Water Chloride Provides Partial Protection during Chronic Exposure to Waterborne Silver in Rainbow Trout (*Oncorhynchus mykiss*) Embryos and Larvae

Colin J. Brauner[1,*]
Jonathan Wilson[2]
Collins Kamunde[3]
Chris M. Wood[3]
[1]Department of Zoology, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada; [2]Centro de Investigação Marinha e Ambiental, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal; [3]Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

Accepted 5/28/03

ABSTRACT

Rainbow trout embryos and larvae were continuously exposed (at 12.5°C) to waterborne silver in a flow-through setup, from fertilization to swim-up, at nominal silver concentrations of 0, 0.1, or 1.0 μg/L total silver (as $AgNO_3$) at three different water Cl^- levels (30, 300, and 3,000 μM, added as KCl). Exposures were conducted in synthetic soft water (hardness 20 mg $CaCO_3$/L generated from reconstituted reverse osmosis freshwater). Continuous exposure to 1.0 μg/L total silver for 58 d at 30 μM water Cl^- resulted in a pronounced ionoregulatory disturbance (as indicated by a reduction in whole body Na^+,K^+-ATPase activity, unidirectional Na^+ uptake [J_{in} Na^+], and whole body Na^+ and Cl^- levels) and a reduction in extractable protein and wet weight. Thus, the mechanism of chronic silver toxicity appears to be similar to that observed during acute silver exposure in juvenile and adult fish, specifically an ionoregulatory disturbance. Higher water Cl^- levels (300 and 3,000 μM Cl^-) offered some degree of protection from the ionoregulatory disturbance, with only minor protective effects in terms of mortality. The protective effects of water Cl^- on the toxicity of silver (as $AgNO_3$) appear to be far less during chronic than during acute exposure. Mortality and larval Na^+ concentration, J_{in} Na^+, and Na^+,K^+-ATPase activity all appear to be correlated with silver body burden and calculated water Ag^+ during chronic silver exposure. Thus, there appears to be potential to model chronic toxicity but not simply by recalibration of an acute model. A chronic model must be based on real chronic data because the protective effects of various ligands appear to be quantitatively very different from those in the acute situation.

* Corresponding author; e-mail: brauner@zoology.ubc.ca.

Physiological and Biochemical Zoology 76(6):803–815. 2003. 1522-2152/2003/7606-2175$15.00

Introduction

Of the species of silver that can exist in solution, the silver ion (Ag^+) is by far the most toxic to aquatic vertebrates and invertebrates and is used frequently in toxicity testing for this reason. In juvenile and adult trout and fathead minnows, as well as in crayfish, acute exposure (24–96 h) to Ag^+ exerts its toxic effects through the direct impairment of gill Na^+,K^+-ATPase activity and inhibition of carbonic anhydrase activity, which disrupts ionoregulation (Wood et al. 1996; Morgan et al. 1997; McGeer and Wood 1998; Grosell et al. 2002). In freshwater, this leads to a reduction in plasma Na^+ and Cl^- levels, which initiates a suite of secondary effects, ultimately leading to circulatory collapse and death (Wood et al. 1999).

Most natural waters contain natural ligands, such as Cl^-, dissolved organic matter (DOM), and sulfide, that have a relatively high affinity for Ag^+ and greatly reduce the toxicity of silver to aquatic organisms (Le Blanc et al. 1984; McGeer and Wood 1998; Bury et al. 1999*a*, 1999*b*; Karen et al. 1999; Bianchini et al. 2002). For example, when juvenile rainbow trout were exposed to a total silver concentration of 3.0 μg/L (as $AgNO_3$) in reconstituted reverse osmosis water with 50 μM Cl^-, there was an 82% impairment of gill Na^+,K^+-ATPase activity within 50 h. Exposure to 1,500 μM Cl^- offered complete protection of Na^+,K^+-ATPase activity (at the same total silver concentration and exposure duration; McGeer and Wood 1998) and increased the LC_{50} value from 7.5 to 25.6 μg/L total silver (Bury et al. 1999*a*).

A similar relationship is observed in juvenile rainbow trout exposed to DOM measured as dissolved organic carbon (DOC). Juvenile trout exposed to 3.7 μg/L total silver as $AgNO_3$ for 6 h in soft water in the presence of 0.3 mg/L DOC exhibited a more than 50% reduction in gill Na^+,K^+-ATPase activity, whereas this effect was abolished when DOC levels were increased to 2.5 mg/L (Bury et al. 1999*a*). The LC_{50} value in 0.3 mg/L DOC was 7.5 μg/L total silver, and this was increased to 18.4 and 27.7 μg/L total silver at 1.6 and 5.8 mg/L DOC, re-

Table 1: Measured water Cl^- and total silver concentration in filtered (0.45 μm) and unfiltered experimental water, as well as calculated Ag^+ concentration calculated using MINEQL+ (Schecher and McAvoy 1994)

Treatment (Nominal Total Silver and Cl^-)	Measured Total Silver in Water (Unfiltered; μg/L)	Calculated Ag^+ (μg/L)	Measured Dissolved Silver in Water (Filtered; μg/L)	Total Water Cl^- (μM)
0 μg/L Ag, 30 μM Cl^-	<.05	<.05	…	…
.1 μg/L Ag, 30 μM Cl^-	.087 ± .004	.0087 ± .0004	…	…
1.0 μg/L Ag, 30 μM Cl^-	.902 ± .054	.226 ± .001	.811 ± .041	…
0 μg/L Ag, 300 μM Cl^-	<.05	<.05	…	346.2 ± 15.6
.1 μg/L Ag, 300 μM Cl^-	.085 ± .013	.0085 ± .0013	…	325.3 ± 13.5
1.0 μg/L Ag, 300 μM Cl^-	.866 ± .054	.173 ± .018	.782 ± .031	361.0 ± 8.3
0 μg/L Ag, 3,000 μM Cl^-	<.05	<.05	…	3,632.6 ± 264.2
.1 μg/L Ag, 3,000 μM Cl^-	.091 ± .002	.0059 ± .0001	…	3,768.9 ± 168.0
1.0 μg/L Ag, 3,000 μM Cl^-	1.228 ± .164	.0958 ± .0128	1.302 ± .031	4,550.6 ± 189.4

spectively. Clearly, both Cl^- and DOC provide a great deal of protection against silver-induced toxicity during acute exposure in freshwater fishes.

Early life stages are often the most sensitive to metal and contaminant exposures, and this appears to be true for silver (Davies et al. 1978; Nebeker et al. 1983). The mechanism of silver-induced toxicity during chronic exposure to silver (as $AgNO_3$) from fertilization to posthatch and swim-up in trout appears to be similar to that during acute silver exposure (Guadagnolo et al. 2001; Brauner and Wood 2002*a*, 2002*b*). Exposure to 1.0 μg/L total silver as $AgNO_3$ in hard water from fertilization to posthatch resulted in an elevation in whole body cortisol and ammonia levels in larval trout (Brauner and Wood 2002*a*), as has been observed during acute silver exposure in juvenile fish (Webb and Wood 1998). Higher levels (10 μg/L total silver as $AgNO_3$) are associated with an ionoregulatory impairment based on reduced whole body Na^+,K^+-ATPase activity levels, unidirectional Na^+ uptake rates (J_{in} Na^+), and whole body Na^+ levels (and, to a lesser degree, Cl^- levels; Guadagnolo et al. 2001; Brauner and Wood 2002*a*, 2002*b*).

Relatively little is known about the mitigative effects of ligands on chronic silver toxicity, despite the great diversity of ligands that exist in natural water systems that could potentially protect organisms from natural and anthropogenic sources of metal toxicity. In a recent study, DOC at 12 mg/L (in the form of Aldrich humic acid) greatly reduced mortality during silver exposure (10 μg/L total silver as $AgNO_3$ in hard water) up to hatch, but not following hatch (Brauner and Wood 2002*b*). Interestingly, DOC did not appear to protect against the silver-induced ionoregulatory disturbance, and it was concluded that any protective effects of DOC during chronic silver exposure appear to be far less than that observed during acute exposure. There are no published studies investigating the effect of water Cl^- on silver toxicity in early life stages of freshwater fishes.

