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TOXICITY, SILVER ACCUMULATION AND METALLOTHIONEIN INDUCTION IN FRESHWATER RAINBOW TROUT DURING EXPOSURE TO DIFFERENT SILVER SALTS

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Abstract—Static-renewal 168-h toxicity tests of silver nitrate ($AgNO_3$), silver chloride ($AgCl_n$), and silver thiosulfate ($Ag(S_2O_3)_n$) with juvenile rainbow trout (*Oncorhyncus mykiss*) were performed by standard methods. Because of low solubility of AgCl(s), bioassays for $AgCl_n$ were performed in two separate ways. In one test series, AgCl(s) was added to freshwater and in another, $AgCl_n$(aq) was generated by adding $AgNO_3$ to freshwater supplemented with 50 mM NaCl. Concentrations of Ag and metallothionein (MT) were analyzed in gills and livers of fish that survived the exposures. Although Ag added as $AgNO_3$ was found to be highly toxic to rainbow trout (168-h LC50 = 9.1 μg Ag L^{-1}), the toxicities of the other Ag salts were low. The 168-h LC50 for $Ag(S_2O_3)_n$ was 137,000 μg Ag L^{-1} and no mortality was observed in $AgCl_n$ (100,000 μg Ag L^{-1}). Exposure to $AgNO_3$, $Ag(S_2O_3)_n$, or $AgCl_n$ caused accumulation of Ag and induction of MT. Highest Ag levels were found in livers of trout exposed to 164,000 μg Ag L^{-1} as $Ag(S_2O_3)_n$. In these fish, the hepatic Ag concentration was increased 335 times from the control value. The MT levels in gills and liver increased with the water Ag concentration and the highest level of MT was found in liver of fish exposed to $Ag(S_2O_3)_n$.

Keywords—Silver Toxicity Accumulation Metallothionein Fish

INTRODUCTION

The free silver ion, Ag^+, is known to be highly toxic to freshwater animals [1–4]. The U.S. Environmental Protection Agency (EPA) has therefore imposed strict water quality criteria for Ag [5]. These criteria are based on total recoverable Ag, rather than the concentration of Ag^+. The photographic industry, which is an important source of Ag in effluents, discharges Ag almost entirely as silver thiosulfate ($Ag(S_2O_3)_n$) [6]. This complex readily dissolves in water, but its high chemical stability ($AgS_2O_3^-$: log K = 8.8; $Ag(S_2O_3)_2^{3-}$: log K = 13.6) effectively prevents the formation of Ag^+ [6,7]. Furthermore, as the Ag^+ is extremely reactive, it complexes with high affinity to anionic sites in sediments and dissolved particles [8]. In fact, the binding of Ag^+ to inorganic and organic matter is so strong that only picomolar concentrations of Ag^+ are left in natural waters [9]. These concentrations correspond to less than 2% of the 96-h LC50 for $AgNO_3$ to fish [2]. Thus, it appears that a pollution prevention regulation based on total recoverable Ag may not represent the critical component associated with Ag toxicity (i.e., free silver ion in the aquatic environment).

Although the toxicity of Ag^+ (tested as $AgNO_3$) to freshwater organisms is relatively well documented, less is known about the toxicity of the major dissolved Ag species, $Ag(S_2O_3)_n$ and Ag chlorides ($AgCl_n$). Available information suggests that $Ag(S_2O_3)_n$ is several thousand times less toxic to fathead minnows (*Pimephales promelas*) than $AgNO_3$ and that $AgCl_n$ is at least 300 times less toxic than $AgNO_3$ [3,10]. Although these pioneer studies indicate that the free Ag^+ is the Ag congener of concern for freshwater environments, there is a need to complement the data and establish LC50 values for $Ag(S_2O_3)_n$ and $AgCl_n$.

Another concern is the potential accumulation of Ag in biota. In particular, can Ag be taken up and accumulated from the more abundant $Ag(S_2O_3)_n$ and $AgCl_n$? Can chronic exposure to sublethal concentrations of waterborne Ag cause a buildup of Ag in tissues leading to long-term physiological effects? There is very little information about the uptake and accumulation of different species of waterborne Ag in freshwater fish. Exposure of brown trout (*Salmo trutta*) to trace levels of waterborne ^{110m}Ag, in the form of AgCN, resulted in a slow accumulation of radioactivity in the body with an extrapolated time to equilibrium of up to 2.5 years [11]. The liver was the primary organ for Ag accumulation, accounting for 70% of the total radioactivity in the fish. Largemouth bass (*Micropterus salmoides*) exposed to $AgNO_3$ during a 4-month period showed a markedly elevated level of Ag in gills, pooled internal organs, and carcass [12]. However, in contrast to the study of Garnier et al. [11] using ^{110m}AgCN, the bass did not continue to increase the body burden of Ag after the first 2 months of exposure. Finally, there is evidence that waterborne $Ag(S_2O_3)_n$ can be a source for Ag uptake in fish. Fathead minnows appeared to accumulate Ag during a 10-week exposure to fluctuating concentrations of $Ag(S_2O_3)_n$ (<20–60 μg L^{-1}) [13].

