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# EFFECTS OF ALTERING FRESHWATER CHEMISTRY ON PHYSIOLOGICAL RESPONSES OF RAINBOW TROUT TO SILVER EXPOSURE

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**Abstract**—The influence of different water $Cl^-$ (50–600 μM), $Ca^{2+}$ (50–1,500 μM), $Na^+$ (50–1,500 μM), or dissolved organic carbon (DOC, 0.31–5 mg/L) levels on silver-induced physiological and biochemical perturbations of rainbow trout were investigated. Fish were acclimated to soft water (50 μM; $Cl^-$, $Ca^{2+}$, and $Na^+$), then exposed to 3.7 μg/L Ag (as $AgNO_3$) for 6 h, which resulted in a reduction in $Na^+$ influx from the water, an inhibition of gill sodium- and potassium-activated adenosine triphosphatase ($Na^+/K^+$-ATPase) activity, and an accumulation of silver on the gills. Increasing the water $Cl^-$ or DOC levels ameliorated the silver toxicity. However, increasing water $Ca^{2+}$ or $Na^+$ concentration did not reduce the silver-induced physiological and biochemical perturbations. The free silver ion ($Ag^+$) concentrations (calculated from MINEQL+, a geochemical speciation computer program) showed a negative correlation with the $Na^+$ influx rates and gill $Na^+/K^+$-ATPase activity. However, gill silver levels did not correlate to $Ag^+$ concentrations and no correlation was found between gill silver levels and either $Na^+$ influx rates or gill $Na^+/K^+$-ATPase activity. These results support the notion that the [$Ag^+$] concentration is of major importance when assessing silver toxicity in fish, and that this should be taken into account in regulatory strategies for silver in the natural environment.

**Keywords**—Silver toxicity Water chemistry Bioavailability Rainbow trout Adenosine triphosphatase

## INTRODUCTION

Silver, when presented in a form that provides substantial amounts of the free silver ion ($Ag^+$), is one of the most toxic metals to aquatic organisms, with a median lethal concentration (LC50) value for freshwater fish in the range of 6.5 to 70 μg/L [1–9]. The primary mechanism of toxicity is the disruption of branchial ion transport [10], which results in death from the consequences of ionoregulatory failure [11,12]. Rainbow trout exposed to 10 μg/L $AgNO_3$ show an inhibition of active branchial $Na^+$ and $Cl^-$ influx, which is total and complete within 8 h of exposure [10,12]. Diffusive $Na^+$ and $Cl^-$ effluxes are not substantially affected [10,12]. Kinetic analysis reveals that the affinity of the $Na^+$ transport mechanism is not affected by silver, but that a reduction occurs in the maximal transport capacity [10]. In conjunction with the reduction in $Na^+$ influx, the activity of sodium- and potassium-activated adenosine triphosphatase ($Na^+/K^+$-ATPase), which is the major enzyme involved in sodium transport across the basolateral membrane of the gill, is inhibited.

In the case of silver, speciation governs toxicity [6,9]. For example, $Ag(S_2O_3)_n$ and $AgCl_{(s)}$ have LC50 values that are 13,000- (96-h LC50 value of 161,000 μg Ag/L) and >10,800-fold (no mortalities after 96 h at 100,000 μg Ag/L), respectively, greater than that of $AgNO_3$, a salt that readily dissociates to $Ag^+$ in freshwater [8]. The toxicities of metals to freshwater fish can be influenced by water hardness, pH, alkalinity, $Cl^-$, and dissolved organic matter [13–16]. The effect of these parameters on toxicity can be explained by either rendering the metal inactive by complexation or by direct competition between the ligand and the metal at the site of toxic action, that is, the gill. Additionally, $Ca^{2+}$ may stabilize the gill membrane, limiting the diffusive loss of electrolytes [17]. Ligands that form silver complexes, such as chloride and dissolved organic carbon (DOC), have been shown to ameliorate $AgNO_3$ toxicity in rainbow trout [18,19]. Analysis of the water chemistry, using computer-based geochemical modeling programs such as MINEQL+ [20], demonstrates that silver toxicity to rainbow trout is directly related to the concentration of [$Ag^+$] [9,21].

Currently, the freshwater acute ambient water quality criterion for silver for the United States [22] recognizes only one geochemical variable that may modify toxicity, "hardness" (representative of water calcium levels in most natural freshwater). Hardness is incorporated in the following equation, designed to help regulatory authorities calculate the maximum total recoverable silver

$$\text{maximum total recoverable Ag } (\mu g/L) = \exp[1.72(\ln \text{hardness}) - 6.52] \quad (1)$$

Recently, Hogstrand et al. [8] and Galvez and Wood [9] questioned the validity of this hardness equation, highlighting the fact that it was strongly influenced by data from only one study [4] and its derivation selectively ignored other available data that did not show a protective effect of hardness. Reanalysis of some of the data presented by Lemke [4] showed a strong correlation between water chloride and toxicity rather than between water hardness and toxicity [8]. These studies highlight a need for a reevaluation of the functional dependence of the silver criterion equation on water quality parameters.