The objectives of this study were to investigate whether Cl^- offers protection during chronic silver exposure (from fertilization to swim-up) in rainbow trout, given the pronounced protection observed during acute silver exposure (Galvez and Wood 1997; McGeer and Wood 1998; Bury et al. 1999*a*, 1999*b*). Developing embryos and larvae were exposed to silver (as $AgNO_3$) at nominal concentrations of 0, 0.1, and 1.0 μg/L total silver (as $AgNO_3$) in reconstituted reverse osmosis water at 30, 300, or 3,000 μM Cl^-. Measurements used to assess toxicity were mortality, time to hatch and swim-up, as well as extractable protein and ionoregulatory indicators such as whole embryo/larval J_{in} Na^+, Na^+,K^+-ATPase activity levels, and Na^+ and Cl^- levels. Total silver concentrations of 0.1 and 1.0 μg/L were chosen because the former is the Canadian Water Quality Guideline, representing both an acute and chronic value (Canadian Council of Ministers of the Environment 1995), and the latter is known to induce significant effects in developing rainbow trout embryos (Guadagnolo et al. 2000, 2001). Water Cl^- levels of 30, 300, and 3,000 μM Cl^- were chosen on the basis of the study of McGeer and Wood (1998). During acute exposure of juvenile rainbow trout to 3 μg/L total silver, 30 μM Cl^- offered very little protection of gill Na^+,K^+-ATPase activity; 300 μM Cl^- offered about 50% protection, whereas 3,000 μM Cl^- is twofold greater than the concentration that provided complete protection of gill Na^+,K^+-ATPase activity. The ultimate goal of this and future studies is to develop a model to predict chronic silver toxicity during exposure to a variety of surface water types.

Material and Methods

Rainbow trout embryos were purchased from Rainbow Springs Trout Hatchery in October 1999 and then transported to McMaster University. Embryos were fertilized at 6°C (at the hatchery) and warmed to 12.5°C over a 3-h duration in a darkened 20-L holding tank supplied with dechlorinated Hamilton hard water (Na^+, 0.60 mM; Cl^-, 0.70 mM; Ca^{2+}, 1.0 mM; Mg^{2+}, 0.2 mM; DOC, 2.86 mg/L; hardness, 120 mg $CaCO_3$/L). Water

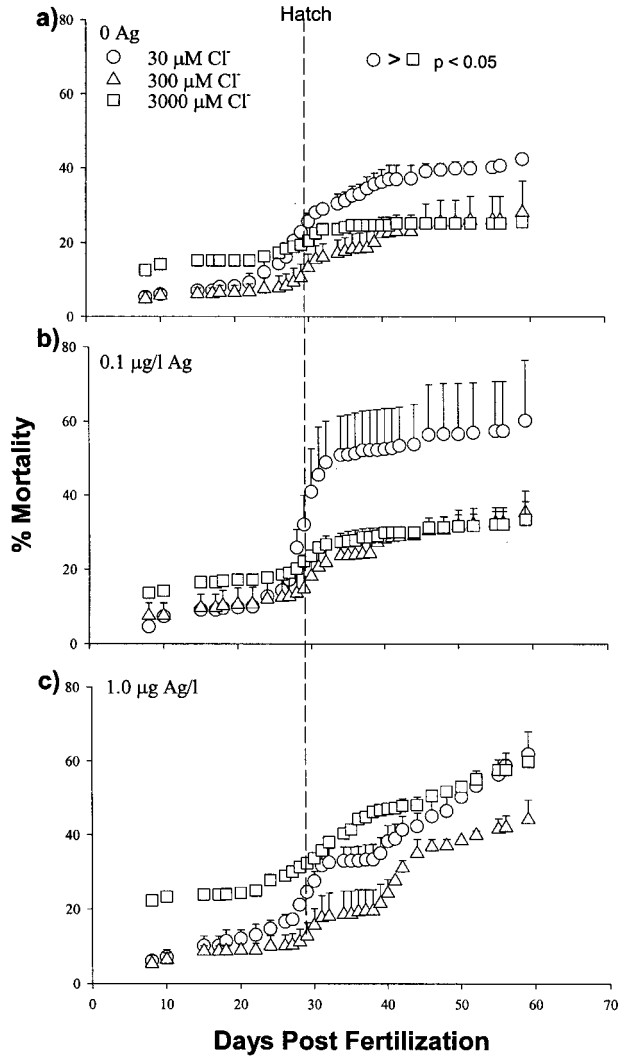

Figure 1. Percent cumulative mortality in rainbow trout embryos and larvae during continuous flow-through exposure to different water Cl^- levels, from fertilization to swim-up during exposure to silver (as $AgNO_3$) at (*a*) 0 μg/L, (*b*) 0.1 μg/L, and (*c*) 1.0 μg/L total silver ($n = 2$, and vertical bars indicate SD). Dashed line indicates 50% hatch. Water Cl^- levels consist of 30 μM Cl^- (*circles*), 300 μM Cl^- (*triangles*), and 3,000 μM Cl^- (*squares*).

temperature was maintained at 12.5° ± 0.2°C over the duration of the study. Following hatch, the photoperiod was 12L : 12D; however, because the larvae resided in opaque containers, the light levels to which they were exposed were very low.

Experimental Protocol

Within 3 h following fertilization, embryos were randomly distributed to one of three flow-through silver exposure concentrations (nominal of 0, 0.1, and 1.0 μg/L total silver, as $AgNO_3$; Fisher Scientific) at three water chloride levels (nominal of 30, 300, and 3,000 μM). All exposures were conducted in a synthetic soft water composed of reconstituted reverse osmosis (RO; Andersen, Dundas, Ontario) water (Na^+, 0.04 mM; Cl^-, 0.030 mM; Ca^{2+}, 0.04 mM; Mg^{2+}, 0.04 mM; DOC, 0.3 mg/L; hardness, 20 mg $CaCO_3$/L) that was generated from Hamilton tap water. Measured silver levels in unfiltered water from each treatment and in filtered water (Acrodisc 0.45-μm polyethersulfone inline filters; Gelman) from the nominal 1.0 μg/L total silver treatment as well as water Cl^- levels were measured every 7 d and are presented in Table 1. Embryos were exposed to treatments in duplicate. The first replicate contained 200 embryos (in 0.5-L chambers), and the second contained 700 embryos (in 2-L chambers). Mortality and time to hatch and

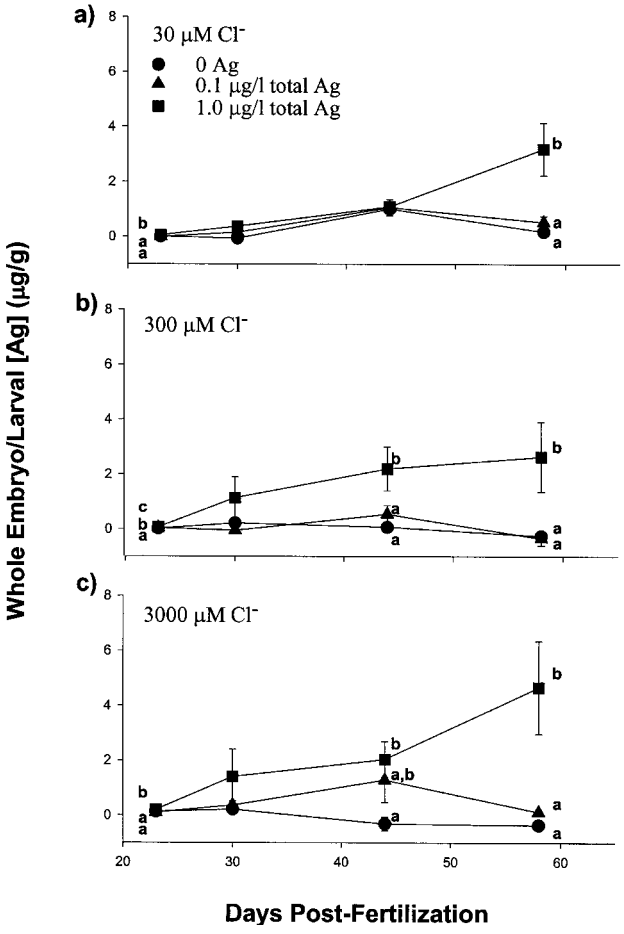

Figure 2. Whole embryo/larval silver accumulation in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. Circles represent controls, triangles represent 0.1 μg/L total silver, and squares represent 1.0 μg/L total silver. Data for days 23 and 30 represent values for embryos, whereas those on days 44 and 58 represent values for larvae. Letters that differ within a given time period indicate statistically significant differences ($P < 0.05$; $n = 10$).