Exposure of fish to waterborne metals often evokes adaptive responses, such as reduced metal uptake, replacement of destroyed enzymes, and increased intracellular capacity to sequester the metal [14–16]. Metallothionein (MT) is a small, cysteine-rich, intracellular protein that avidly binds metals of groups IB and IIB of the Periodic System [17,18]. This protein has the unusual property of being induced by increased levels of intracellular copper, zinc, cadmium, or mercury. The newly synthesized apo-MT will bind the excess metal and may thereby reduce the chance of metal toxicity [19–21]. In mammalian cells in vitro, Ag seems to be one of the more potent inducers of MT [22], but nothing is known about the ability of Ag to elicit MT synthesis in nonmammalian species. Thus, in order to assess the ability of fish to detoxify accumulated Ag, it is necessary to establish whether or not Ag is an inducer of MT synthesis.

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The first objective of the present study was to determine the 96-h and 168-h LC50s of $AgNO_3$, $Ag(S_2O_3)_n$, and $AgCl_n$ to freshwater rainbow trout (*Oncorhynchus mykiss*). Based on the findings from the experiments with $AgCl_n$, we question current water criteria used by the EPA to regulate maximum recoverable Ag [5,23]. Although these criteria are based on water hardness as the single modulator of Ag toxicity, we suggest that the $[Cl^-]$ may be a more important variable in ameliorating acute toxicity of Ag. Our second objective was to study the abilities of the different forms of Ag to accumulate in fish and to evoke MT synthesis. This objective was accomplished by analysis of Ag and MT in gills and liver of surviving fish from the bioassays.

MATERIALS AND METHODS

Animals

Rainbow trout (1–4 g) were purchased from a local hatchery (Rainbow Springs Hatchery, Thamesford, ON, Canada). The fish were held in a 100-L polyethylene tank for at least 2 weeks prior to experimentation. The tank was supplied at a rate of 900 ml min^{-1} with a flow-through of dechlorinated, aerated Hamilton city tap water ($[Na^+]$ = 0.6 mM; $[Cl^-]$ = 0.7 mM; $[Ca^{2+}]$ = 1.0 mM; $[HCO_3^-]$ = 1.9 mM; pH 7.9–8.2). The temperature was 15°C. Fish were fed dry trout pellets (Martin's Feed Mill, Elmira, ON, Canada) to satiation three times a week. No food was given during the last 2 days prior to experimentation.

Bioassay for $AgNO_3$

Bioassay methods followed the general principles outlined by Sprague [24]. Fourteen 50-L polyethylene bioassay tanks were filled with dechlorinated Hamilton city tap water. Each tank was equipped with a lid and an airline. To 13 of these tanks, $AgNO_3$ (analytical grade, Sigma Chemical, St. Louis, MO, USA) was added from a stock solution to yield total Ag concentrations ranging from 0.1 to 1,000 μg L^{-1} in a semilogarithmic series. One tank, which contained tap water only, served as control. Ten fish were put into each bioassay tank at the start of the test. Eighty percent of the water was replaced daily and a water sample was taken before and after each daily water renewal for analysis of total Ag. There was no measurable change in the total dissolved Ag concentrations between water renewals. The fish were monitored at 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 24 h, and three times daily during the following 6 days. Cessation of opercular movements was used as the criterion for mortality. Dead fish were removed continuously. Fish were not fed during the bioassay.

On day 7, the experiment was terminated and surviving fish from selected tanks were sampled for Ag and MT in gills and liver. These fish were killed by a blow to the head. Gills and liver were excised and placed in preweighed polypropylene vials. The vials were then weighed again, and frozen in liquid nitrogen. The samples were stored at −80°C until analysis.

Bioassay for $Ag(S_2O_3)_n$

The experimental setup and protocol was largely as described above for the bioassay for $AgNO_3$. Silver thiosulfate was synthesized by mixing AgCl with $Na_2S_2O_3 \cdot 5H_2O$ (BDH, Toronto, ON, Canada) in a molar ratio of 1:4. The 16 concentrations of $Ag(S_2O_3)_n$ tested ranged from 32 μg Ag L^{-1} to 1,000,000 μg Ag L^{-1}. There was on average a 7.0% decrease in the measured total dissolved Ag concentrations between water renewals. The effect of the highest $Na_2S_2O_3$ concentration used (37 mM L^{-1}), was tested in four replicate exposure tanks. Duplicate tanks containing dechlorinated tap water only served as controls.

Bioassay for $AgCl_n$

Toxicity of $AgCl_n$ was tested in freshwater, and freshwater supplemented with 50 mM NaCl (brackish water). In plain freshwater, the bioassay was carried out using AgCl(s) (Sigma). Nine concentrations were tested in freshwater, ranging nominally from 0.1 to 1,000 μg Ag L^{-1} in a semilogarithmic series, but it was clear that a particulate suspension was being tested, because of the low solubility of AgCl(s) salt in freshwater. The low solubility of $AgCl_n$ was reflected in a 20% decrease in the ''concentrations'' of total dissolved/suspended Ag between water renewals. To overcome this problem, we adopted the approach used by LeBlanc et al. [3]. Fish were acclimated for 2 weeks to freshwater supplemented with 50 mM NaCl, and then exposed to $AgNO_3$ in this medium. A total of 11 concentrations, ranging from 100 to 100,000 μg Ag L^{-1}, were tested under these conditions. Speciation calculations, using MINEQL+ [25] or MINTEQA2 [26] indicated that in the water supplemented with 50 mM NaCl, essentially all Ag in the test medium was present as $AgCl_n$. Data obtained from computer modelling was supported by analysis of Ag in the water. The measured concentrations of total dissolved Ag were very close to the nominal and they decreased no more than 4.4% between water renewals. However, with increasing [Ag], an increasing percentage of the $AgCl_n$ precipitated in the form of cerargyrite. In all other aspects, the bioassays for $AgCl_n$ were performed as described above for $AgNO_3$.

