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PHYSIOLOGICAL EFFECTS OF DIETARY SILVER SULFIDE EXPOSURE IN RAINBOW TROUT

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Abstract—Silver accumulation was examined in juvenile rainbow trout during a 58-d feeding study with silver sulfide (Ag_2S) added to the diet at concentrations ranging from 0 to 3,000 mg/kg Ag. Silver in the livers of fish fed the 3,000-mg/kg diet reached a level approximately fourfold higher than the control, representing an initial accumulation rate of 9.5 ng/(g·d). Despite this increase in silver levels in the liver, no influence of liver copper levels was observed. In comparison, there were no significant elevations in silver burdens in the kidneys, gills, or intestines, apart from a transient increase in the gills of fish fed the 3,000-mg/kg Ag diet on day 24 only. Daily food consumption rates were lowered by 14 to 22% in all the Ag_2S treatments relative to control levels, possibly because of the decreased palatability of the Ag-laden diets. However, there were no significant differences in growth rates between any of the treatments for the duration of the study, suggesting a lack of any physiological perturbation by dietary Ag_2S exposure. The results of this study suggest that dietary silver sulfide exposure at or below 3,000 mg/kg Ag is physiologically benign over a 58-d period.

Keywords—Silver sulfide Rainbow trout Accumulation Growth Food consumption

INTRODUCTION

Approximately 700,000 kg of silver enters aquatic environments in the United States on a yearly basis. Of this amount, 267,000 kg (38%) originates from anthropogenic activities, with photographic processing and photographic manufacturing facilities contributing at least 17% [1]. Reportedly, more than 99% of photographic facilities in the United States discharge their effluents into municipal sewers leading to publicly owned treatment works (POTWs) [2]. Schafer et al. [3] recently showed that more than 94% of the silver entering POTWs is removed from the effluent before leaving the facilities, a finding consistent with results of previous studies [4]. Silver in photographic industrial effluents is predominantly discharged as soluble, undissociated silver thiosulfate complexes [4]; however, during secondary waste treatment at POTWs, silver thiosulfate is converted to chemically inert silver sulfide ($\log K = 19.2$). Because of the low solubility of Ag_2S in water (solubility coefficient = 3×10^{-10} mg/L in natural waters), most of the silver entering POTWs is incorporated into sludge, which is later shipped away as solid waste. Discharged silver is largely in the form of colloidal silver sulfide and silver chloride compounds, soluble organic silver complexes, and silver bound to organic particles [1]. In any case, once in the natural environment, silver is expected to exist in either a colloidal or particulate phase [5] and is quickly scavenged by suspended sediments [6], ending up in the benthic portion of receiving waters.

In comparison to our considerable knowledge of the magnitude and effects of waterborne silver uptake, relatively little is known about the extent of uptake and toxicity of silver by means of food in aquatic organisms [7]. Garnier and Baudin [8] found that bioavailable silver was readily accumulated in

planktonic organisms but was not transferred throughout the food chain. However, evidence exists that carnivorous fish fed silver contaminated fish may accumulate silver through their food [9]. In addition, teleost fish may inadvertently ingest silver-laden sediments while feeding on benthic organisms, another source of dietary silver. Although bioaccumulated Ag is expected to be bound to sulfhydryl groups on proteins, silver may be further reduced to Ag_2S once internalized. Studies on bivalves suggest that up to 80% of their bioaccumulated silver is associated with sulfides and that only 20% is bound to protein [10]. Consequently, either direct or indirect exposure of fish to dietary Ag_2S is of environmental relevance and warrants investigation.