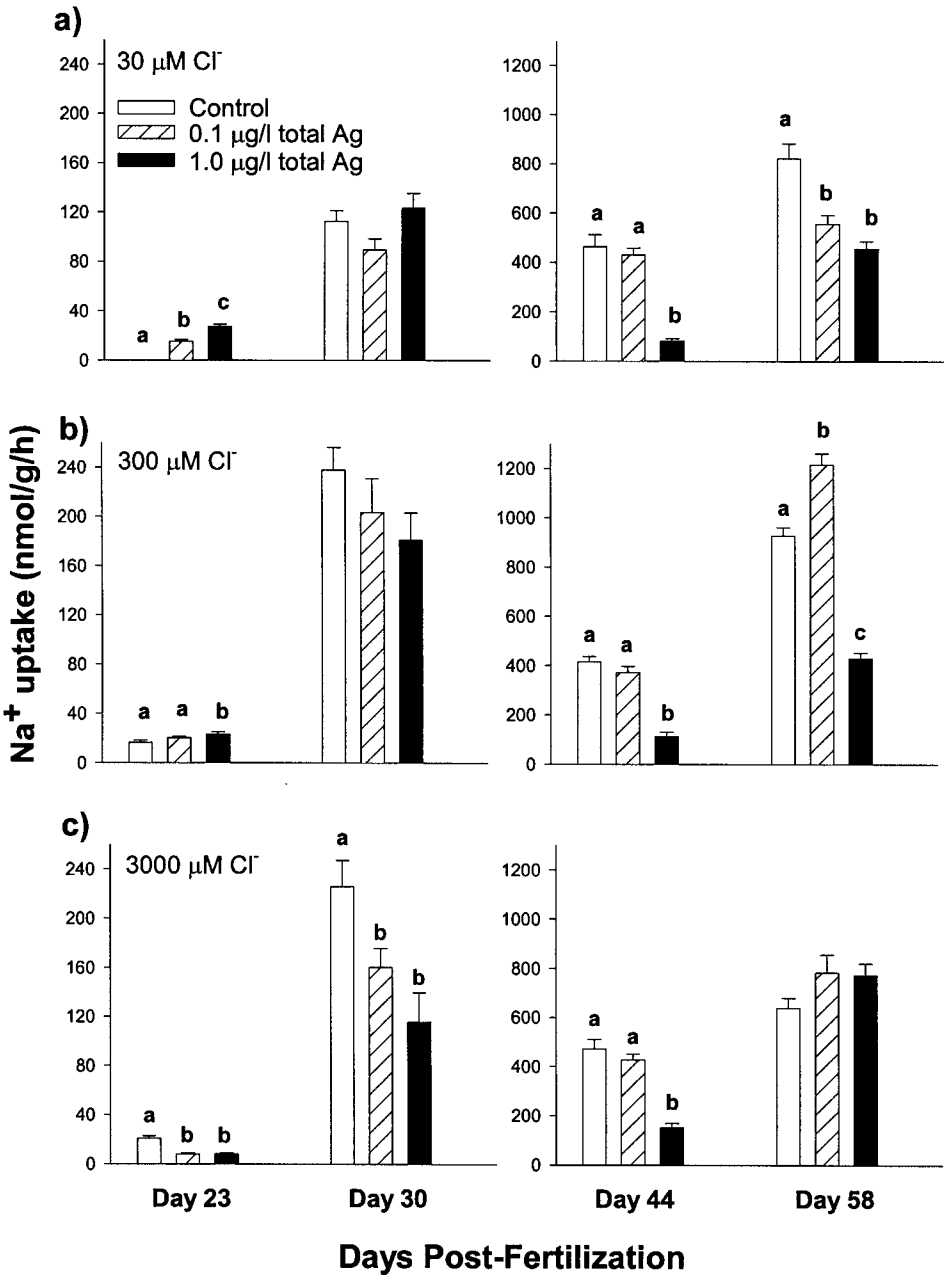

Figure 3. Whole embryo/larval unidirectional Na^+ uptake (J_{in} Na^+) in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. Open bars represent controls, hatched bars represent 0.1 μg/L total silver, and solid bars represent 1.0 μg/L total silver. Data for days 23 and 30 represent values for embryos, whereas those on days 44 and 58 represent values for larvae. Letters that differ within a given time period indicate statistically significant differences ($P < 0.05$; $n = 10$).

swim-up were measured in both replicates, whereas the second replicate was the source for embryos and larvae terminally sampled throughout the experiment. All containers were opaque to keep embryos in the dark during development.

Acidified stock solutions (0.5% HNO_3 [v/v] trace metal grade HNO_3; Fisher Scientific) of $AgNO_3$ and KCl in distilled water were made up in 1-L brown glass bottles at 1,000 times the final desired exposure concentration. No KCl was added to the nominal 30 μM Cl^- treatment because this was the composition of the reconstituted RO water. A peristaltic pump was used to

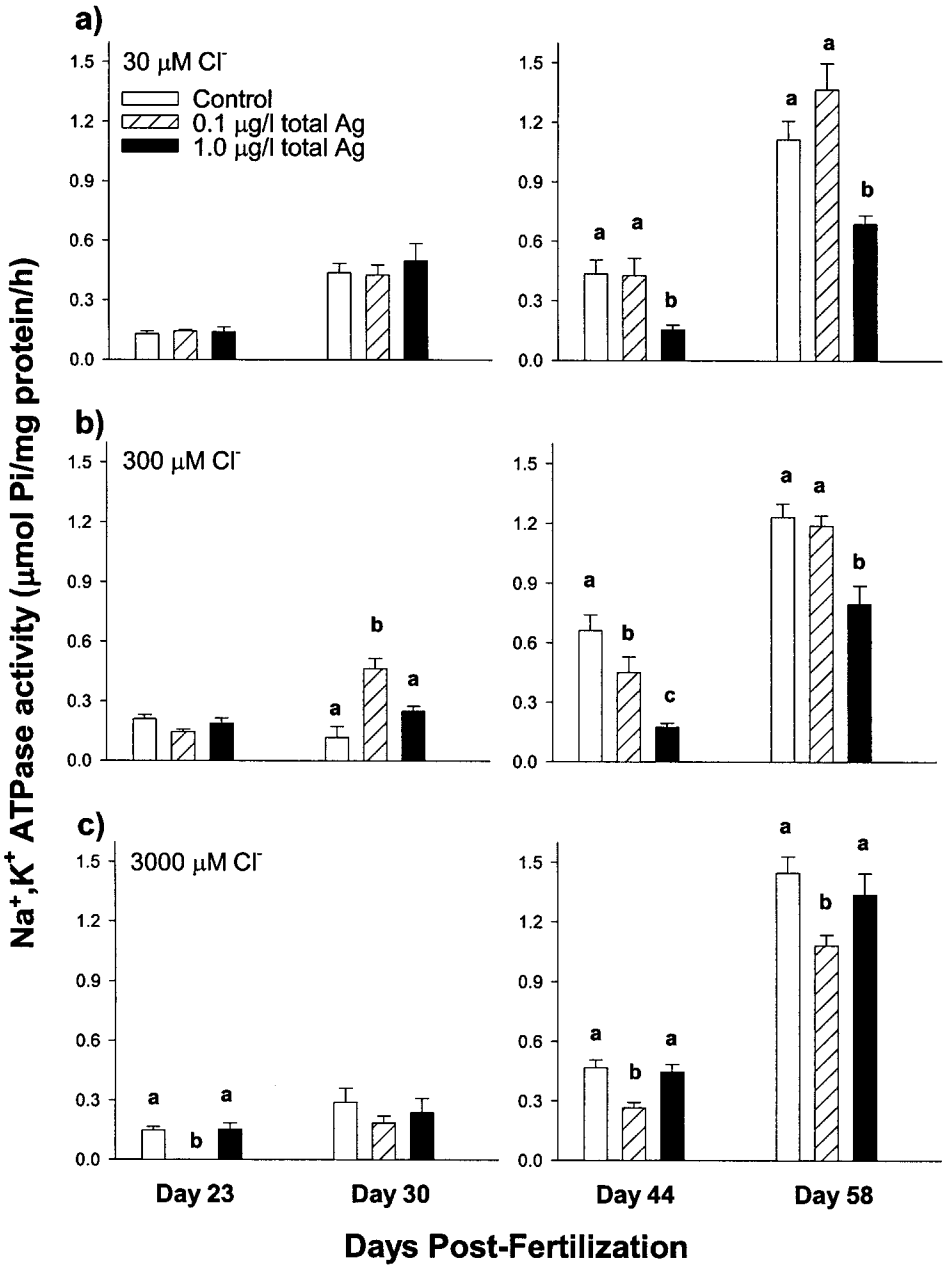

Figure 4. Whole embryo/larval Na^+,K^+-ATPase activity levels (standardized for protein concentration) in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. See Figure 3 legend for further information.

deliver stock solutions to 150-mL header tanks at a flow rate of 0.1 mL/min and mixed with temperature-controlled reconstituted RO water at 100 mL/min. Aeration was used to facilitate mixing, and the overflow from each header tank was split so that each exposure chamber had an individual flow-through source. In the 0.1 μg/L total silver treatment, 10 $\mu Ci^{110m}Ag$/L (Amersham International, Courtaboeuf, France) was added to the stock solution so that the final silver concentration in the exposure chamber could be determined by radioisotopic dilution (cf. Guadagnolo et al. 2000, 2001). This greatly increased the accuracy of silver detection.