Analysis of silver in water

Water samples from the bioassays were acidified by the addition of 0.5% (v/v) of HNO_3 (trace metal grade; J.T. Baker, Missisauga, ON, Canada). The samples were analyzed for Ag by atomic absorption spectroscopy (AAS), using a graphite furnace or an air–acetylene flame as atomizer (Varian AA 1275). With the graphite furnace and a multiple injection procedure, the detection limit for the method was 0.1 μg Ag L^{-1}. Presented values for water Ag concentrations are the measured average concentrations of total dissolved/suspended Ag during each test. Measured concentrations of Ag were also used for determinations of LC50 values.

Analysis of Ag and MT in gills and livers

Gills and livers (10–100 mg) were homogenized individually in 1.00 ml of 50 mM Tris-HCl, pH 8.0, at 0°C, using a glass–Teflon homogenizer. A 400-μl aliquot of each homogenate was withdrawn and saved in Eppendorf tubes at −80°C for subsequent preparation for Ag analysis. The rest of the homogenate was centrifuged at 10,000 *g*, 4°C, for 20 min. The supernatant was transferred to Eppendorf tubes and stored at −80°C until analyzed for MT content.

Homogenates from gills and livers were digested in acid-washed glass tubes for 1 hour with five volumes of 70% HNO_3 at 120°C. The samples were then cooled to room temperature and 0.75 volumes of H_2O_2 was added. The digests were evaporated to dryness at 120°C. Five milliliters of 0.5% HNO_3 was finally added to the digestion tubes and Ag was analyzed by atomic absorption spectroscopy as described above. No solid material was present in the final digest.

Metallothionein levels were analyzed with a double antibody radioimmunoassay (RIA), using rabbit antiserum raised against MT from perch, *Perca fluviatilis*, as the first antibody, ^{125}I-labelled rainbow trout MT as tracer, and goat anti-rabbit IgG as the second antibody [27]. The MT (I and II) from rainbow

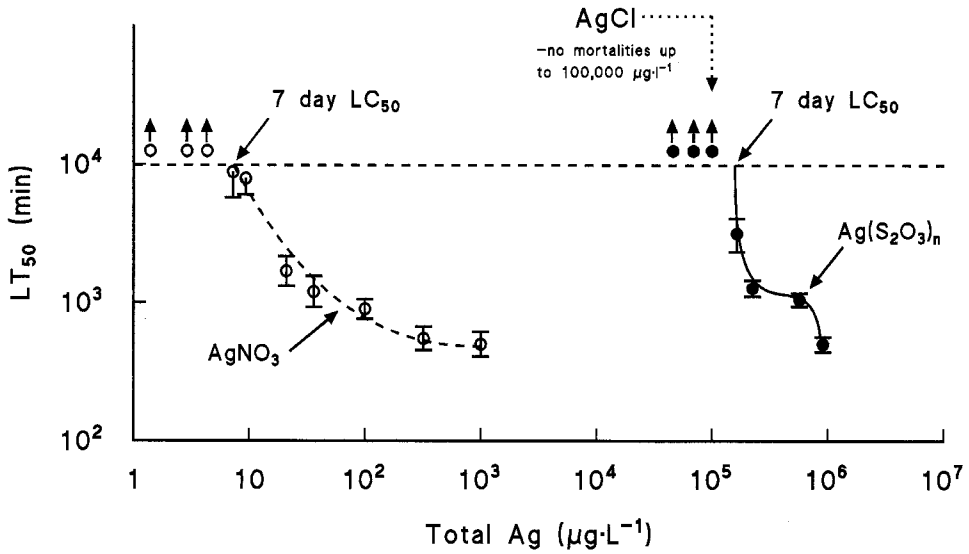

Fig. 1. Toxicity curves for $AgNO_3$, $AgCl_n$, and $Ag(S_2O_3)_n$ with juvenile rainbow trout. The data are presented as the median lethal times (LT50) ± 95% CL ($N = 10$) at each test concentration. Lines were fitted by eye. The 168-h median lethal concentrations (LC50) from the $AgNO_3$ and $Ag(S_2O_3)_n$ tests are indicated. No mortalities were observed in fish exposed to an $AgCl_n$ level of 1,000 μg Ag L^{-1} added as AgCl(s) in freshwater or 100,000 μg Ag L^{-1} added as $AgNO_3$ in brackish water (50 mM NaCl).

trout, used as tracer, was purified according to Olsson and Haux [28] with the modifications described by Hogstrand and Haux [27] for perch MT. A 10,000-*g* supernatant prepared from the livers of Cd-injected rainbow trout was used as MT standard. The MT content of the standard was calibrated against a standard curve prepared from purified rainbow trout MT [27,29]. The working range of the RIA was 10 to 100 ng rainbow trout MT per assay tube, which corresponds to 0.6 to 6 μg g^{-1} liver wet weight.