The published proceedings of a recent Society of Environmental Toxicology and Chemistry workshop, the *Reassessment of Metals Criteria for Aquatic Life Protection* [23], stressed the necessity of new approaches in the regulation of metals. The calculation of metal–gill binding constants for a

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number of metals [24–27] has enabled the gill to be considered as another ligand in computer-based geochemical models. Assuming that acute metal toxicity will depend on the metal–gill surface interaction [13], prediction of toxicity based on water geochemistry and gill metal burden may be possible. At present, only copper toxicity to rainbow trout and brook trout has been directly correlated to gill metal accumulation [27]. Consequently, more data are required concerning the relationship between metal toxicity and metal speciation and gill metal accumulation. The present study provides information concerning the influence of water chemistry on silver toxicity in rainbow trout. Physiological ($Na^+$ influx from the water) and biochemical (gill $Na^+/K^+$-ATPase activity) parameters are good indicators of the sublethal effects of metals [28], and were measured to evaluate the effects of silver speciation (by changing water $Cl^-$, $Ca^{2+}$, $Na^+$, and DOC levels within the freshwater range) on silver toxicity in rainbow trout. The relationship between these indicators and gill silver accumulation was also assessed.

## MATERIALS AND METHODS

### *Fish husbandry*

Juvenile rainbow trout were obtained from Humber Springs Hatchery, Orangeville, Ontario, Canada, and were initially kept in flowing dechlorinated Hamilton (ON, Canada) tap water (0.6 mM $Na^+$, 0.8 mM $Cl^-$, 1 mM $Ca^{2+}$, pH 8.0, 10–15°C). The fish were fed trout food (Martin Mills, Tavistock, ON, Canada) at a ration of 1 to 2% of their body weight daily, except on the day prior to transfer to experimental boxes. Soft water was generated by reverse osmosis (RO, Andersen, Dundas, ON, Canada) and the fish were acclimated to this over a 2-week period. The final ion concentration in the holding tank was adjusted by the addition of dechlorinated tap water to give approximately 50 μM $Na^+$, 50 μM $Cl^-$, 50 μM $Ca^{2+}$, and 0.3 mg DOC/L at pH 6.8 and 11 to 14°C. The fish were maintained in this medium for at least 4 weeks before experimentation.

### *Silver exposure*

For each test at a particular water composition six or eight fish, average weight 8.83 ± 3.84 g (±SD), were transferred to black Perspex boxes containing 2.5 L of RO water adjusted to 50 μM $Na^+$, 50 μM $Cl^-$, and 50 μM $Ca^{2+}$ by addition of the salts $Ca(NO_3)_2$, NaCl, and KCl, respectively, to create a defined medium. The fish were maintained in these containers for 24 h prior to the experiment, with each box receiving aeration with a flow through of between 22.9 and 44.2 ml/min.

Each experiment lasted for 6 h. On commencement of the experiment the flow to the boxes was stopped and the water was spiked with the appropriate salts. Silver was added as $AgNO_3$ to give a concentration of 3.7 μg Ag/L, which was verified by analysis (see below). The water $Cl^-$, $Ca^{2+}$, $Na^+$, and DOC concentrations were varied individually by the addition of KCl, $Ca(NO_3)_2$, $Na_2SO_4$, or commercial humic acid (Aldrich, St. Quentin Fallavier, France) to give concentrations in the range of 50 to 600 μM $Cl^-$, 50 to 1,500 μM $Ca^{2+}$, 50 to 1,500 μM $Na^+$, and 0.3125 to 5 mg/L DOC, respectively. All values were verified by analysis (see below). No silver was detected in control media (detection limit 0.2 μg/L). Once the boxes had been spiked the flow was turned on and the concentrations of salts, DOC, and silver were maintained by the addition of the relevant concentrated stock solution to the inflow via a peristaltic pump. After 4 h the flow was stopped and $Na^+$ influx was measured over the next 2 h.

### *$Na^+$ influx measurements*

The $Na^+$ influx rate from the water was determined by the addition of 1 μCi $^{22}Na^+$/L. The radioisotope was allowed to mix for 15 min and then a 13-ml water sample was taken. A further water sample was taken at the end of the experiment. After 2 h of exposure to the isotope, the fish were euthanized by an overdose of anesthetic (MS-222, Syndel Pharmaceuticals, Vancouver, BC, Canada) and washed in 3 mmol/L NaCl for 1 min to displace surface-bound $^{22}Na^+$. This was followed by two 1-min washes in distilled water. The fish were blotted dry with a paper towel, weighed, and then whole-body radioactivity was measured on a gamma counter (Packard, Downers Grove, IL, USA). Water [$Na^+$] and [$Ca^{2+}$] were measured by an atomic absorption spectrophotometer (Varian AA 1275, Mississauga, ON, Canada), water [$Cl^-$] via the colorimetric mercuric thiocyanate method [29], DOC by a Rosemount Analytical DC-180 automated TOC analyzer (Folio Instruments, Kitchener, ON, Canada), and silver by graphite furnace atomic absorption spectrophometry (AAS) (Varian AA 1275 fitted with a CTA-95 atomizer).