Each day, mortality, percent hatch, and percent swim-up were determined, and following hatch, degree of yolk sac absorption was visually estimated. Dead embryos or larvae were

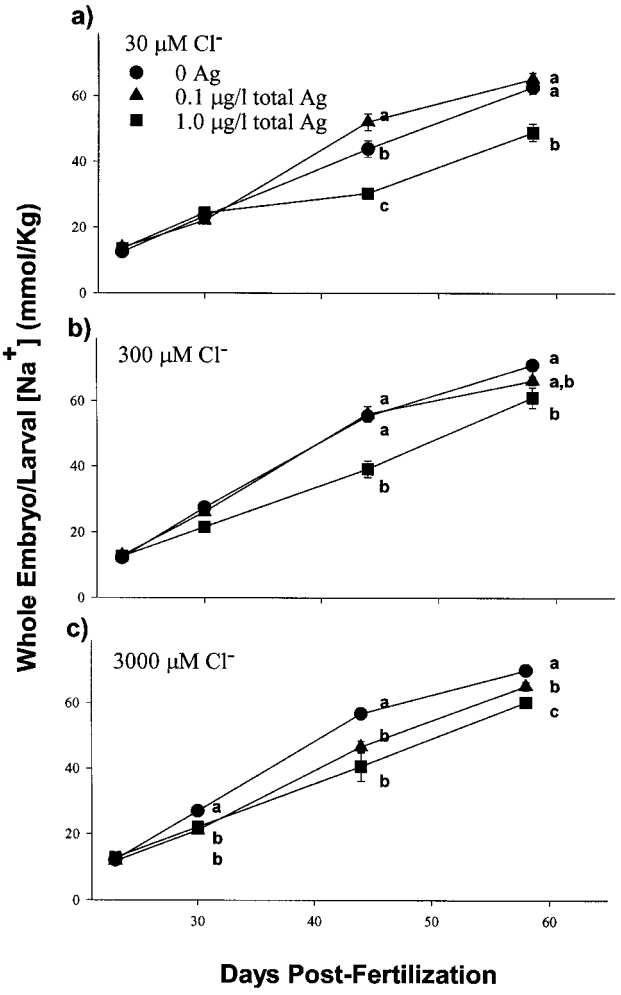

Figure 5. Whole embryo/larval Na^+ concentration in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. See Figure 2 legend for further information.

removed immediately from the exposure chambers and discarded. When possible, cessation of heartbeat was the criterion for mortality; however, before heartbeat could be observed, opaqueness of embryos was used as the criterion. On days 23, 30, 44, and 58 postfertilization, 50 live embryos or larvae from each treatment were collected, euthanized in tricaine methanesulfonate (MS-222; 0.1 g/L buffered with $NaHCO_3$), blotted dry, weighed, placed individually into Eppendorf tubes, and then frozen in liquid nitrogen. These samples were used for the determination of whole embryo/larval Na^+,K^+-ATPase activity levels, extractable protein and Na^+, Cl^-, and total silver concentrations as described below. An additional 20 embryos were removed at each sampling time for determination of whole embryo/larval unidirectional Na^+ uptake (J_{in} Na^+) as described below.

Water Analysis

Flow rates of water and stock solutions were checked daily, and water samples were collected from each exposure chamber weekly. Samples were immediately acidified with 0.5% (v/v) trace metal grade HNO_3 (Fisher Scientific) and stored in the dark for up to 1 wk before analysis. Atomic absorption spectrophotometery (AAS; Varian AA 1275, Mississauga, Ontario) was used to measure water [Na^+] for use in unidirectional Na^+ uptake calculations, and an associated graphite furnace atomizer (Varian GTA-95) was used for silver analyses, with a detection limit of about 0.05 μg/L total silver.

Total silver in the 0.1 μg/L exposure water was measured using radioisotopic dilution where the stock solution silver concentration was determined using AAS, and the radioactivity

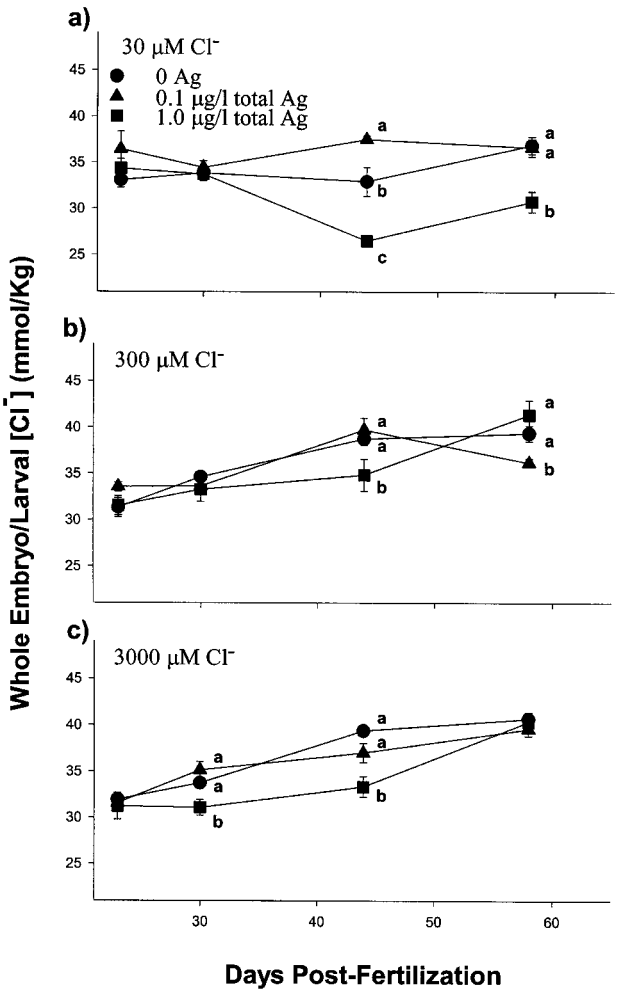

Figure 6. Whole embryo/larval Cl^- concentration in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. See Figure 2 legend for further information.

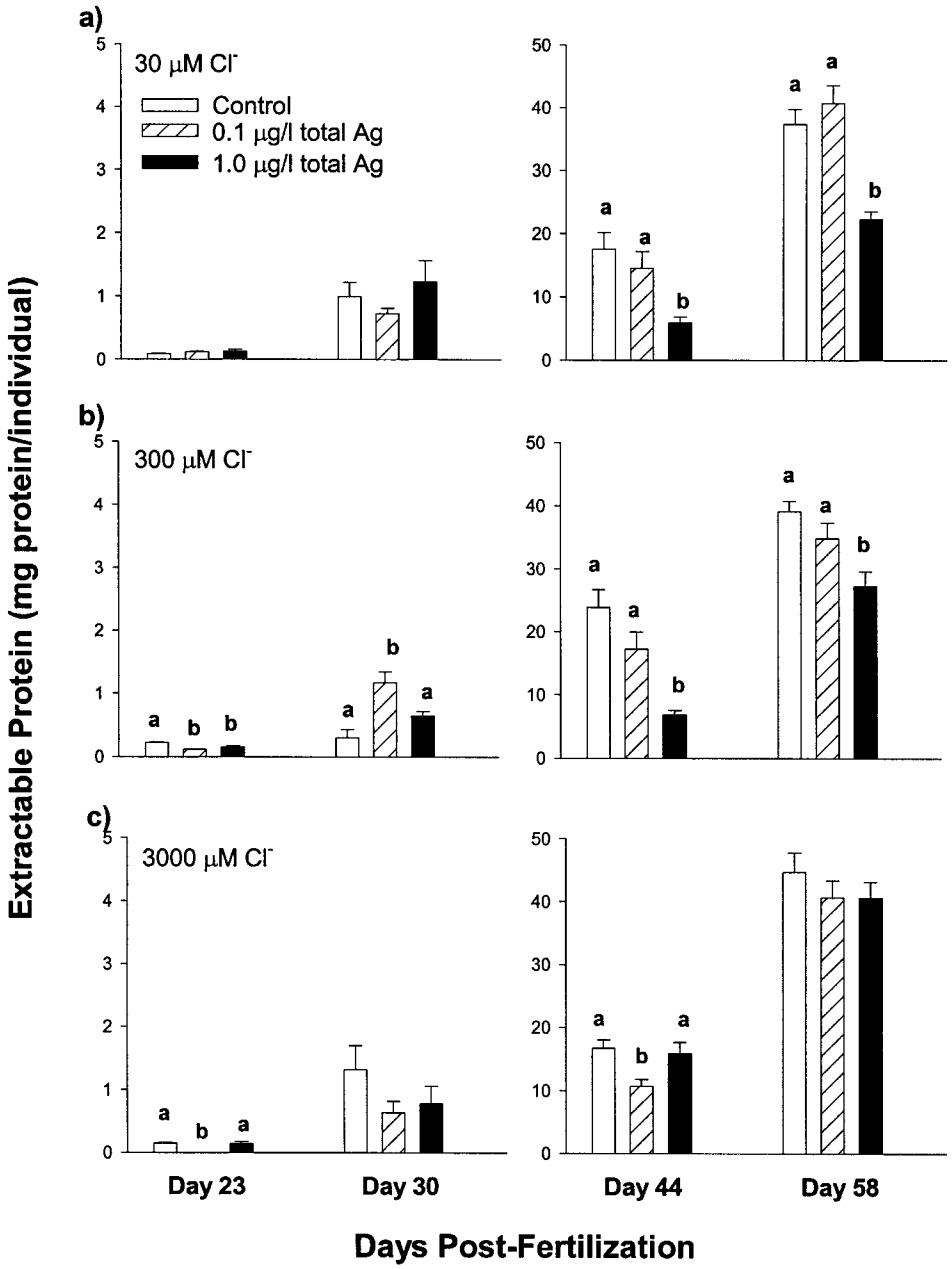

Figure 7. Whole embryo/larval extractable protein levels in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. See Figure 3 legend for further information.