Statistical methods

Median lethal time (LT50) values were obtained from mortality plots by standard log-probit analysis [24], employing the nomographic method by Litchfield and Wilcoxon [30] to calculate 95% confidence limits. Toxicity curves were plotted as log LT50 versus log concentration (e.g., Fig. 1) to check whether or not acute mortality had ceased (i.e., whether true incipient LC50 values were achieved) [24]. The partial mortality data at 94 h and 168 h were used to calculate the 96- and 168-h LC50 values ± 96% confidence limits, again by log-probit analysis. All calculations were based on mean concentrations measured in the tanks, not nominal values. Differences between groups in tissue concentrations of Ag and MT were tested with the Mann–Whitney *U* test or where appropriate with ANOVA followed by the Tukey HSD test.

RESULTS

Silver nitrate in freshwater (yielding 60% $AgCl^0$, 34% Ag^+, and 5% $AgCl_2$ by MINEQL+) was by far the most toxic form of Ag tested (Fig. 1). The mean 96- and 168-h LC50 values for $AgNO_3$ to juvenile rainbow trout were 11.8 (95% confidence limits: 10.9–13.8) μg Ag L^{-1} and 9.1 (7.3–11.3) μg Ag L^{-1}, respectively. The 96-h LC50 for $Ag(S_2O_3)_n$ was 161,000 (146,000–177,000) and the 168-h LC50 was 137,000 (118,000–159,000) μg Ag L^{-1}. Sodium thiosulfate itself was not toxic at 37 mM. Thus, $Ag(S_2O_3)_n$ was about 15,000 times less toxic than Ag added as $AgNO_3$.

Silver chlorides, introduced as AgCl(s) in freshwater, or by adding $AgNO_3$ to water containing 50 mM NaCl, exerted no observed toxicity. After 7 days of exposure, there were no mortalities up to the highest ''concentrations'' tested. For AgCl(s) in freshwater, this ''concentration'' was 1,000 μg Ag L^{-1}, although essentially no Ag was present in solution after filtration. The highest [$AgCl_n$] tested in 50 mM NaCl was 100,000 μg Ag L^{-1}. Speciation calculations using the computer programs MINEQL+ [25] or MINTEQA2 [26] indicated that, although addition of $AgNO_3$ to 50 mM NaCl yields $AgCl_n$ almost exclusively, large quantities of cerargyrite are formed at higher ''concentrations'' of Ag. At the highest level of $AgCl_n$ tested, 100,000 μg Ag L^{-1}, the calculated concentration of dissolved $AgCl_n$ was about 120 μg Ag L^{-1}. Thus, dissolved $AgCl_n$ is at least 10 times less toxic than Ag added as $AgNO_3$ and particulate $AgCl_n$ appears to be nontoxic at environmentally realistic concentrations.

The observed ameliorating effect of Cl^- on Ag toxicity led us to critically reanalyze one of the most detailed $AgNO_3$ toxicity studies in the literature. Lemke [31] reported the results of an EPA-sponsored interlaboratory comparison, in which the acute toxicity of $AgNO_3$ to rainbow trout varied markedly between laboratories (96-h LC50: 11.8–280.0 μg Ag L^{-1}). Upon plotting the data tabulated by Lemke [31] against water [Ca^{2+}] and [Cl^-], respectively, we found no correlation between the 96-h LC50 and water [Ca^{2+}] but a highly significant correlation with the [Cl^-] of the water (Fig. 2).

Analysis of Ag in gills and liver from fish that survived the 168-h bioassays demonstrated that Ag, added as $AgNO_3$, $Ag(S_2O_3)_n$, AgCl(s) in freshwater, or $AgNO_3$ in 50 mM NaCl ($AgCl_n$) accumulated in the body (Figs. 3–5). The highest level of tissue Ag was observed in livers of fish exposed to $Ag(S_2O_3)_n$, but $AgCl_n$-exposed fish also exhibited a marked increase in hepatic Ag burden (Figs. 4 and 5). The level of Ag in gills was generally lower than that in the liver, with the exception for $AgCl_n$-exposed fish. Little Ag accumulated in fish exposed to 1,000 μg Ag L^{-1} as AgCl(s) in freshwater, indicating that particulate AgCl is not readily available for uptake by rainbow trout (Fig. 5). The MT levels in gills and liver reflected the concentration of dissolved Ag in water (Figs. 3 to 5). The concentration of MT was much higher in liver than in gills even in fish exposed to $AgCl_n$ in 50 mM NaCl, where the branchial

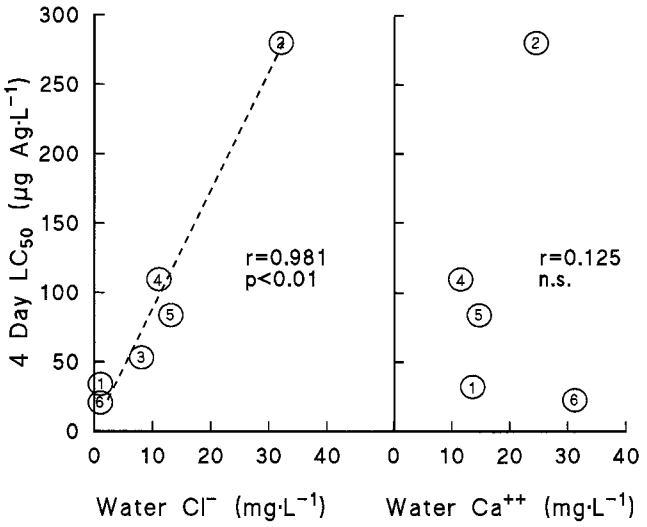

Fig. 2. Plot of data published by Lemke [31] on the toxicity of $AgNO_3$ to juvenile rainbow trout, indicating the close correlation between toxicity and water [Cl^-], and the lack of importance of water [Ca^{2+}] in modifying the 96-h LC50. Numbers refer to coded laboratories in the original report.