This article presents results obtained during a 58-d dietary exposure of juvenile rainbow trout to diets enriched in Ag_2S over a broad range of concentrations. The main objectives were to assess the bioavailability of dietary Ag_2S by measuring tissue burdens of silver in gills, kidney, intestine, and liver and to use growth and feeding rates as indicators of possible physiological effects of these exposures. In addition, copper concentrations in the liver were monitored to determine whether bioaccumulated silver was able to impair the homeostatic control of this biologically essential metal. Previous studies have shown that silver (as AgNO_3) can interfere with copper metabolism in rats [11]. Dietary concentrations used in this study ranged from 3 to 3,000 mg/kg Ag (as Ag_2S). Sediment concentrations for rivers receiving industrial effluents (e.g., Genesee River, NY, USA) may be as high as 55.4 mg/kg Ag [12]; the 3,000-mg/kg Ag diet was chosen to represent a worst-case scenario because the concentration is 100 times higher than that found at contaminated sites. Furthermore, the two lowest dietary concentrations (3 and 30 mg/kg Ag) represent levels typically found in many species of freshwater bivalves near sites of sewage outflow [13].

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Table 1. Mean daily food consumption, specific growth rates, and food conversion efficiencies for juvenile trout fed diets with silver concentrations ranging from 0 to 3,000 mg/kg (as Ag₂S)^a

	Silver concn. (mg/kg wet weight)				
	Control	3.0	30.0	300.0	3,000.0
Food consumption, ^b % wet weight/d	2.82A	2.22B	2.42AB	2.26AB	2.33AB
Specific growth rate, ^c % wet weight/d	1.37A	1.05B	1.81AB	1.05AB	1.47AB
Food conversion efficiency	48.6A	47.3B	74.8AB	46.5AB	63.1AB

^a Sample size used to derive these data decreased throughout the experiment because of fish sampling.

^b Significant differences ($p < 0.05$) in food consumption among treatment means exist wherever common letters are not shared.

^c No significant differences in specific growth rate were observed among treatments.

MATERIALS AND METHODS

Diet preparation

Silver-enriched trout diets were prepared from commercial trout food (Martin's Feed, Tavistock, ON, Canada) pulverized into a fine powder using a household blender. Silver sulfide (99.9%) (Ag₂S, lot JN 02006 BN, Aldrich Chemical, Milwaukee, WI, USA), oven-dried to a constant weight, was added to the commercial feed to achieve nominal concentrations of 3, 30, 300, or 3,000 mg Ag/kg. This formulation was mixed thoroughly for ~30 min, extruded using a pasta maker, and then shaped into pellets (average weight = 0.018 g per pellet). The extruded feed was dried at 50°C for ~36 h, sieved using a fine-mesh sieve to remove small fragments, and refrigerated until use. A control diet was also formulated in the same manner except that no silver was added.

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) ($n = \sim 500$) weighing 2 to 3 g were obtained from Humber Springs Hatchery (Orangeville, ON, Canada). Fish were held for at least 2 weeks in a circular polyethylene tank (~400 L capacity). The tank was supplied with aerated, dechlorinated Hamilton tap water at a flow rate of 1,000 ml/min. The chemical composition of the water was as follows (in mM): Na⁺, 0.6; Cl⁻, 0.7; Ca²⁺, 1.0; Mg²⁺, 0.2; and K⁺, 0.05; titratable alkalinity to pH 4.0 of 1.0, and pH was 7.8 to 8.0. Water temperature was kept at ambient conditions, which varied between 8 and 13°C. The fish were fed to satiation once daily with commercial trout pellets (Martin's Feed). Food consumption was monitored visually to ensure that all food offered to the fish was consumed; daily consumption in each treatment was recorded.