Whole-body $Na^+$ influx was calculated from the formula

$$\text{whole-body } Na^+ \text{ influx} = \frac{q}{\text{SA} \cdot \text{t} \cdot \text{wt}} \quad (2)$$

where $q$ represents the counts per minute (cpm) for the whole body, $t$ is the time, wt is the wet weight of the fish, and SA is the specific activity of the water calculated from

$$\text{SA} = [(\text{cpm}_i/[\text{Na}]_i) + (\text{cpm}_f/[\text{Na}]_f)]/2 \quad (3)$$

where $\text{cpm}_i$ represents the initial cpm per milliliter in the water, $\text{cpm}_f$ represents the final cpm per milliliter in the water, and $[Na^+]_i$ and $[Na^+]_f$ represent the initial and final sodium concentrations of the water, respectively.

### *Gill silver accumulation*

At the end of a separate set of experiments that followed the same protocol, the fish were euthanized by an overdose of MS-222 and the gills were rapidly excised, washed in distilled water, and blotted dry. The wet weight of the gills was determined and a volume five times this weight of 10% (v/v) $HNO_3$ was added. The gills were digested for at least 3 h at 80°C, vortexed, and allowed to settle. A 100-μl aliquot of the supernatant was diluted with 0.9 ml of nanopure water and then analyzed for silver concentration by graphite furnace AAS (as above).

### *Gill $Na^+/K^+$-ATPase activity*

At the end of a separate set of experiments that followed the same protocol, the gills were perfused with phosphate-free Cortland buffer (modified from [30]), so as to minimize contamination with red blood cells. One or two gill arches were placed in 0.5 ml of SEI buffer (0.25 M sucrose, 0.02 M $Na_2$-ethylenediaminetetraacetic acid [EDTA], 0.1 M imidazole, adjusted to pH 7.1 with HCl) and immediately frozen in liquid nitrogen.

The gill samples were thawed and kept on ice throughout the following procedure. The gills were sonicated by two bursts at 30% of maximum with a sonic dismembrator. The resulting mixture was centrifuged at 3,000 rpm for 5 min and the supernatant was removed. The protein content was determined

using Bradford reagent (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as a standard. Before the assay, the protein content was adjusted to 1 mg/ml and, to enable maximum substrate accessibility, 0.2 mg saponin per mg protein was added.

The $Na^+/K^+$-ATPase assay followed methods similar to those of Bonting et al. [31], where the difference in the concentrations of inorganic phosphate ($P_i$) liberated by the gill homogenate in two defined media, one containing $K^+$ and the other containing ouabain (a specific $Na^+/K^+$-ATPase inhibitor) and without $K^+$, represents $Na^+/K^+$-ATPase activity. The reaction mixture consisted of 100 mM NaCl, 30 mM imidizole/HEPES pH 7.4, 5 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $Na_2$-EDTA. Before the assay, 3 mM $Na_2$-ATP was added and the pH was adjusted to 7.4 with Tris. Medium A also consisted of 10 mM KCl, whereas in medium E, KCl was omitted and 1.67 mM ouabain was added. The tubes were vortexed and placed in a water bath at 30°C. The reaction was stopped after 20 min by the addition of 1 ml of ice-cold 8.6% (w/v) trichloroacetic acid after which 1 ml of a solution containing 0.66 mM $H_2SO_4$, 9.2 mM $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, and 0.33 mM $FeSO_4 \cdot 7H_2O$ was added. The solution was left for an additional 20 min and the concentration of liberated $P_i$ was measured colormetrically at 700 nm as the reduced phosphomolybdate complex. The $Na^+/K^+$-ATPase activity was calculated from the difference between the concentration of $P_i$ in the A and E media.

### *Statistics*

A one-way analysis of variance (ANOVA) was used to test the difference between the $Na^+$ influx rates for fish not exposed to silver but in different media (i.e., different control series, SPSS for Windows 6, Chicago, IL, USA). A one-way ANOVA followed by a least significant difference (LSD) test (SPSS for Windows 6) was used to test the difference between $Na^+$ influx rates for fish exposed to silver in different media (i.e., where $Cl^-$, $Ca^{2+}$, or DOC were varied) and the combined $Na^+$ influx rates from the appropriate controls series (i.e., the $Na^+$ influx rates for the silver exposed fish at each [$Cl^-$] were compared to the combined $Na^+$ influx rates for the $Cl^-$ controls). An independent *t* test (SPSS for Windows 6) was used to test the differences between the $Na^+$ influx rate for silver-exposed fish and the controls at corresponding water [$Na^+$]. Kinetic parameters ($V_{max}$ and $K_m$) were calculated after fitting the data to the Michaelis–Menten equation, using nonlinear regression data analysis. Comparison between $V_{max}$ and $K_m$ for silver- and non-silver-exposed fish was determined by a *t* test [32]. One-way ANOVA followed by an LSD test (SPSS for Windows 6) was used to determine differences between the gill silver burden and the gill $Na^+/K^+$-ATPase activity for silver-exposed fish and control fish. Linear regression analysis was calculated using SSPS for Windows 6.

## RESULTS

### *Influence of silver*

Exposure to 3.7 µg/L Ag (added as $AgNO_3$) strongly inhibited $Na^+$ influx and gill $Na^+/K^+$-ATPase activity, and significantly elevated gill total silver levels, at least at the lowest concentrations of $Cl^-$, $Na^+$, $Ca^{2+}$, and DOC (Figs. 1 to 4). The influence of increasing concentrations of these constituents on these effects of silver are discussed below.