Ag concentration was very high. Exposure to $Ag(S_2O_3)_n$ (Fig. 4) or $AgCl_n$ in 50 mM NaCl (Fig. 5) resulted in markedly elevated levels of hepatic MT. Particulate silver chloride exposure in freshwater at 1,000 µg Ag L^{-1} did not affect MT concentrations, which is consistent with the low accumulation of Ag in this group (Fig. 5).

Considering the high concentrations of Ag employed in some of the exposures, in particular where the effects of $Ag(S_2O_3)_n$ and $AgCl_n$ were studied, it could be contemplated that Ag accumulation may be a function of the ionic Ag^+ concentration rather than the concentrations of the prevalent Ag complexes. To investigate this possibility the tissue Ag concentrations of all sampled fish, exposed to Ag in any form, were plotted against the Ag^+ concentrations of the water, as calculated by MINEQL+ [25]. These plots showed that there were no significant correlations between the levels of Ag in liver ($r^2 = 0.1$) or gills ($r^2 = 0.4$) and the concentration of ionic Ag^+ in the water.

DISCUSSION

Although relatively good information is available about the acute toxicity of $AgNO_3$ to fish [1–4], little data is available on the relative toxicity of different Ag salts [3,10]. Most notably, no LC50 value has been published previously for $Ag(S_2O_3)_n$, which is the form of Ag discharged from photographic processing facilities. The present study provides 96- and 168-h LC50 values for $Ag(S_2O_3)_n$ to rainbow trout and demonstrates that $Ag(S_2O_3)_n$ is more than four orders of magnitude less toxic than $AgNO_3$. Although LC50 values could not be determined for $AgCl_n$, we conclude that for rainbow trout, dissolved $AgCl_n$ is at least 10 times less toxic than $AgNO_3$, that particulate AgCl is nontoxic even at ''industrial concentrations,'' and that that the level of total Ag required to produce toxicity from $AgCl_n$ in freshwater and brackish water exceeds 100,000 µg Ag L^{-1}. The data emphasize the remarkable effect that speciation has on Ag toxicity and highlight the dramatic difference between the extremely toxic $AgNO_3$ and the nontoxic $Ag(S_2O_3)_n$ and $AgCl_n$.

The toxicity data for $AgNO_3$ from the present study compare well with data published by other workers. Literature 96-h LC50