(total counts per minute [cpm]) of ^{110m}Ag was measured on a gamma counter (Packard Instruments, Downers Grove, Ill.). The specific activity of the stock solution was calculated (cpm/μg silver) and divided into the total silver activity within the exposure tank to determine the final silver exposure concentration. Total Cl^- of the exposure water was measured using the colorimetric mercuric thiocyanate method (Zall et al. 1956).

Ion and Ionoregulatory Measurements

Unidirectional Na^+ uptake (J_{in} Na^+) in whole embryos and larvae was determined using ^{22}Na and 2–6-h flux periods in 60-mL Nalgene bottles according to the method of Brauner and Wood (2002*a*, 2002*b*). Whole embryo/larval Na^+, Cl^-, and total silver concentrations were determined by thawing previ-

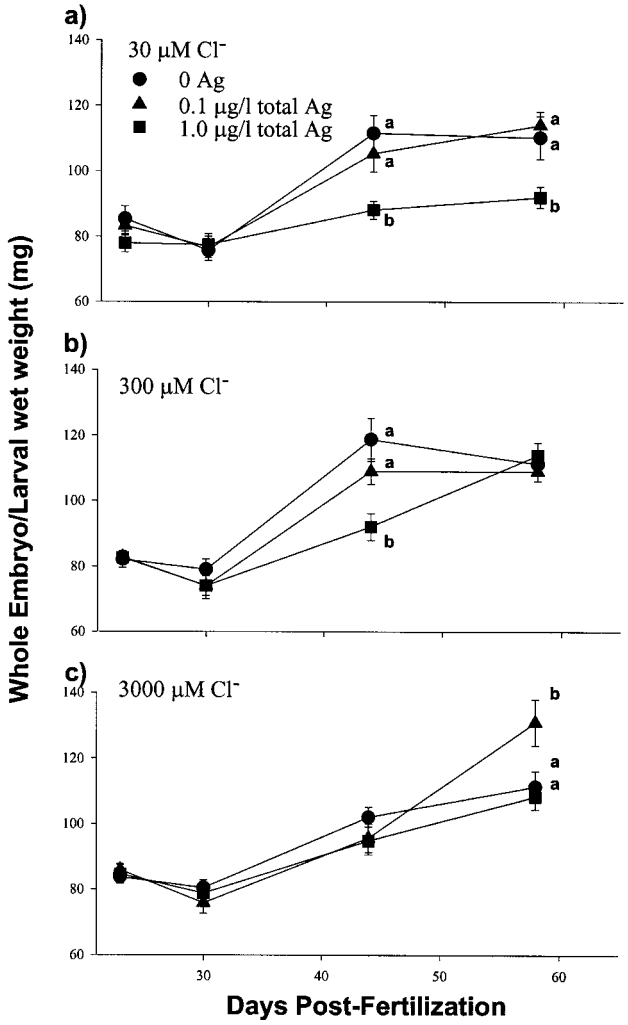

Figure 8. Whole embryo/larval wet weight of rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in synthetic soft water, from fertilization to swim-up at (*a*) 30 μM Cl^-, (*b*) 300 μM Cl^-, and (*c*) 3,000 μM Cl^-. See Figure 2 legend for further information.

ously frozen embryos or larvae in 1.5-mL Eppendorf tubes, adding 150 μL of 1.0 N HNO_3, and placing each sample in a drying oven at 60°–70°C for 24 h. The samples were vigorously vortexed and crushed when necessary to permit thorough digestion. Tubes were centrifuged at 10,000 *g* for 2 min, and the supernatant was diluted for measurement of silver using graphite furnace AAS. The supernatant was also used for measurement of [Na^+] using flame AAS and [Cl^-] by the colorimetric mercuric thiocyanate method (Zall et al. 1956).

The method of McCormick (1993) was used to measure Na^+,K^+-ATPase activity in crude homogenates of whole embryos or larvae, using a plate reader (Molecular Devices). Activity was expressed as the concentration of inorganic phosphate liberated per hour, standardized to protein content (which was measured using Bradford reagent [Bio-rad, Richmond, Calif.]), and bovine serum albumin as a standard.

Statistics

Time to 50% hatch and 50% swim-up were determined by log-probit analysis. Within a given water Cl^- treatment, statistical differences among mean values for the different silver exposure concentrations were compared using a one-way ANOVA on different days, followed by a Student-Newman Keuls post hoc test. Differences in mortality were tested among treatments up to hatch and from hatch to the end of the experiment using a one-way ANOVA on the number of mortalities per day (i.e., on noncumulative mortality). Values are presented as mean $\pm$ 1 SE and $n = 10$ throughout, unless otherwise indicated. The level of statistical significance for all analyses was $P < 0.05$.

Results

Water Chemistry

Measured water total silver levels were generally within 20% of nominal values (Table 1). There were no differences between filtered (0.45 μM) and unfiltered values, indicating that all silver was effectively dissolved. Measured water Cl^- levels tended to be somewhat higher than nominal values (Table 1). Speciation analyses were conducted using MINEQL+ (Schecher and

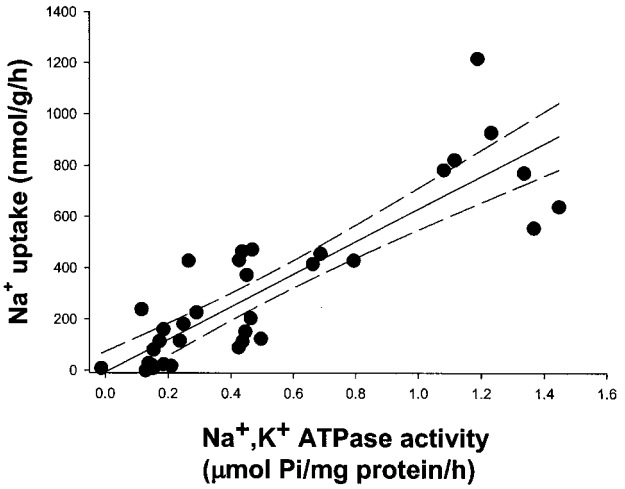

Figure 9. Relationship between whole body Na^+,K^+-ATPase activity level and Na^+ uptake rate (J_{in} Na^+) in developing embryos and larvae for all treatments and times combined. Individual points represent mean values ($n = 10$; with SEMs for both Na^+,K^+-ATPase and J_{in} Na^+ contained within the diameter of the point), and dashed lines indicated 95% confidence intervals about the regression. $y = 636.45x - 7.49$; $r^2 = 0.750$.

McAvoy 1994), and Ag^+ levels for the various treatments are reported in Table 1.

Mortality and Time to Hatch

Mortality (calculated as the number dead per day) before hatch was not statistically different among different water Cl^- levels at the silver concentrations tested in this study (Fig. 1). Control mortality reached 20% in the absence of silver exposure, which may have been influenced by the daily checking and removing of dead embryos.

Following hatch, noncumulative mortality in control embryos (no additional silver; Fig. 1*a*) was significantly greater in embryos reared at 30 μM Cl^- than 3,000 μM Cl^-, indicating that 30 μM Cl^- alone is a challenge for these larvae (Fig. 1). Noncumulative mortality in the 0.1 and 1.0 μg/L total silver group was not statistically different among the water Cl^- treatments, indicating that water Cl^- levels offered little protection against silver-induced mortality.

There were no statistically significant differences in time to 50% hatch among any of the treatments (pooled value = 28.7 ± 0.44 d) or in time to 50% swim-up (pooled value = 45.9 ± 0.46 d). By day 58, all larvae that were alive had reached swim-up.

