for the same tissue.
ver alone, and 48.0% in daphnids exposed to silver plus sulfide. In the absence of sulfide, "gills" and carcass accounted for 5.2% and 28.2% of the whole body silver burden, respectively. In the presence of sulfide, these compartments accounted for 5.5% and 21.5%, respectively. In both cases, the remaining silver burden (18.8% in the absence of sulfide and 25.0% in the presence of sulfide) could be attributed to losses of part of tissues during dissection. This loss corresponded to only 5.2% of the whole body mass. No significant difference ($P > 0.05$) in the carcass silver burden was observed in the absence and in the presence of reactive sulfide. After the 5-h depuration period, a significant ($P < 0.05$) drop in silver concentration in all tissues was observed. For all tissues, no significant differences ($P > 0.05$) in the silver burden were observed in the absence and in the presence of reactive sulfide after the 5-h depuration period (Fig. 2).

Autoradiograms show that radioactive silver is most accumulated in the daphnid compound eyes and into the digestive tract, in either the absence or the presence of reactive sulfide (Fig. 3). It must be considered
here that the compound eyes were part of the carcass when daphnids were dissected to measure silver tissue distribution. Based on the autoradiograms, the high silver accumulation in the compound eyes could account for the high contribution of carcass for the whole body silver burden.

Quantitative data obtained with phosphor screens confirm those obtained from dissections. Daphnids exposed to silver in the presence of zinc sulfide clusters accumulated twice the radioactivity of those exposed to silver alone, and radioactivity in the digestive tract was five to six times higher than the values found for the whole body (Fig. 4).

4. Discussion

Recently, it has been demonstrated that metastable sulfide complexes occur commonly in oxic surface freshwaters at picomolar to nanomolar concentrations (see Bianchini and Bowles, 2002 for review). This finding is extremely important for the environmental toxicology of metals, because sulfide has a very high binding affinity for metal cations (log $K$ for Ag(I) = 13.6; Paquin et al., 1999), and has been identified as an important silver-binding ligand in oxic surface waters (Bianchini and Bowles, 2002; Bianchini et al., 2002; Bowles et al., 2002). The observed sulfide concentrations are high enough to remove silver from other ligands and high enough to potentially prevent silver toxicity in natural environments, because silver is generally present in these media at concentrations lower than the sulfide (Bianchini and Bowles, 2002).

Previous studies indicate that various chemical ligands have a protective effect against the toxicity of silver to aquatic organisms (McGeer et al., 2000; Wood, 2001; Bury et al., 2002). Previously, we demonstrated acute silver toxicity to $D. magna$ was decreased by about 5.5-fold in the presence of environmentally realistic levels of sulfide ($\sim 25$ nM) in receiving waters, while 250 nM sulfide effectively abolished acute Ag toxicity (Bianchini et al., 2002). Despite this protective effect of sulfide, silver accumulation was much higher in the presence of $\sim 25$ nM of reactive sulfide. Results obtained in the present study are in accord; silver was quickly accumulated in both the presence and absence of 25 nM sulfide, but whole body silver burden was about two-fold higher in the presence of sulfide. The possibility that the silver was weakly bound to the daphnid exoskeleton was negated because the radiotracer silver was not displaced with an excess of non-radiolabeled silver. Also, we previously demonstrated that silver accumulation in dead $D. magna$ (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 (Bianchini et al., 2002). Based on these findings, we conclude that silver is incorporated by daphnids rather than merely adsorbed on the exoskeleton. Higher accumulation of silver in the presence of ligands, as demonstrated here, is not exclusive to the presence of reactive sulfides. For example, thiosulfate and chloride increased silver uptake by the green alga Chlamydomonas reinhardtii; silver–thiosulfate complexes appear to be transported across the plasma membrane via sulfate/thiosulfate transport systems (Fortin and Campbell, 2001). Greater silver uptakes in the presence of chloride and thiosulfate have been reported in freshwater fish gills, and attributed to diffusion of the neutral silver chloride and silver–thiosulfate complexes (Wood et al., 1996b; Wood et al., 2002).

The results of the present study suggest that the higher silver accumulation in daphnids exposed to silver in the presence of reactive sulfide can be explained by an entirely different mechanism—a higher rate of silver ingestion by daphnids under this situation. This interpretation is based on the fact that an almost twofold high silver burden was observed at the digestive tract of daphnids exposed to waterborne silver in the presence of reactive sulfide (Fig. 2). Results obtained from autoradiography under the same experimental conditions also support this finding. Furthermore, the digestive tract represented only about 12% of the body weight but accounted for about 48% of the silver body burden in either the absence or the presence of sulfide, while “gills” accounted only for ∼5%. The other compartment which accumulated more silver was the carcass, accounting for ∼25% of the silver body burden. It must be noted that the compound eyes were part of the carcass in the present study.

The compound eyes of daphnids accumulated high amounts of silver in either the absence or the presence of sulfide, and thus contributed a substantial portion of the carcass burden, presumably due to the high affinity of the compound eyes for Ag. The cyclopic compound eye of D. magna is highly mobile, being rotated by six muscles arranged as three bilateral pairs (Consoli et al., 1987) and is functionally important in visually evoked behaviour (Sims and Macagno, 1985). The daphnid compound eye is made up of many separate ommatidia, each equipped with a lens, and an optic nerve fiber (Nassel et al., 1978). The major lens proteins of invertebrates and vertebrates are a surprisingly diverse group of multifunctional proteins, the crystallins (Tomarev and Piatigorsky, 1996). In most teleost fish, gamma-crystallin is the most abundant lens protein (Pan et al., 1995), whereas in invertebrates relatively little is known about the crystallins. However, it has been shown that the major cephalopod (squid, octopus, and cuttlefish) crystallins (S-crystallins) have, like fish crystallins, been recruited from a stress protective metabolic enzyme, glutathione S-transferase (Tomarev and Piatigorsky, 1996). Compounds and enzymes involved in protection against oxidative stress, such as glutathione and glutathione S-transferase, are generally rich in SH-groups; indeed gamma-crystallins exhibit a high-methionine content (Pan et al., 1995). This could explain the high accumulation of silver observed in the daphnid compound eye in either the absence or the presence of sulfide.