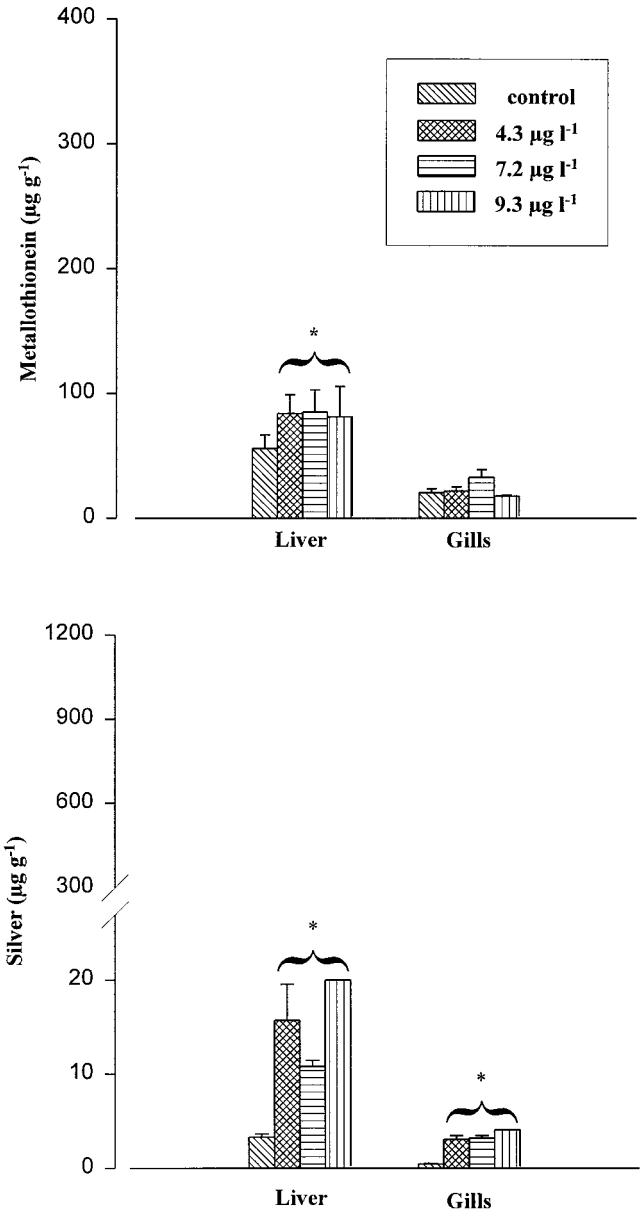

Fig. 3. Metallothionein (MT) and silver concentrations in liver and gills of juvenile rainbow trout, exposed for 7 days to $AgNO_3$ at 4.3 ($N = 9$), 7.2 ($N = 5$), and 9.3 ($N = 2$) µg Ag L^{-1} (measured average Ag concentrations). A control group was held under identical conditions ($N = 10$). Vertical error bars denote one-way SE. The hepatic levels of MT and Ag were elevated ($p < 0.05$) in exposed fish, tested as one single group against the controls (Mann–Whitney U test). Using the same approach of analysis, the Ag concentration of gills from exposed fish was higher than in control fish.

values for $AgNO_3$ to rainbow trout range from 6.5 to 280.0 µg Ag L^{-1} [2,31,32]. The 96-h LC50 for $AgNO_3$ obtained in the present study was 11.8 µg Ag L^{-1}. Acute toxicity data for $AgNO_3$ have also been published for fathead minnows. This species does not seem to differ from the rainbow trout in terms of sensitivity to $AgNO_3$ [2,3,31]. As far as we are aware, there are no previously published LC50 values for $Ag(S_2O_3)_n$ to fish. However, earlier data strongly suggest that $Ag(S_2O_3)_n$ is much less toxic than $AgNO_3$. Terhaar et al. [10] found 25% mortality in fathead minnows after 4 days of exposure to 250,000 µg Ag L^{-1} as $Ag(S_2O_3)_n$. Using the same fish species, LeBlanc et al. [3] observed no mortality after 4 days exposure to $Ag(S_2O_3)_n$

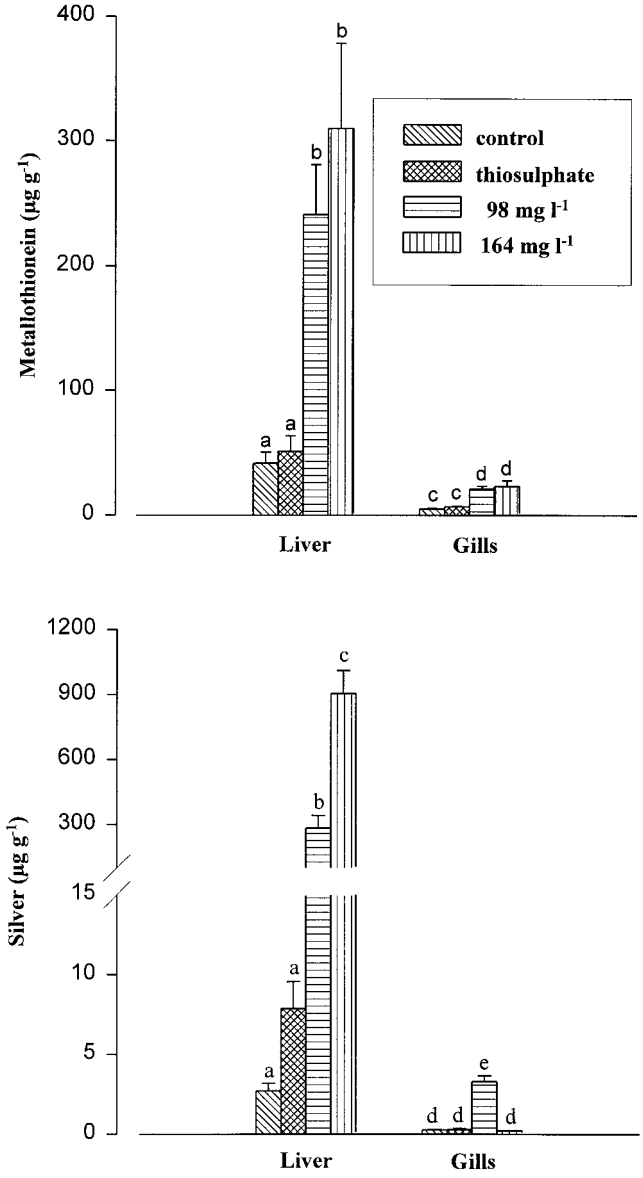

Fig. 4. Metallothionein (MT) and silver concentrations in liver and gills of juvenile rainbow trout, exposed for 7 days to $Ag(S_2O_3)_n$ at 98 ($N = 17$), or 164 ($N = 3$) mg Ag L^{-1} (measured Ag concentrations). A control group was held under identical conditions ($N = 10$ for MT and 23 for Ag). In addition, one group of fish was exposed to 37 mM of $Na_2S_2O_3$ ($N = 5$ for MT and 8 for Ag). Vertical error bars denote one-way SE. Groups with common letter superscripts were not statistically different in a Tukey HSD test following a one-way ANOVA.