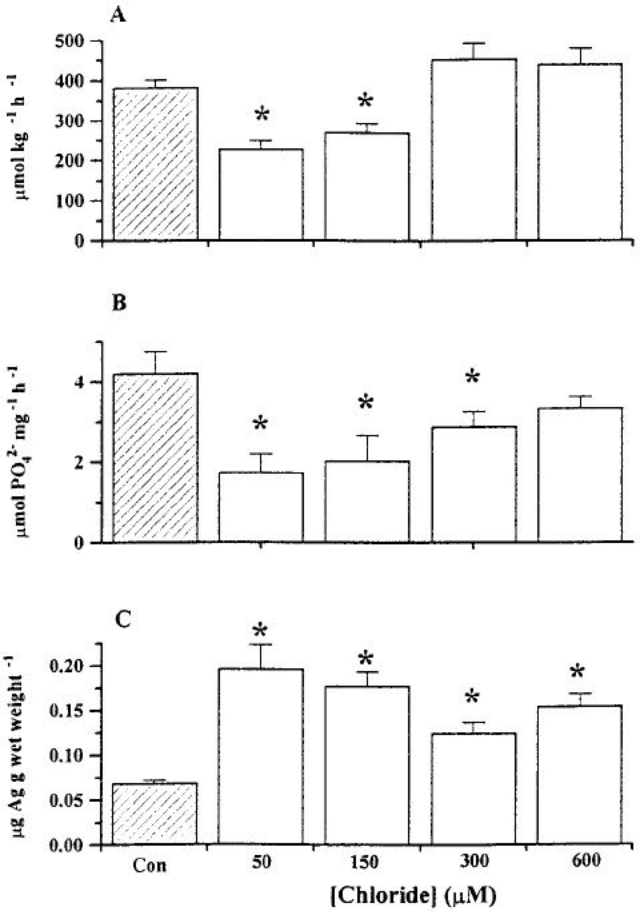


Fig. 1. Influx rates of $Na^+$ from the water (**A**), gill sodium- and potassium-activated adenosine triphosphatase activity (**B**), and gill silver levels (**C**) of control rainbow trout (Con) and those exposed to 3.7 µg Ag/L (as $AgNO_3$) at different water chloride levels. Other water ion concentrations remained constant (50 µM $Na^+$, 50 µM $Ca^{2+}$, 0.3 mg/L DOC). Values are mean ± SEM ($n$ = 6–14); asterisks indicate significant difference from controls (one-way analysis of variance followed by a least significant difference test, $p < 0.05$).

### *Influence of chloride*

The chloride levels were chosen to ensure that no cerargyrite formed and that fish were exposed to varying concentrations of aqueous $Ag^+$, and aqueous silver chloride species, AgCl and $AgCl_2^-$, as calculated from MINEQL+ [20]. Increasing the concentration of water $Cl^-$ reduced the degree of inhibition of $Na^+$ influx induced by silver exposure, with the rate returning to normal at a water [$Cl^-$] of 300 µM (Fig. 1A). Increasing water [$Cl^-$] also reduced the degree of inhibition of gill $Na^+/K^+$-ATPase activity in a dose-dependent manner (Fig. 1B). However, these water $Cl^-$ concentrations did not prevent the accumulation of silver on the gills (Fig. 1C).

### *Influence of calcium*

Increasing the water $Ca^{2+}$ concentrations to either 50, 600, or 1,500 µM had no effect on the degree of inhibition of $Na^+$ influx or gill $Na^+/K^+$-ATPase activity induced by silver exposure in rainbow trout (Fig. 2A and B). Silver accumulated on the gills of fish exposed to silver at 50 and 600 µM $Ca^{2+}$ (Fig. 2C). However, less silver accumulated on the gills of fish exposed to silver at 1,500 µM $Ca^{2+}$.