Silver Accumulation

By day 23 of silver exposure, whole embryo silver accumulation was statistically greater in the 1.0 μg/L silver treatment relative to controls at all water Cl^- levels, although the total accumulation at this stage was relatively low (Fig. 2). There were no statistically significant differences in silver content among treatments immediately following hatch (day 30 postfertilization), but by days 44 and 58, silver content of the 1.0 μg/L total silver group was significantly greater than controls at all water Cl^- exposure levels, except for the 30 μM Cl^- treatment on day 44. Qualitatively, accumulation during exposure to 1.0 μg/L total silver over time was similar at all water Cl^- levels.

Ionoregulation

J_{in} *Na^+*. Before hatch, J_{in} Na^+ levels were low; however, on day 23 postfertilization, there was a statistically significant elevation in J_{in} Na^+ during exposure to 0.1 and 1.0 μg/L total silver relative to controls at 30 μM Cl^-, as well as a significant elevation in J_{in} Na^+ in the 1.0 μg/L treatment relative to controls at 300 μM (Fig. 3). For most remaining sampling times, there was a significant reduction in J_{in} Na^+ during exposure to 1.0 μg/L total silver relative to controls at the different water Cl^- concentrations, except for day 30 in the 30 and 300 μM Cl^- treatments and day 58 in the 3,000 μM Cl^- treatment, when no statistically significant differences were observed among the silver exposure concentrations. Thus, in general, water chloride

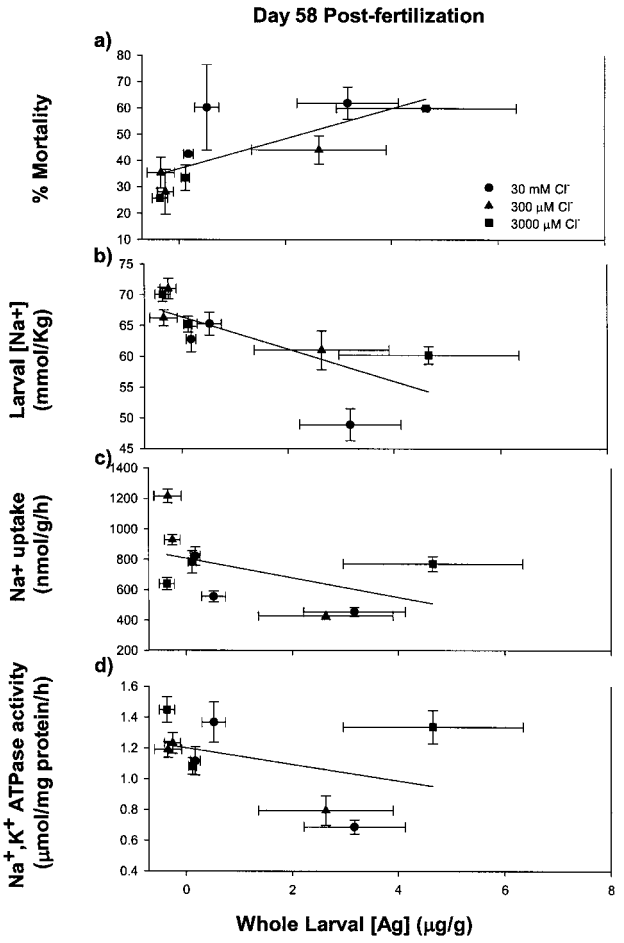

Figure 10. Relationship between whole body silver concentration on day 58 postfertilization and (*a*) cumulative percent mortality ($y = 5.73x + 36.90$; $r^2 = 0.559$), (*b*) larval [Na+] ($y = -2.59x + 66.37$; $r^2 = 0.541$), (*c*) Na^+ uptake ($y = -64.05x + 806.32$; $r^2 = 0.228$), and (*d*) whole larval Na^+,K^+-ATPase activity ($y = -0.053x + 1.20$; $r^2 = 0.150$). Individual points represent mean values ($n = 10$; with SEMs). Circles represent data obtained at 30 μM Cl^-, triangles at 300 μM Cl^-, and squares at 3,000 μM Cl^-. Removal of data for the 3,000 μM Cl^- treatment (all silver treatments) significantly improves the relationships to $y = -169.4x + 900.56$, $r^2 = 0.698$ (*c*) and $y = -0.156x + 1.218$, $r^2 = 0.808$ (*d*). This omission has statistically insignificant effects on the relationships in *a* and *b*.

levels up to 3,000 μM Cl^- did not appear to protect against exposure to 1.0 μg/L total silver with the exception of day 58.

Na^+,K^+-ATPase Activity. Up to day 30 postfertilization, there were only minor significant differences in Na^+,K^+-ATPase specific activity levels among silver and water Cl^- treatments (Fig. 4). However, by days 44 and 58 postfertilization, there was a statistically significant reduction in enzyme specific activity level during exposure to 1.0 μg/L total silver relative to controls at 30 and 300 μM Cl^-. This effect was not observed in 3,000 μM

Cl^- on days 44 and 58 postfertilization, indicating that water Cl^- levels at this level may have been protective. Interestingly, however, 0.1 μg/L total silver resulted in a significant reduction in Na^+,K^+-ATPase specific activity relative to controls in 3,000 μM Cl^- on these days.

Whole Embryo/Larval [Na^+] and [Cl^-]. No significant differences were observed in either embryonic Na^+ (Fig. 5) or Cl^- (Fig. 6) before hatch (day 23 postfertilization). Following hatch, there was a significant reduction in larval [Na^+] at 1.0 μg/L total silver relative to controls at all Cl^- levels, except for the 30 and 300 μM Cl^- treatments on day 30 (Fig. 5). On days 44 and 58, the greatest difference in [Na^+] between controls and the 1.0 μg/L total silver-exposed group was at 30 μM Cl^-, indicating that water Cl^- may have been protective of Na^+ loss during silver exposure. However, the 3,000 μM Cl^- treatment was also the only treatment that resulted in a significant reduction in larval [Na^+] in the 0.1 μg/L total silver treatment relative to controls (except for 30 μM Cl^- on day 43 postfertilization).

Changes in larval Cl^- levels were less clear (Fig. 6). In general, there was a significant reduction in larval Cl^- levels during exposure to 1.0 μg/L total silver relative to controls at 3,000 μM Cl^- on day 30. This trend was observed at all water Cl^- levels on day 43 but only in the 30 μM Cl^- treatment on day 58 postfertilization. These data indicate that later in development, water Cl^- is partially protective of silver-induced body Cl^- loss.

Growth and Extractable Protein. In general, changes in extractable protein from whole embryos or larvae during exposure to the various silver and water Cl^- combinations were qualitatively similar to those observed for Na^+,K^+-ATPase activity (Fig. 7). Following hatch, on days 44 and 58, there was a statistically significant reduction in whole larval extractable protein relative to controls during exposure to 1.0 μg/L total silver at water Cl^- concentrations of 30 and 300 μM; however, there was no difference at 3,000 μM Cl^- consistent with a protective effect of water Cl^- during silver exposure. Interestingly, as with Na^+,K^+-ATPase activity, there was a statistically significant reduction in extractable protein in larvae exposed to 0.1 μg/L total silver on day 44 in the 3,000 μM Cl^- treatment.

There were no significant reductions in larval wet weight associated with silver exposure until day 44, at which time values in the 1.0 μg/L total silver-exposed group were significantly less than control and 0.1 μg/L total silver treatments at 30 and 300 μM Cl^- (Fig. 8). On day 44, there were no significant differences among treatments at 3,000 μM Cl^-. On day 58, wet weight was significantly lower in 1.0 μg/L total silver relative to the control and 0.1 μg/L total silver treatments at 30 μM Cl^- but not at 300 or 3,000 μM Cl^-. These data again indicate that higher water Cl^- levels appear to be protective of silver-induced reductions in growth.