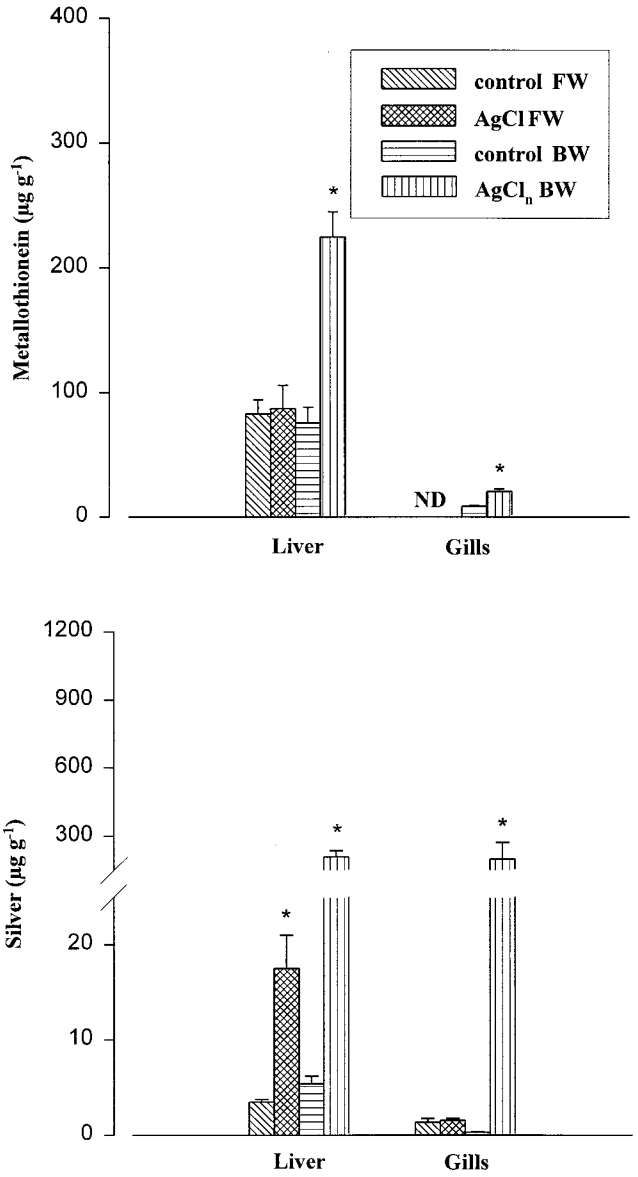

Fig. 5. Metallothionein (MT) and silver concentrations in liver of juvenile rainbow trout, exposed for 7 days to AgCl(s) at 1.0 mg Ag L^{-1} (nominal) added to freshwater (FW; $N = 10$) or to $AgNO_3$ at 100,000 μg total Ag L^{-1} (nominal) in freshwater supplemented with 50 mM NaCl (brackish water, BW; $N = 10$). Gill MT was not determined (ND) in the freshwater experiment. The AgCl(s) dissolved poorly in freshwater. Instead, addition of AgCl(s) to freshwater produced a dense particulate suspension. The $AgNO_3$ added to brackish water also produced some particulate Ag (see text). Using the MINEQL+ aquatic chemistry program [25], the concentration of dissolved Ag in the brackish water experiment was calculated to be 120 μg L^{-1}, almost entirely as $AgCl_n$. The control groups were held under identical conditions either in freshwater or in brackish water ($N = 10$). Vertical error bars denote one-way SE. An * denotes a statistically significant difference from the control at $p < 0.05$ in the Mann–Whitney U test.

at 280,000 μg Ag L^{-1}. Hence, $Ag(S_2O_3)_n$ may be even less toxic to fathead minnows than to rainbow trout.

In the present study, all fish survived for 7 days in $AgCl_n$ levels up to 100,000 μg Ag L^{-1} in 50 mM NaCl and we were thus unable to establish any mortality data for $AgCl_n$. In the only previous study of $AgCl_n$ toxicity to fish in brackish water, 4,600 μg Ag L^{-1} caused 40% mortality of fathead minnows during 4 days of exposure [3]. Computer simulation of water quality data by MINEQL+ [25] or MINTEQA2 [26] suggests that the speciation of Ag was similar as that in the present study. However, LeBlanc et al. [3] used a flow-through exposure system and exposures in the present study were semistatic. Because precipitation of cerargyrite is a time-dependent process, the difference in exposure regime may explain the difference in results. Nevertheless, both studies clearly demonstrate that $AgCl_n$ is very unlikely to cause acute toxicity to fish in any freshwater or brackish water system.

Silver toxicity has earlier been considered to be modulated primarily by the hardness of the water. The astonishing protective effect of the water [Cl^-] prompted us to review and reanalyze data on Ag toxicity presented in previous studies. Of special interest was an interlaboratory comparison on the tox-

icity of $AgNO_3$, in which different laboratories obatined reported LC50 values that ranged from 11.8 to 280.0 μg Ag L^{-1} for the same species [31]. The author tentatively attributed this variation in toxicity to differences in water hardness. However, the possible influence of $[Cl^-]$ was overlooked. When the toxicity data from Lemke [31] were plotted against the $[Cl^-]$ of the water a strong and highly significant linear relationship was found (Fig. 2). In contrast, there was no correlation between toxicity and water total Ca concentration, generally considered to be the major component of water hardness. The data published by Lemke [31] were used as part of the input data set by the EPA [31] in deriving the hardness-based equation that is used to regulate the maximum total recoverable Ag levels:

$$\text{Maximum total recoverable Ag } (\mu g\ L^{-1}) = e^{[1.72(\ln \text{hardness}) - 6.52]}$$

The basis for this relationship is unclear, and the results from both the present and previous studies suggest that water $[Cl^-]$ may be a prominent modulator of Ag toxicity. Water $[Cl^-]$ should likely be included in future modifications of this relationship. The results of the present study also demonstrate that dissolved but complexed Ag (e.g., $Ag(S_2O_3)_n$ or $AgCl_n$) and suspended particulate Ag (e.g., cerargyrite) are far less toxic than the free ionic Ag^+, and therefore question a criterion based on total recoverable Ag.