Experimental design

After acclimation, ~90 fish were transferred to each of five rectangular self-cleaning tanks. Each 60-L tank was aerated and supplied with a continuous flow of water at a rate of 500 ml/min. Fish were fed the commercial diet for an additional week, after which the study was started by switching to one of the experimental diets. The feeding regimen during the experimental phase was identical to that of the acclimation period. Although the tanks were self-cleaning, feces were siphoned daily from each tank after feeding to minimize coprophagy. The daily food consumption (FC) was calculated for each treatment on the basis of the percentage of weight consumed per day. Tank biomass was determined approx. every 2 weeks by placing fish in a large plastic colander and weighing the colander with and without the fish. Tank biomass (in g) was calculated as the difference between these weights. These

data were used to calculate specific growth rates (SGRs, in % wet weight/d). Specific growth rate values were obtained from the slopes of regression lines through the natural logarithm of fish weight versus time. Gross food conversion efficiencies (CEs) were calculated for each treatment using the following formula:

$$CE = 100(SGR/FC) \quad (1)$$

On days 24, 43, and 58, eight fish per treatment were randomly selected. Fish were killed with a quick blow to the head, dried, and weighed, and then the liver, intestine (from the pyloric ceca to the anus), entire gill basket, and kidney were dissected from each fish for silver analysis. Gills were rinsed in 18-M Ω deionized water to remove any loosely bound particulate matter. The intestinal tract was flushed with Cortland saline [14] and scraped with a fine spatula to remove any undigested feed or feces. All tissues were blotted dry, weighed, placed in centrifuge tubes, frozen in liquid nitrogen, and then stored at -70°C until further processing.

Gills (0.06–0.37 g), kidney (0.01–0.33 g), intestine (0.01–0.07 g), and liver (0.02–0.33 g) were digested in acid-washed glass test tubes with 2 ml of concentrated trace-metal-grade HNO₃ (Baker, Toronto, ON, Canada) and 400 μ l of H₂O₂ (Caledon Chemicals, Georgetown, ON, Canada) at 120°C. Digests were evaporated without allowing the digested residue to become ashed. Tissues were then reconstituted with 2 to 5 ml of 0.5% HNO₃ (exact volume noted) and analyzed for silver by graphite furnace atomic absorption (Varian GTA-95). In addition, a subsample of the reconstituted liver digest was used for analysis of copper in liver by flame atomic absorption (Varian AA 1275).

Specific growth rates are expressed as the percentage of weight per day \pm SE. Values between treatments were statistically compared using an analysis of covariance in JMP 3.1 (SAS Institute, Cary, NC, USA). Tissue metal burden levels and food consumption rates for each treatment are expressed as the mean \pm SE. Mean values were statistically compared using a one-way analysis of variance in the SPSS (version 8) statistical package. This was followed by the Tukey-Kramer post hoc test, which allows multiple comparisons between treatments. The level of statistical significance for all analyses was $p < 0.05$.

RESULTS

Food consumption rates were monitored in each dietary Ag₂S treatment and the relevant control group. Food consumption rates decreased between 14 and 22% in fish fed the silver-laden diets when compared with the control. However, apart from the 3-mg/kg Ag group, these reductions in food consumption were not statistically significant (Table 1). No

Table 2. Mean intestinal silver concentrations (\pm SE) ($n = 8$) of juvenile trout on days 24, 43, and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 mg/kg (as Ag_2S)^a

Day	Silver concn. (mg/kg wet weight)				
	Control	3.0	30.0	300.0	3,000.0
24	0.26 (0.10)	0.12 (0.02)	0.13 (0.03)	0.15 (0.02)	1.24 (0.65)
43	0.24 (0.04)	0.17 (0.03)	0.19 (0.04)	0.30 (0.14)	0.38 (0.14)
58	0.11 (0.01)	0.10 (0.02)	0.61 (0.43)	0.19 (0.06)	1.80 (1.38)

^a No significant differences among treatments ($p < 0.05$).

significant differences in SGR were observed among groups, and no clear trend was noted between the level of Ag_2S in the diet and mean growth rates. Similarly, food conversion efficiencies were not affected in a dose-dependent manner (Table 1).