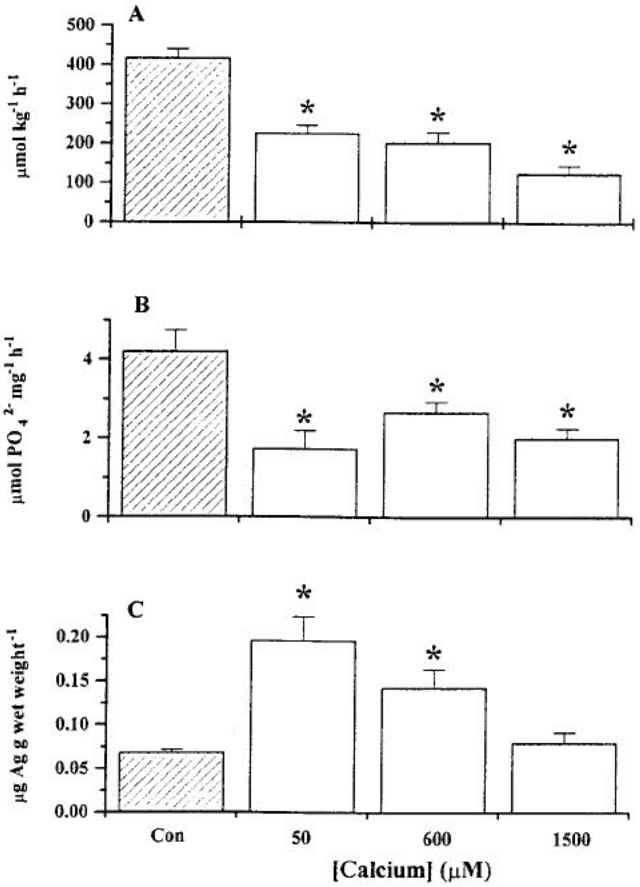


Fig. 2. Influx rates of $Na^+$ from the water (A), gill sodium- and potassium-activated adenosine triphosphatase activity (B), and gill silver levels (C) of control rainbow trout (Con) and those exposed to 3.7 µg Ag/L (as $AgNO_3$) at different water calcium levels. Other water ion concentrations remained constant (50 µM $Na^+$, 50 µM $Cl^-$, 0.3 mg/L DOC). Values are mean ± SEM ($n$ = 8–14); asterisks indicate significant difference from controls (one-way analysis of variance followed by a least significant difference test, $p < 0.05$).

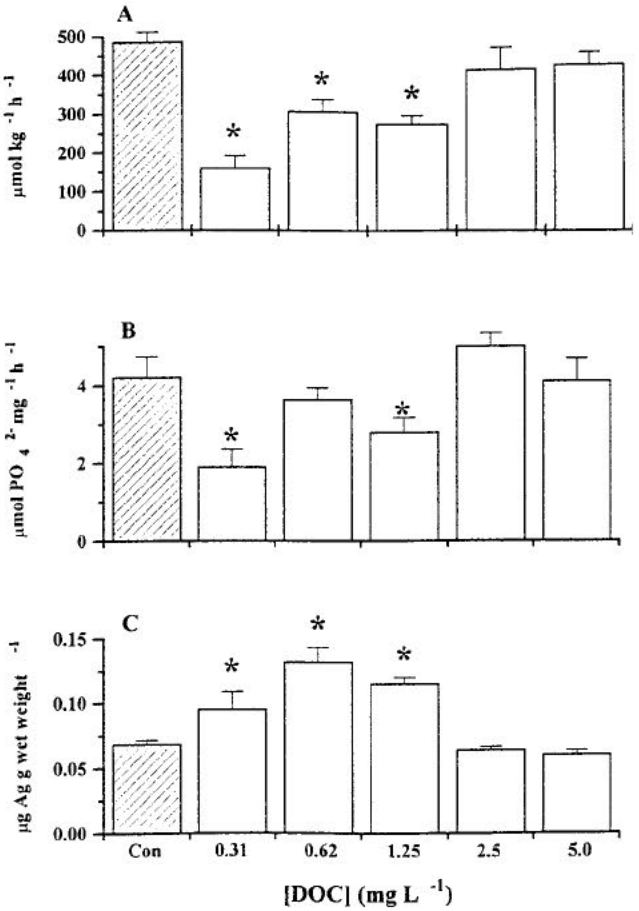


Fig. 3. Influx rates of $Na^+$ from the water (A), gill sodium- and potassium-activated adenosine triphosphatase activity (B), and gill silver levels (C) of control rainbow trout (Con) and those exposed to 3.7 µg Ag/L (as $AgNO_3$) at different water dissolved organic carbon (DOC) levels. Other water ion concentrations remained constant (50 µM $Na^+$, 50 µM $Cl^-$, 50 µM $Ca^{2+}$). Values are mean ± SEM ($n$ = 6–19); asterisks indicate significant difference from controls (one-way analysis of variance followed by a least significant difference test, $p < 0.05$).

### *Influence of dissolved organic carbon*

Increasing the concentration of water DOC reduced both the degree of inhibition of $Na^+$ influx and the inhibition of gill $Na^+/K^+$-ATPase activity induced by silver exposure in fish. At a level of 2.5 mg DOC/L, the $Na^+$ influx rates, gill $Na^+/K^+$-ATPase activity, and level of silver accumulated on the gills of silver exposed fish all returned to control values (Fig. 3).

### *Influence of sodium*

Increasing the concentration of external $Na^+$ increased the $Na^+$ influx rates (Fig. 4A). However, an increased external [$Na^+$] did not affect the inhibition induced by $Ag^+$. For example, at 50 µM $Na^+$, the whole-body $Na^+$ influx rates in silver-exposed fish were reduced by 40.6% when compared to control fish, whereas at 1,500 µM the degree of inhibition in silver-exposed fish was 41.2%. Kinetic analysis showed a reduction in $V_{max}$, whereas $K_m$ was unaffected (Table 1). Increasing the water $Na^+$ concentrations did not affect the degree of inhibition of gill $Na^+/K^+$-ATPase activity or the accumulation of silver on the gills (Fig. 4B and C).

### *Correlation analysis*

The $Ag^+$ levels were calculated from the geochemical modeling program MINEQL+ [20]. This program possesses fixed stability constants (log $K$) for $AgCl_n$ species but not for Ag–DOC complexes. Consequently, for the purpose of our calculations we added the log $K$ values for Ag–DOC complexes derived by Janes and Playle [26] into this program's database.

The $Ag^+$ concentration showed a good negative correlation with the $Na^+$ influx rates (Fig. 5A; $r = -0.637$, $p = 0.026$) and a strong correlation with gill $Na^+/K^+$-ATPase activity (Fig. 5B; $r = -0.870$, $p < 0.001$). However, no significant correlation was found between water $Ag^+$ levels and gill silver concentration (Fig. 5C; $r = 0.396$, $p = 0.144$). No significant correlation was found between gill silver levels and $Na^+$ influx rates (Fig. 6A; $r = -0.239$, $p = 0.455$) and gill $Na^+/K^+$-ATPase activity (Fig. 6B; $r = -0.442$, $p = 0.10$).