The exact form of the metal sulfides in natural aquatic systems has not been established. However, zinc sulfide (ZnS, and Zn,S,−) clusters in aqueous solution could form as base units (estimated molecular mass = 350-700 Da (Luther et al., 1999)). Ag displaces and replaces Zn in the clusters (Bowles et al., 2002). Thus, it is possible that daphnids are eating silver bound to sulfide clusters as a colloidal material or attached to natural organic matter. Uptake of Ag(I) from colloidal macromolecular organic matter has been reported in the brown shrimp Penaeus aztecus (Lauma et al., 2002). Daphnids are zooplanktonic crustaceans and members of the filter feeding Cladocerans. The diets of suspension-feeding zooplankton are complex mixtures of phytoplankton, detritus, bacteria, and protists (Lampert, 1987; Sterner and Hessen, 1994). D. magna feeds non-selectively over a broad range of particle sizes, and are unable to handle and reject poor-quality particles individually (DoMott, 1995). In addition, both oral and anal intakes of water are known to take place in crustaceans, including daphnids (Fox, 1952). Thus, even in the absence of food (i.e., the test conditions used here), daphnids are constantly pumping water through the digestive tract, a situation that could favor an increased rate of ingestion of silver. Based on the mean digestive tract wet weight (0.38 mg) and the silver concentrations in the water (0.93 μg Ag/L) and the digestive tract (3.05 μg Ag/g) of daphnids, it is estimated that silver contained in 1.29 mL of experimental medium would be necessary to account for the mea-
sured amount of silver accumulated in the digestive tract after 1-h period of exposure in the presence of sulfide. In the absence of sulfide, the required volume would be 0.74 mL. If all silver ingested over the 1-h exposure period had been accumulated in the digestive tract, the water flux necessary to provide this would be of 0.74–1.29 mL/h.

This dose of Ag could be provided by either grazing on colloidal silver or by anal and oral drinking, or by both. Direct measurements of intake of fluid into the gut in *D. magna* indicated that in 10 min a weight of fluid equivalent to 3.1% of the body weight was taken into the gut (Stobhart et al., 1977). This suggests that a weight of fluid equivalent to 18.6% of the body weight, i.e., only ~0.38 µL in a typical 3.11 mg daphnia, would be taken into the gut over the 1-h exposure period. Thus, it seems that most of the silver accumulated into the gut was not provided by anal and oral drinking. In contrast, estimates of maximum filter feeding rates of daphnids are generally in the range of 1–4 mL per adult per h (Lampert, 1987), so grazing is the most likely explanation for the amount of silver accumulated in the digestive tract of *D. magna*.

According to Bianchini and Bowles (2002), at least three fates for the silver bound to sulfide inside daphnids should be considered. Firstly, the complexed silver ingested may be merely excreted as faeces. In this case, a higher whole body accumulation can be explained by a simple accumulation of complexed silver in the digestive tract, due to the time lag for the passage of material through the whole digestive tract. Secondly, complexed silver ingested may be adsorbed onto the digestive tract. Thirdly, complexed silver may be absorbed into the body by the digestive tract and kept in circulation or stored in internal organs. Our results show that faster silver depuration was observed in daphnids exposed to silver in the presence of reactive sulfide for 1 h and depurated for 5 h in clean water (Fig. 1), supporting the idea that ingested silver bound to sulfide is quickly and easily removed from the body. This assumption is based on the fact that over the first 80 min of depuration in clean water, virtually no silver was depurated from daphnids exposed to silver in the absence of sulfide, whereas by the end of this 80 min period, the levels of accumulated silver in daphnids exposed to silver in the presence of reactive sulfide was virtually the same as in the absence of reactive sulfide (Fig. 1). After this period, silver depuration under both conditions occurred at about the same rate until the end of the experiment. These data suggest that the "extra" amount of silver accumulated in the presence of reactive sulfide (compared to that observed in the absence of reactive sulfide), is readily depurated and that the silver depurated comes essentially from the digestive tract.

Furthermore, the digestive tract was the most important compartment where silver was accumulated, accounting for ~48% of the whole body silver burden in either the absence or the presence of sulfide. The much higher concentration of silver in the digestive tract compared to that observed in the "gills" under both experimental conditions could suggest that the uptake and consequently the internal distribution of silver in daphnids may differ from patterns in other freshwater organisms such as the freshwater crayfish *Cambarus diogenes diogenes* (Grosell et al., 2002) and the teleost fish *Oncorhynchus mykiss* (Wood et al., 1996a; Webb and Wood, 1998). In these organisms, the gill appears to be the major (if not only) site of uptake and toxicity during waterborne exposure to silver. However, the high concentration of silver (0.19 µg Ag/g) observed in "gills" of daphnids after 1-h exposure to 0.93 µg Ag/L in the absence of sulfide indicates that this tissue could play an important role in silver uptake in this organism. Such high silver concentration was observed in gills of the freshwater crayfish, but only after 96 h of exposure to waterborne silver at 8.4 µg Ag/L.

In summary, our results indicate that the first fate for silver proposed above (Bianchini and Bowles, 2002) is the most likely, i.e., the complexed silver ingested is merely excreted as faeces. In this case, a higher whole body silver accumulation in the presence of reactive sulfide is explained by a simple accumulation of complexed silver in the digestive tract.

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