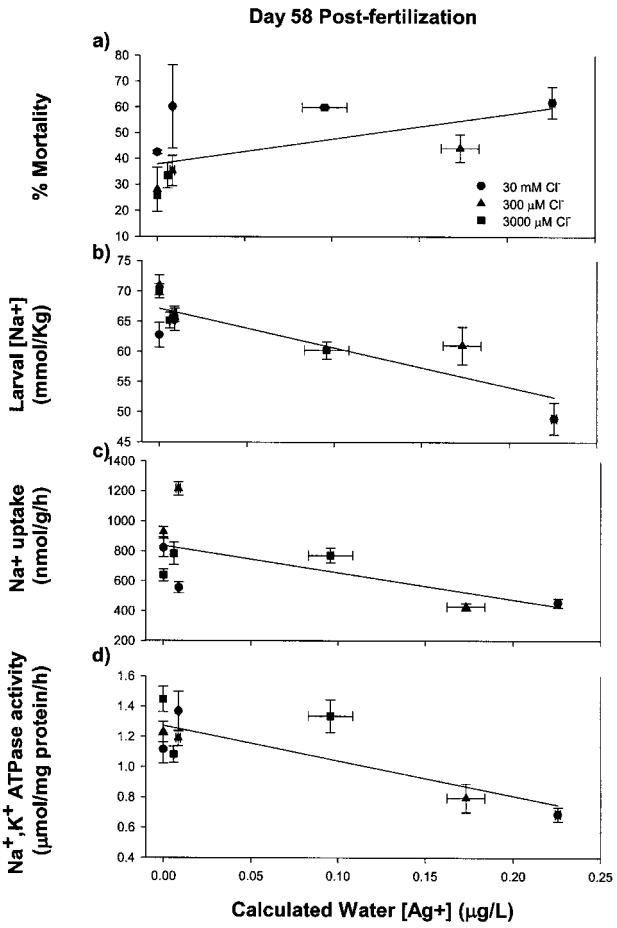

Figure 11. Relationship between calculated water Ag^+ concentration on day 58 postfertilization and (*a*) cumulative percent mortality ($y = 97.2x + 37.9$; $r^2 = 0.354$), (*b*) larval [Na+] ($y = -65.0x + 67.1$; $r^2 = 0.748$), (*c*) Na^+ uptake ($y = -1{,}801.7x + 836.6$; $r^2 = 0.397$), and (*d*) whole larval Na^+,K^+-ATPase activity ($y = -2.35x + 1.27$; $r^2 = 0.624$). See Figure 3 legend for further information.

Discussion

Use of Whole Body Na^+,K^+-ATPase to Assess Ionoregulatory Status in Early Life Stages

In this and previous studies (Brauner and Wood 2002*a*, 2002*b*), whole body Na^+,K^+-ATPase activity has been assumed to be correlated with gill, yolk sac, and skin Na^+,K^+-ATPase activity. Thus, a reduction in whole body Na^+,K^+-ATPase activity levels associated with silver exposure is assumed to be indicative of some level of ionoregulatory impairment. Although Na^+,K^+-ATPase is found throughout the body in virtually all cells, support for using whole body levels as an indicator of ionoregulatory status in early life stages is offered by Figure 9, where there is a clear relationship ($r^2 = 0.75$) between whole body Na^+,K^+-ATPase activity and J_{in} Na^+ when all data are plotted for all treatments (total silver as $AgNO_3$ of 0, 0.1, and 1.0 μg/

L at 30, 300, or 3,000 μM water Cl^-) and days (23, 30, 44, and 58 d postfertilization) combined. Although whole body Na^+,K^+-ATPase activity levels increase exponentially during early stages of development, this is correlated with a similar increase in J_{in} Na^+ (Brauner and Wood 2002*a*). Thus, changes in whole body Na^+,K^+-ATPase activity levels, relative to appropriate controls, appear to be very useful as an indicator of ionoregulatory status, particularly following hatch when activity levels and J_{in} Na^+ are increasing rapidly (Rudy and Potts 1969; Eddy and Talbot 1985; Brauner and Wood 2002*a*, 2002*b*).

As early as 2 d posthatch, it is clear that there is extensive Na^+,K^+-ATPase immunoreactivity in the gills and skin in rainbow trout (data not shown). With further development (16 d posthatch), there is increased labeling in the gills and a reduction in labeling in the skin. These data are consistent with the theory that the gills play a major role in ionoregulation early in development (Rombough 1999, 2002). Extensive labeling was also observed in the kidney (data not shown), and although this contributes to ionoregulatory ability, it will not influence J_{in} Na^+. Clearly, a reduction in whole body Na^+,K^+-ATPase activity associated with an experimental treatment will underestimate the magnitude of ionoregulatory disturbance at the gills but appears quite useful as a qualitative index of ionoregulatory impairment in early life stages.

Effect of Water Cl^- in Mitigating Silver Toxicity

In general, by days 44 and 58 postfertilization, exposure to silver at 1.0 μg/L total silver (as $AgNO_3$) at the lowest water Cl^- level (30 μM) resulted in an accumulation of whole body silver and a reduction in J_{in} Na^+, Na^+,K^+-ATPase activity level, whole body Na^+ and Cl^- levels, as well as extractable protein and larval wet weight relative to controls (Figs. 2–8). These data indicate that the mechanism of toxicity during chronic silver exposure is associated with a large ionoregulatory disturbance, as has been observed in other studies investigating chronic silver toxicity (Guadagnolo et al. 2001; Brauner and Wood 2002*a*, 2002*b*). Furthermore, an ionoregulatory disturbance is the primary mechanism of silver toxicity during acute silver exposure (Wood et al. 1996; Morgan et al. 1997; McGeer and Wood 1998), indicating some similarities between acute and chronic silver exposure.

An increase in water Cl^- offered partial protection against the ionoregulatory disturbance associated with silver exposure that is most clear on day 58 postfertilization, where J_{in} Na^+, whole body Na^+,K^+-ATPase activity, larval Cl^-, extractable protein, and wet weight were no longer significantly different between controls and the 1.0 μg/L total silver treatment, and the disturbance in larval [Na^+] had been partially restored. This occurs at a time at which gill chloride cell proliferation is likely fairly extensive (Rombough 1999). Interestingly, however, the protection of water Cl^- on silver-induced ionoregulatory disturbance did not appear to be transmitted to mortality, where relatively little protection was observed.

The greatest mortality during exposure to 0.1 μg/L total silver was observed in larvae at the lowest water Cl^- level (30 μM) when control mortality was high (Fig. 1), and increased water Cl^- levels appeared to offer some reduction in mortality relative to controls (Fig. 1). However, in embryos and larvae exposed to 1.0 μg/L total silver, similar levels of mortality were observed at 30 and 3,000 μM water Cl^-, where the latter was hypothesized to offer the greatest level of protection. Given that the ionoregulatory disturbance associated with exposure to 1.0 μg/L total silver in 3,000 μM water Cl^- was not mitigated until day 58 postfertilization, it may be that, had the experiment proceeded longer, greater differences in the level of protection offered by water Cl^- would have been observed at higher water Cl^- levels. Regardless, the level of protection afforded by Cl^- during long-term exposure to silver is much less than that seen during acute silver exposure, in which LC_{50} values are increased by four- to fivefold over this range in water Cl^- (McGeer and Wood 1998; Bury et al. 1999*b*).

In similar studies designed to investigate the protective effects of DOC on long-term silver exposure in rainbow trout from fertilization to swim-up, DOC offered only partial protection of mortality and minimal protection against the silver-induced ionoregulatory disturbance, indicating that DOC is far less protective during chronic than during acute silver exposure (Brauner and Wood 2002*b*). Whether this greatly reduced level of protection by water Cl^- and DOC during silver exposure is associated specifically with early life stages or merely related to the difference in duration of silver exposure, remains to be determined.

Prediction of Chronic Silver Toxicity

Geochemical type models ("Biotic Ligand Models") are presently being used to estimate acute metal toxicity by predicting gill metal accumulation (Janes and Playle 1995; Playle 1998; MacRae et al. 1999; Paquin et al. 1999), and recently, an entire issue in *Comparative Biochemistry and Physiology* (vol. 133C, pp. 1–343) was devoted to this topic. These models take into account the chemistry of the water and the gill microenvironment to predict the speciation state of the metal and interactions of the metal with ligands present in the water and binding sites on the gill. The assumption is that acute toxicity is proportional to the gill metal burden, and this has been validated to different degrees for Cu, Cd, and Ni (Playle et al. 1993; MacRae et al. 1999; Meyer et al. 1999). During acute exposure to silver, however, gill metal burden does not appear to predict silver toxicity (McGeer and Wood 1998; Bury et al. 1999*b*; Wood et al. 1999). A recent model has been developed that predicts acute silver toxicity on the basis of the predicted binding of Ag^+ to gill Na^+,K^+-ATPase (the site at which silver exerts its primary toxic effect) rather than predicted gill silver accu-

mulation, and this is presently being verified for a greater range of laboratory and natural waters (McGeer et al. 2000).

Although gill silver burden does not appear to predict silver toxicity during acute exposure, data in this study indicate that whole body silver accumulation may indeed predict chronic silver toxicity in early life stages of rainbow trout. Figure 10 represents the relationship between whole larval silver concentration on day 58 of silver exposure and cumulative mortality to that point for all treatments combined ($r^2 = 0.559$; Fig. 10*a*). Given that the primary mechanism of silver toxicity appears to be due to ionoregulatory disturbance, it is not surprising that a correlation also exists between larval silver accumulation and whole body Na^+ concentration ($r^2 = 0.541$; Fig. 10*b*). Mortality and whole body Na^+ concentration result from the cumulative effects of long-term silver exposure. Measurements such as whole body Na^+,K^+-ATPase activity and J_{in} Na^+ are more instantaneous measurements of silver-induced ionoregulatory disturbances and, not surprisingly, exhibit weaker relationships with body silver levels (Fig. 10*c*, 10*d*). However, when the data for the highest water Cl^- treatment (much higher than that found in most natural freshwaters) is removed from the analyses, the relationship between larval silver concentration and whole body Na^+,K^+-ATPase activity and J_{in} Na^+ are greatly strengthened ($r^2 = 0.6981$ and 0.808, respectively). Thus, silver accumulation during long-term exposure appears to be correlated with impairment of Na^+,K^+-ATPase activity and J_{in} Na^+, which ultimately leads to a reduction in whole body Na^+ concentration and mortality.