## DISCUSSION

Altering silver speciation by the addition of chloride or DOC ameliorated the silver-induced physiological (whole-body $Na^+$ influx rate) and biochemical (gill $Na^+/K^+$-ATPase activity) disturbances seen in silver-exposed fish. In contrast, increasing water calcium or sodium levels, procedures that do not affect silver speciation, did not prevent these disturbances. Analysis of the silver species present, by the geochemical

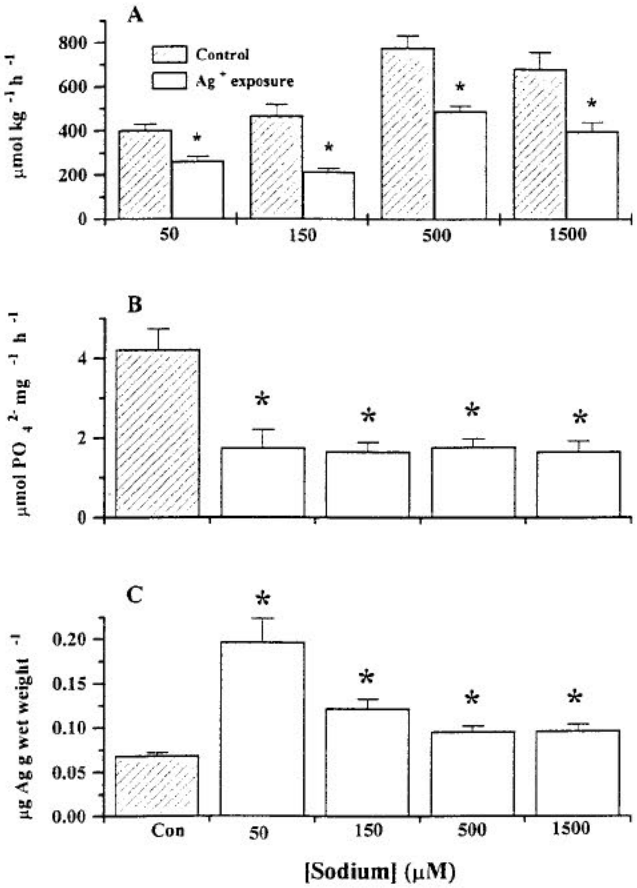


Fig. 4. Influx rates of $Na^+$ from the water (A), gill sodium- and potassium-activated adenosine triphosphatase activity (B), and gill silver levels (C) of control rainbow trout (hatched columns in A and Con in B and C) and those exposed to 3.7 µg Ag/L (as $AgNO_3$) at different water sodium levels. Other water ion concentrations remained constant (50 µM $Ca^{2+}$, 50 µM $Cl^-$, 0.3 mg/L DOC). Values are mean ± SEM ($n$ = 6–18); asterisks indicate significant difference from controls (in A independent $t$ test, $p < 0.05$ and in B and C one-way analysis of variance followed by a least significant difference test, $p < 0.05$).

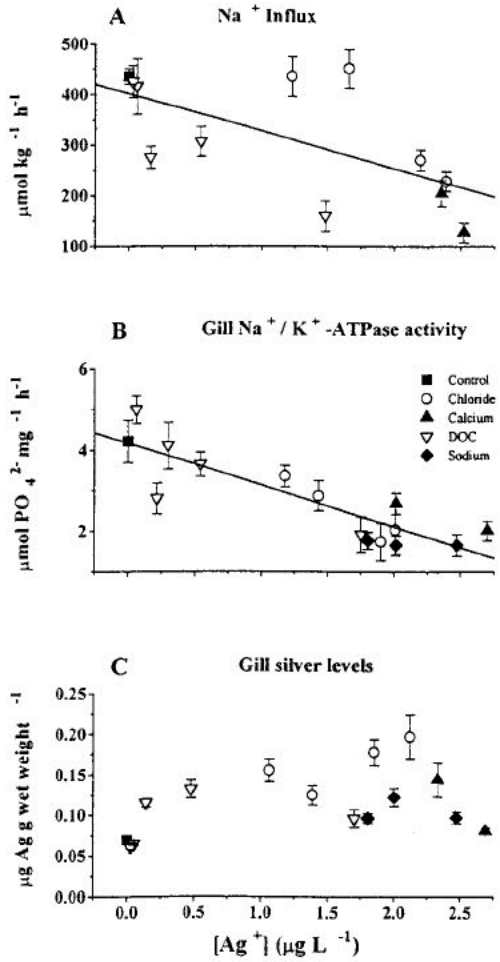


Fig. 5. Correlation analyses of the free Ag ion ($Ag^+$) concentration (calculated from [20]) versus $Na^+$ influx rates from the water (A), gill sodium- and potassium-activated adenosine triphosphatase ($Na^+/K^+$-ATPase) activity (B), and gill silver levels (C). Lines indicate linear regression with coefficients of (A) $r = -0.637$, $p = 0.026$; (B) $r = -0.87$, $p < 0.001$; (C) $r = 0.396$, $p = 0.144$.

modeling program MINEQL+ [20], showed that a negative correlation occurred between the [$Ag^+$] and $Na^+$ influx rates, as well as between the [$Ag^+$] and gill $Na^+/K^+$-ATPase activity. These findings concur with the LC50 values for rainbow trout in different water chemistries [9,21] and the mechanism of silver nitrate toxicity [10], which have both been attributed solely to $Ag^+$. The toxicity of other metals to aquatic biota may also be a function of the free metal ion concentration [13,14].