No significant differences were observed in the concentrations of silver accumulated in the intestinal tissue among any of the fish fed the silver-enriched diets and controls. Intestinal silver concentrations typically ranged between 0.10 and 0.30 mg/kg Ag in all of the treatments, except in fish fed the 3,000-mg/kg Ag diet, which had a mean measured value of 1.80 mg/kg Ag by day 58 (Table 2). Despite a sixfold increase in intestinal silver concentration for this group (above control levels), the elevation was not statistically significant because of large variability among individuals.

In comparison, control silver levels in gills were one order of magnitude lower than in the intestine. Silver burdens in gill in the 3,000-mg/kg Ag group were significantly elevated ($p < 0.05$) compared to all other treatments on day 24 but not on day 43 of the experiment (Table 3). Fish fed the 3,000-mg/kg Ag diet exhibited moderate yet significant ($p < 0.05$) accumulation of silver in their livers only at day 43. Hepatic silver concentrations for this treatment (0.52 ± 0.09 mg/kg Ag) were approximately fivefold higher than control levels (Fig. 1). However, by day 58, silver levels in the liver of fish fed the control and 3,000-mg/kg Ag diets were not significantly different. Hepatic copper levels were also monitored to assess whether accumulated silver disrupted regulation of this essential metal. Figure 2 shows steady accumulation of hepatic copper between days 24 and 58 in all treatments, regardless of the silver concentration in the diet. Apart from a twofold elevation on day 43 in hepatic copper levels in the fish fed the 300-mg/kg Ag diet relative to controls, there appears to be no significant effect of dietary silver on copper levels in the liver.

Table 3. Mean gill silver concentrations (\pm SE) ($n = 8$) of juvenile trout on days 24, 43, and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 mg/kg (as Ag_2S)^a

Day	Silver concn. (mg/kg wet weight)				
	Control	3.0	30.0	300.0	3,000.0
24	0.054 (0.005)A	0.431 (0.252)A	0.101 (0.059)A	0.673 (0.374)AB	2.470 (1.200)B
43	0.060 (0.01)	0.053 (0.01)	0.126 (0.03)	0.139 (0.05)	1.043 (0.92)

^a Significant differences ($p < 0.05$) among treatment means exist wherever common letters are not shared. No significant differences were observed between treatments on day 43.

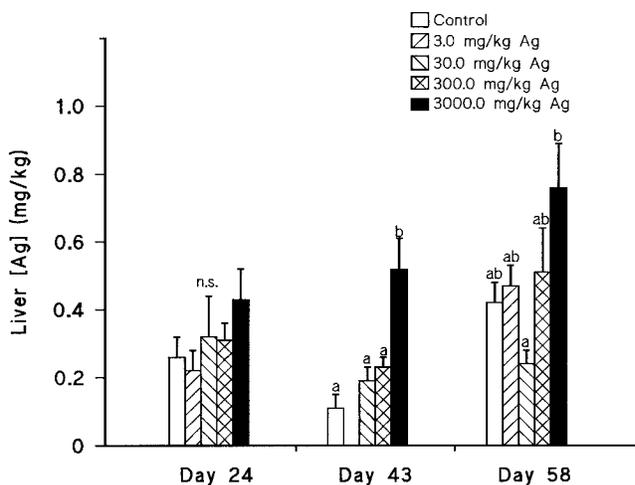


Fig. 1. Mean liver silver concentrations \pm SE ($n = 8$) (mg/kg wet weight) of juvenile trout on days 24, 43, and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 mg/kg (as Ag_2S). Significant differences ($p < 0.05$) among treatment means exist wherever common letters are not shared. n.s. = not significant.

In addition, silver concentrations in the kidney showed no discernable trend throughout the 58-d exposure, although silver levels in the kidneys of fish fed the 3,000-mg/kg Ag diet tended to be higher (0.49 ± 0.24 vs 0.08 ± 0.01 mg/kg in control) (Fig. 3). Interestingly, kidney silver concentrations were elevated in all treatments above 30 mg/kg Ag (compared to controls) on day 24, although this increase was not significant. However, kidney silver levels for fish fed the 30- and 300-mg/kg Ag diets decreased thereafter.