Bioassay endpoints, based on acute testing, do not always reflect the potential chronic hazard of toxicants. Acute toxicity to fish is usually caused by damage to gill functions (and sometimes structure) resulting in fatal osmo- and/or gas-regulatory dysfunctions [16]. However, both field studies and laboratory studies show that chronic exposure to metals may cause not only accumulation of metals but also long-term effects on growth, fecundity, and survival [33–37]. Therefore, investigation of whether Ag can be taken up and accumulated by fish from Ag^+, $AgCl_n$, and $Ag(S_2O_3)_n$ in the water and what, if any, effects accumulated Ag might have are of interest. All three forms of dissolved Ag tested were taken up by the fish and accumulated in the tissue. Interestingly, extremely high levels of Ag were found in livers of fish exposed to $AgCl_n$ and the hepatic Ag accumulation in $Ag(S_2O_3)_n$-exposed fish was remarkable. After 7 days of exposure to $Ag(S_2O_3)_n$ at the 96-h LC50 level, the concentration of Ag in the liver was increased 335 times as compared with the control and the Ag was 5 times more concentrated in the liver than in the exposure water. These data show that Ag can be taken up by fish during $Ag(S_2O_3)_n$ exposure, but the toxicity of Ag to internal organs seems to be low under these conditions. In two recent studies, we investigated the physiological effects of sublethal concentrations of Ag added as $AgNO_3$ and $Ag(S_2O_3)_n$, respectively [38,39]. Although the key toxic mechanism of Ag added as $AgNO_3$ appeared to be a profound disruption of Na^+ and Cl^- regulation and an associated fluid-volume disturbance, no such effects could be detected in fish exposed to a 3,000 times greater concentration of $Ag(S_2O_3)_n$.

Exposure to $Ag(S_2O_3)_n$ and $AgCl_n$ elicited production of MT in the liver and there was also a significant induction of branchial MT. The induction of MT, caused by these Ag salts, was remarkably large compared with the response reported for other metals. For example, exposure of juvenile rainbow trout for 12 weeks to waterborne $CuSO_4$ at 20 μg L^{-1} (28% of the 120-h LC50) increased the hepatic MT level by 230% [40]. Sixteen weeks of exposure to a mixture of Zn, Cu, and Cd, at a level corresponding to 32% of the 168-h LC50 ([Zn] = 215 μg L^{-1}; [Cu] = 11 μg L^{-1}; [Cd] = 0.54 μg L^{-1}), induced hepatic MT in rainbow trout by approximately 320% [41]. In the present study, exposure to $Ag(S_2O_3)_n$ for 7 days at 71% of the 168-h LC50 resulted in a 480% increase in the hepatic MT content. Furthermore, exposure to sublethal 120 μg Ag L^{-1}, as waterborne $AgCl_n$, induced MT in the liver by 200% during the 7-day period.

The reason for the much higher MT content in fish exposed to $Ag(S_2O_3)_n$ and $AgCl_n$ than in $AgNO_3$-exposed fish is probably directly related to the extremely high toxicity of the Ag^+ ion. The only conditions where the Ag^+ concentrations were high were in the exposures to $AgNO_3$. Tissue Ag data showed that although Ag accumulated in both gills and liver of fish exposed to Ag added as $AgNO_3$, the tissue levels of Ag were much higher in fish exposed to the considerably higher concentrations of waterborne $Ag(S_2O_3)_n$ and $AgCl_n$. In fish exposed to $AgNO_3$, the fish simply died before significant amounts of Ag accumulated in the body. Exposure to $AgCl_n$ in freshwater resulted in accumulation of Ag in the liver, whereas there was no increase in the hepatic MT level. A similar Ag accumulation in the liver was observed in fish exposed to Ag^+ (added as $AgNO_3$) and these fish did show some limited induction of MT. The difference in MT response between these two tests was probably that the hepatic MT concentration in control fish from the test with $AgCl_n$ in freshwater was higher than the MT concentration in the control for the Ag^+ experiment. Thus, in the fish exposed to $AgCl_n$ in freshwater, no additional MT was needed to sequester the accumulated Ag.

The physiological significance of MT induction is more difficult to assess. Metallothionein is known to reduce the toxicity of metals such as cadmium [19,20] and copper [21], but similar data are missing concerning Ag. The present study demonstrates that Ag is a potent inducer of MT, which may suggest that the liver has inherent protection against chronic Ag toxicity. This would explain the apparent lack of toxicity from the high hepatic Ag loads observed in fish exposed to $Ag(S_2O_3)_n$ or $AgCl_n$.

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