Metal accumulation by the fish depends on initial adsorption to the gill apical surface and subsequent transfer of the metal across the gill into the body. Compounds present in the water column may prevent accumulation by complexing the metal or competing with its binding sites. Dissolved organic carbon forms complexes with $Ag^+$, which prevents silver accumulating on the gills, thus reducing the degree of inhibition of $Na^+$ influx and gill $Na^+/K^+$-ATPase by $Ag^+$. This accounts for the ameliorating effect that DOC has on silver toxicity [18,19]. The DOC concentration required to prevent gill silver accumulation in the present study was 10 times lower than that reported by Janes and Playle [26]. This may reflect a difference between the number or composition of binding sites of the two types of DOC used, or the difference in silver concentrations used (in the present study, 3.7 µg Ag/L and in the former study 11.9 µg Ag/L).

Table 1. Affinity constant ($K_m$) and maximum transport rate ($V_{max}$) for $Na^+$ influx rate from the water in control and silver-exposed trout. Asterisk indicates significant difference between silver-exposed and nonsilver-exposed trout ($t$ test [32], $p < 0.05$)

| | $V_{max}$ (µmol kg/h) | $K_m$ (µM) |
|---|---|---|
| Control | 755.0 ± 92.4 | 53.9 ± 31.7 |
| Ag exposed | 450.1 ± 100.6* | 60.4 ± 62.4 |

An anomaly exists for chloride, which has been shown to reduce silver toxicity in rainbow trout [6,8], but does not prevent the accumulation of silver in the gills or liver (Fig. 1) [8,9,21,33]. The site of toxic action in fish is assumed to be the gill $Na^+/K^+$-ATPase [10], and more specifically the $Mg^{2+}$-dependent part of the $Na^+/K^+$-ATPase cycle [34], which is located on the intracellular side of the basolateral membrane of the chloride cells (CC), which are the major ion-transporting cells of the gill (see review by Perry [35] for the role of CC in the gill). The correlation between $Ag^+$ and inhibition of gill $Na^+/K^+$-ATPase activity suggests that $Ag^+$ must first enter the

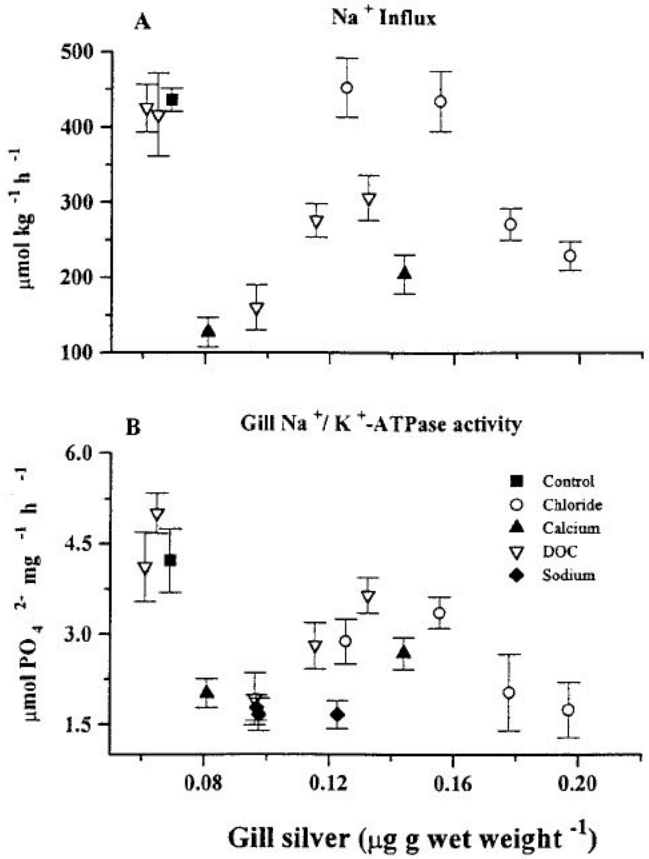


Fig. 6. Correlation analyses of gill silver levels versus whole body $Na^+$ influx rates from the water (**A**) and gill sodium- and potassium-activated adenosine triphosphatase ($Na^+/K^+$-ATPase) activity (**B**). Regression coefficients are (**A**) $r = -0.239$, $p = 0.455$ and (**B**) $r = 0.442$, $p = 0.099$.

CC, probably via an apical channel, before exerting its toxic action. An increase in water chloride levels will result in a decrease in the $Ag^+$ levels and a concomitant increase in $AgCl_n$ species, thereby reducing the portion of silver entering as $Ag^+$. The uncharged AgCl may enter passively, accumulating in all cell types of the gill (the CC constitute only 10%, or less, of the total gill cell population [35]). Moreover, Simkiss and Taylor [36] estimated that $HgCl_2^0$ could penetrate lipid membranes $10^6$ times faster than $Hg^{2+}$. Similarly, $AgCl_n$ could be more persistent than $Ag^+$. Consequently, it is not the amount of silver on the gill that is important for toxicity, but where and how it accumulates.