DISCUSSION

Previous studies have shown that various species of waterborne silver are able to accumulate in tissues, especially the liver, at extremely high rates. In a recent review, Hogstrand and Wood [7] tabulated silver concentration specific accumulation rates (CSARs) (in $\text{ng/g}\cdot\text{d}^{-1}\cdot\text{ppb}^{-1}$) in the liver of juvenile trout during a variety of waterborne silver exposures. By normalizing for both silver concentration and duration of exposure, they were able to rank the bioavailability of different

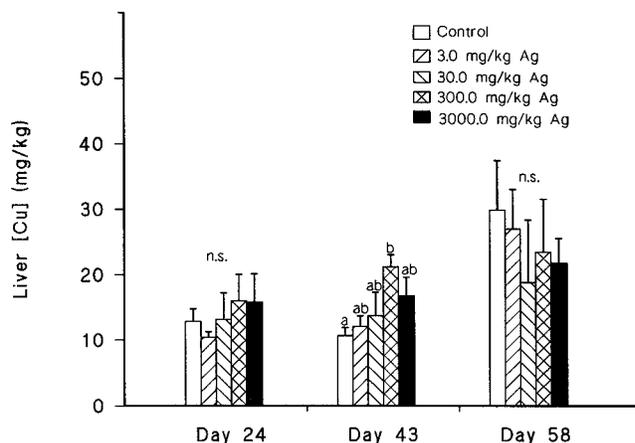


Fig. 2. Mean liver copper concentrations \pm SE ($n = 8$) (mg/kg wet weight) of juvenile trout on days 24, 43, and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 mg/kg (as Ag_2S). Significant differences ($p < 0.05$) among treatment means exist wherever common letters are not shared. n.s. = not significant.

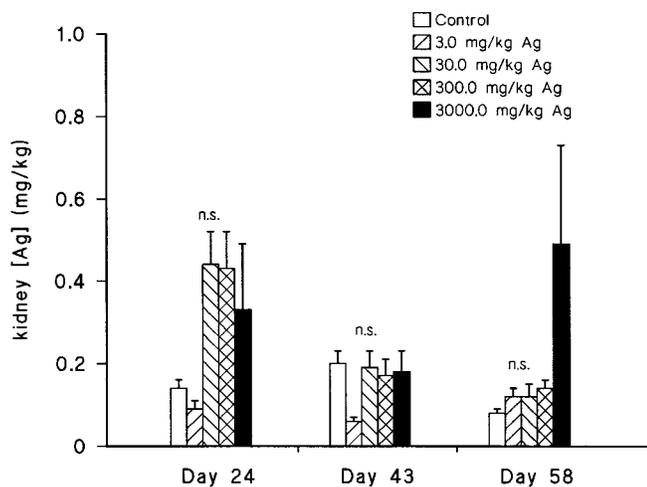


Fig. 3. Mean kidney silver concentrations \pm SE ($n = 8$) (mg/kg wet weight) of juvenile trout on days 24, 43, and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 mg/kg (as Ag_2S). No significant differences were observed between treatments. n.s. = not significant.

waterborne silver species. They found that juvenile trout had CSARs as high as $2,800 \text{ ng/g}\cdot\text{d}^{-1}\cdot\text{ppb}^{-1}$ for AgNO_3 exposures in freshwater. However, if acutely exposed to elevated concentrations of negatively charged AgCl_n^{1-n} ($n > 2$) or $\text{Ag}(\text{S}_2\text{O}_3)_n^{n-}$ ($n > 1$) complexes, CSAR values decreased significantly to between 0.34 to $0.38 \text{ ng/g}\cdot\text{d}^{-1}\cdot\text{ppb}^{-1}$. They suggested that both negatively charged silver species and highly complexed silver (i.e., thiosulfates; $\log K = 8\text{--}14$) were less bioavailable in water than easily dissociable forms of silver (i.e., AgNO_3).