During acute silver exposure, ionoregulatory impairment and mortality is best correlated with the calculated (using MINEQL+; Schecher and McAvoy 1994) water Ag^+ levels (McGeer and Wood 1998; Bury et al. 1999*b*) rather than total silver levels. This also appears to hold true during chronic silver exposure, where there were significant correlations between calculated water Ag^+ concentration and cumulative mortality ($r^2 = 0.354$), larval [Na+] ($r^2 = 0.748$), J_{in} Na^+ ($r^2 = 0.397$), and whole larval Na^+,K^+-ATPase activity ($r^2 = 0.624$) in the last sampling period following 58 d of silver exposure (Fig. 11).

Summary

The mechanism of chronic silver toxicity appears to be impairment of ion uptake leading to a reduction in egg/larval Na^+ and Cl^- concentrations and death, similar to that observed during acute silver exposure. During chronic silver exposure, Ag^+ is the most toxic form of silver, and both Cl^- and DOC offer some degree of protection as observed during acute silver exposure but not nearly to the same degree. There appears to be potential to model chronic toxicity but not simply by recalibration of an acute model. A chronic model must be based on real chronic data because the protective effects of various ligands appear to be quantitatively very different from the acute situation. These data indicate that a model based on silver accumulation and/or calculated water Ag^+ levels may be possible during chronic exposure. Further experiments are being conducted to investigate the relationship between protective effects of other water ligands, such as Ca^{2+}, on silver toxicity to incorporate into a chronic toxicity model.

Acknowledgments

This study was supported by a Natural Sciences and Engineering Research Council of Canada Industrially Oriented Research Grant in conjunction with Kodak Canada Incorporated. We thank Joe Gorsuch for valuable comments and suggestions and Angel Sing and Martin Grosell for valuable technical assistance. We also thank Tammie Morgan for conducting the MINEQL+ silver speciation analyses.

Literature Cited

Bianchini A., K.C. Bowles, C.J. Brauner, J.W. Gorsuch, J.R. Kramer, and C.M. Wood. 2002. Evaluation of the effect of reactive sulfide on the acute toxicity of silver (I) to *Daphnia magna*. 2. Toxicity results. Environ Toxicol Chem 21:1294–1300.

Brauner C.J. and C.M. Wood. 2002*a*. Ionoregulatory development and the effect of chronic silver exposure on growth, survival, and sublethal indicators of toxicity in early life stages of rainbow trout (*Oncorhynchus mykiss*). J Comp Physiol B 172:153–162.

———. 2002*b*. Effect of long-term silver exposure on survival and ionoregulatory development in rainbow trout (*Oncorhynchus mykiss*) embryos and larvae, in the presence and absence of added dissolved organic matter. Comp Biochem Physiol 133C:161–173.

Bury N.R., F. Galvez, and C.M. Wood. 1999*a*. Effects of chloride, calcium, and dissolved organic carbon on silver toxicity: comparison between rainbow trout and fathead minnows. Environ Toxicol Chem 18:56–62.

Bury N.R., J.C. McGeer, and C.M. Wood. 1999*b*. Effects of altering freshwater chemistry on the physiological responses of rainbow trout to silver exposure. Environ Toxicol Chem 18:49–55.

Canadian Council of Ministers of the Environment. 1995. Canadian Water Quality Guidelines: Silver. Canadian Council of Ministers of the Environment, Winnipeg, Manitoba.

Davies P.H., J.P. Goettl, and J.R. Sinley. 1978. Toxicity of silver to rainbow trout (*Salmo gairdneri*). Water Res 12:113–117.

Eddy F.B. and C. Talbot. 1985. Sodium balance in eggs and dechorionated embryos of the Atlantic salmon *Salmo salar* L. exposed to zinc, aluminum, and acid waters. Comp Biochem Physiol 81C:259–266.

Galvez F. and C.M. Wood. 1997. The relative importance of water hardness and chloride levels in modifying the acute

toxicity of silver to rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 16:2363–2368.

Grosell M., C.J. Brauner, S.P. Kelly, J.C. McGeer, A. Bianchini, and C.M. Wood. 2002. Physiological responses to acute silver exposure in the freshwater crayfish (*Cambarus diogenes diogenes*): a model invertebrate? Environ Toxicol Chem 21:369–374.

Guadagnolo C.M., C.J. Brauner, and C.M. Wood. 2000. Effects of an acute silver challenge on survival, silver distribution and ionoregulation within developing rainbow trout eggs (*Oncorhynchus mykiss*). Aquat Toxicol 51:195–211.

———. 2001. Chronic effects of silver exposure on ion levels, survival and silver distribution within developing rainbow trout embryos (*Oncorhynchus mykiss*). Environ Toxicol Chem 20:553–560.

Janes N. and R.C. Playle. 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 14:1847–1858.

Karen D.J., D.R. Ownby, B.L. Forsythe, T.P. Bills, T.W. La Point, G.B. Cobb, and S.J. Klaine. 1999. Influence of water quality on silver toxicity to rainbow trout (*Oncorhynchus mykiss*), fathead minnows (*Pimephales promelas*), and water fleas (*Daphnia magna*). Environ Toxicol Chem 18:63–70.

Le Blanc G.A., J.D. Masone, A.P. Paradice, B. Wilson, H.B. Lockhart, and K.A. Robillard. 1984. The influence of speciation on the toxicity of silver to fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 3:37–46.

MacRae R.K., D.E. Smith, N. Swoboda-Colberg, J.S. Meyer, and H.L. Bergman. 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gills: implication for assessing bioavailable metal. Environ Toxicol Chem 18:1180–1189.

McCormick S.D. 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+ ATPase activity. Can J Fish Aquat Sci 50:656–658.

McGeer J.C., R.C. Playle, C.M. Wood, and F. Galvez. 2000. A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to rainbow trout in fresh waters. Environ Sci Technol 34:4199–4207.

McGeer J.M. and C.M. Wood. 1998. Protective effects of water Cl^- on physiological responses to waterborne silver in rainbow trout. Can J Fish Aquat Sci 55:2447–2454.

Meyer J.S., R.C. Santore, J.P. Bobbitt, L.D. DeBrey, C.J. Boese, P.R. Paquin, H.E. Allen, H.L. Bergman, and D.M. DiToro. 1999. Binding of nickel and copper to fish gills predicts toxicity when water hardness varies, but free-ion activity does not. Environ Sci Technol 33:913–916.

Morgan I.J., R.P. Henry, and C.M. Wood. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. Aquat Toxicol 38:145–163.

Nebeker A.V., C.K. McAuliffe, R. Mshar, and D.G. Stevens. 1983. Toxicity of silver to steelhead and rainbow trout, fathead minnows and *Daphnia magna*. Environ Toxicol Chem 2:95–104.

Paquin P.R., D.M. DiToro, R.S. Santore, D. Trevedi, and K.B. Wu. 1999. A biotic ligand model of the acute toxicity of metals. III. Application to fish and *Daphnia* exposure to silver. EPA 822-E-99-001, Environmental Protection Agency, Washington, D.C.

Playle R.C. 1998. Modelling metal interactions at fish gills. Sci Total Environ 219:147–163.

Playle R.C., D.G. Dixon, and K. Burnison. 1993. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modeling of metal accumulation. Can J Fish Aquat Sci 50:2678–2687.

Rombough P.J. 1999. The gill of larvae: is it primarily a respiratory or an ionoregulatory structure? J Fish Biol 55:186–204.

———. 2002. Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. J Exp Biol 205:1787–1794.

Rudy P.P. and W.T.W. Potts. 1969. Sodium balance in the eggs of the Atlantic salmon, *Salmo salar*. J Exp Biol 50:239–246.

Schecher W.D. and D.C. McAvoy. 1994. MINEQL+ User's Manual. Version 3.01. Environmental Research Software, Hallowell, Maine.

Webb N.A. and C.M. Wood. 1998. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 17:579–588.

Wood C.M., C. Hogstrand, F. Galvez, and R.S. Munger. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. Effects of ionic Ag^+. Aquat Toxicol 35:93–109.

Wood C.M., R.C. Playle, and C. Hogstrand. 1999. Physiology and modelling of mechanisms of silver uptake and toxicity in fish. Environ Toxicol Chem 18:71–83.

Zall D.M., D. Fisher, and M.D. Garner. 1956. Photometric determination of chlorides in water. Anal Chem 28:1665–1678.