The ions $Ag^+$ and $Na^+$ have the same charge, an ionic radius of 1.26 and 0.97 Å, respectively [37], and may share similar uptake pathways across the gill. The exact mechanism of $Na^+$ entry across the apical membrane of the gill is currently under debate. Two mechanisms are hypothesized: entry via a $Na^+$ channel coupled to an $H^+$-ATPase, or via an $Na^+/H^+$-exchanger (see review [38]). If $Ag^+$ enters via either of these processes, then $Na^+$ will compete with $Ag^+$ at the site of entry. Comparing silver-exposed trout to controls at the same [$Na^+$] shows that $Na^+$, up to a concentration of 1,500 µM, did not prevent silver-induced physiological and biochemical disturbances or the accumulation of gill silver. Janes and Playle [26] found that a concentration of 16 mM $Na^+$ was required to prevent gill silver accumulation. This suggests that Ag, which was present at 34 nM (i.e., 3.7 µg/L) in this study, has a very strong affinity for these uptake pathways. The low $K_m$ value (Table 1, 53.9 ± 31.7 µM) for $Na^+$ uptake in the present control fish compared to the $K_m$ value (257.1 ± 89.7 µM) previously reported for trout adapted to Hamilton tap water (moderately hard water, with 12-fold higher $Na^+$ levels, [10]), suggests that the mechanism by which these fish adapt to dilute soft water is via an increase in the affinity of the transport process for sodium. The decrease in $V_{max}$ but constant $K_m$ for $Na^+$ uptake in silver-exposed soft water-acclimated fish is similar to the type of inhibition of $Na^+$ uptake observed in silver-exposed hard water-acclimated fish [10]. This is indicative of a noncompetitive inhibitor, suggesting that $Ag^+$ inhibits transport by binding to the uptake machinery at a site different from that of $Na^+$. These findings have been confirmed in vitro, where silver inhibits $Na^+/K^+$-ATPase by interfering with the $Mg^{2+}$-binding site of the enzyme [34].

Water hardness, changed by altering calcium concentrations, had no effect on the silver-induced physiological and biochemical perturbations in rainbow trout. However, it is unclear why an increase in $Ca^{2+}$ concentration results in a reduction in the accumulation of silver on the gill. This result corroborates the toxicity data, which show that increasing calcium concentrations reduced silver toxicity by only a small extent [9,19]. This modest ameliorating effect was attributed to the stabilizing effect that $Ca^{2+}$ ions have on the gill membrane [17]. The data from this study further question the validity of the hardness equation for the regulation of silver (see Introduction and [21]). Consequently, a new approach is needed for the environmental regulation of silver. The advancement of computer-based geochemical models enables rapid identification of the metal species present. Recently, a free-ion activity model (FIAM) has been derived for determining metal bioavailabilty (defined as the degree to which a metal is available for uptake by the organism) [15,39]. In a critical review of the model, Campbell [15] highlighted a number of studies where metal bioavailability does not conform to the FIAM. One such instance was for the accumulation of Ag by the grass shrimp *Palaemoetes pugio* at four different salinities [40]. The results from the present study also confirm that silver, in the case of rainbow trout in freshwater, does not fit the FIAM. Moreover, based on geochemical modeling, it is the [$Ag^+$] that determines toxicity, although it is not the principal factor in determining metal accumulation (Fig. 5C) [8,9,21].

Another approach that has been proposed for the prediction of aquatic metal toxicity is the gill metal burden model, which relates toxicity to the accumulation of metal on the gill [23]. This model has been verified for copper, where the gill Cu concentration after 24 h correlated well with the eventual LC50 values [27]. However, in the case of silver, gill metal levels after a 6-h exposure to the metal did not correlate with the degree of physiological and biochemical perturbations (indicators of toxicity), a finding that corroborates those of McGeer and Wood [33]. Whether gill silver levels after a different exposure period (e.g., 3, 12, or 24 h) would correlate to toxicity is unclear, and warrants further investigation. However, Hogstrand et al. [8] and Galvez and Wood [9] found no correlation between tissue Ag levels and the 96-h LC50 values. Interestingly, Hollis et al. [41], in a study concerning the effects of aging DOC on Cd and Cu gill accumulation, found a relationship between Cu toxicity and gill metal levels, but this relationship was not apparent for Cd. Taken together, these results show that general assumptions cannot be drawn for all metals. In the case of silver, the correlation between $Ag^+$ (the toxic form of silver) and the site of toxic action illustrates that

this parameter (i.e., gill $Na^+/K^+$-ATPase activity) is a good indicator of the biological impact of silver.

The results from this study confirm that speciation governs silver toxicity. Furthermore, the results illustrate that the gill metal burden model does not relate to silver toxicity, as determined by inhibition of $Na^+$ influx or inhibition of gill $Na^+/K^+$-ATPase activity. However, $[Ag^+]$ is a good indicator of silver toxicity to rainbow trout. A new regulatory approach for silver that considers speciation should be considered.

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