The present study was designed to address whether silver, via dietary Ag_2S exposure, is bioavailable to freshwater teleost fish. It was observed that silver levels in the liver of fish fed the 3,000-mg/kg diet were approximately fourfold higher than control levels by day 43, representing an accumulation rate of $9.5 \text{ ng/g}\cdot\text{d}^{-1}$. However, if normalized for silver level in the diet, the hepatic CSAR value for dietary Ag_2S exposure was only $3.2 \times 10^{-6} \text{ ng/g}\cdot\text{d}^{-1}\cdot\text{ppb}^{-1}$. In comparison, we recently showed that juvenile trout exposed to a dietary exposure of biologically incorporated silver ($\sim 3 \text{ mg/kg Ag}$) exhibited liver concentrations 12-fold higher than those of controls after 3 months [15]. This represents a CSAR of $0.18 \text{ ng/g}\cdot\text{d}^{-1}\cdot\text{ppb}^{-1}$. It can therefore be concluded that the bioavailability of dietary silver is dependent on speciation, similar to the situation seen with waterborne silver exposures. With a $\log K$ of 19.2, Ag_2S is probably not dissociated within the gut lumen during digestion, thus preventing internalization of the metal. This is further substantiated considering the low rates of accumulation of silver in the kidney in response to dietary Ag_2S exposure. Recently, a 28-d bioaccumulation study was performed in the oligochaete *Lumbriculus variegatus* exposed to laboratory-spiked sediments containing $\sim 444 \text{ mg Ag/kg}$ dry weight (as Ag_2S) [16]. Hirsch [16] reported tissue silver levels of 80.3 mg/kg dry weight at the end of the test, representing a CSAR of only $6.4 \times 10^{-3} \text{ ng/g}\cdot\text{d}^{-1}\cdot\text{ppb}^{-1}$.

The elevated levels of silver in the intestine (3,000-mg/kg Ag diet only) are likely due to adsorption of Ag_2S at the apical membrane of the gut epithelium. This outer integument is continually sloughed off as a result of the movement of food through the gut lumen. Consequently, adsorbed silver at the

gut epithelium is not expected to be of physiological relevance to the fish. In contrast, biologically incorporated silver is likely bound to proteins. During digestion, proteases should break down these proteins to form amino acid–silver complexes, which may be absorbed via specific amino acid transporters [17]. This uptake route may explain the higher accumulation rate of biologically incorporated silver seen by Galvez et al. [15] compared with dietary Ag_2S .

No significant differences were observed in growth rates of fish fed either the control diet or diets with Ag_2S levels as high as 3,000 mg/kg Ag , suggesting a lack of any physiological perturbation, which is consistent with results of previous studies [15,16]. The 14 to 22% decrease in food consumption observed between the control and Ag_2S dietary treatments may have been due to a decreased palatability of the silver-laden diet. However, despite this slight decrease in food consumption, no significant effect on SGR was seen. As a result, the gross conversion efficiency of fish fed the 30- and 3,000-mg/kg Ag diets increased slightly compared with the control treatment. This is consistent with growth-ration curves, which typically show that SGRs begin to plateau before maximal ration levels are reached. The sum of these effects (further elevation in food consumption with no additional increase in specific growth rate) explains why gross conversion efficiencies tend to decrease as daily food consumption rates approach satiation [18]. In a study in which brown trout (*Salmo trutta*) were fed a daily ration (5% daily) of juvenile carp preexposed to $4.3 \mu\text{g/L } ^{110\text{m}}\text{Ag}$ (as AgCN) for 3 d, no significant effects on growth rates were observed [8]. Similarly, preliminary results from a 4-month study in which juvenile trout were fed biologically incorporated silver [15] suggest that dietary silver exposure is physiologically benign.

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