

## CHRONIC EFFECTS OF SILVER EXPOSURE ON ION LEVELS, SURVIVAL, AND SILVER DISTRIBUTION WITHIN DEVELOPING RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EMBRYOS

CHRISTINE M. GUADAGNOLO,<sup>†</sup> COLIN J. BRAUNER,<sup>\*‡</sup> and CHRIS M. WOOD<sup>†</sup>

<sup>†</sup>Department of Biology, McMaster University, Hamilton, Ontario L8S 4K, Canada

<sup>‡</sup>Department of Biology, 5500 Campanile Drive, San Diego State University, San Diego, California 92182-4614, USA

(Received 17 September 1999; Accepted 17 July 2000)

**Abstract**—Rainbow trout embryos were chronically exposed to silver (as AgNO<sub>3</sub>) in moderately hard water (120 mg CaCO<sub>3</sub>/L, 0.70 mM Cl<sup>-</sup>, 1.3 mg/L dissolved organic matter, 12.3 ± 0.1 °C) at nominal concentrations of 0.1, 1, and 10 µg/L (measured = 0.117 ± 0.008, 1.22 ± 0.16, and 13.51 ± 1.58 µg/L, respectively) to investigate the effects on mortality, ionoregulation, and silver uptake and distribution of the embryo. Mortalities in the low concentrations (0.1 and 1.2 µg/L) were not significantly different from controls throughout embryonic development (days 1–32 postfertilization). Mortalities of embryos in the 13.5-µg/L treatment reached 56% by day 32 postfertilization (33% when accounting for control mortality), by which time more than 50% of surviving embryos had hatched. Accumulation of silver in whole embryos of 1.2- and 13.5-µg/L treatments reached the highest concentrations of 0.13 and 0.24 µg/g total silver, respectively, by day 32, but whole embryo silver burden was not correlated with mortality. Silver concentrations in different compartments of the whole embryo (chorion, dissected embryo, and yolk) were greatest just before hatch and were higher in the chorion for all experimental treatments. Up to 85% of total whole embryo silver content was bound to the chorion, which acts as a protective barrier during silver exposure. Whole embryo Na<sup>+</sup> concentration in the 13.5-µg/L treatment was significantly reduced relative to controls from days 23 to 32 postfertilization, and levels in the embryo were reduced by 40% at day 32 postfertilization, indicating that silver toxicity in the whole embryo is associated with an ion regulatory disturbance that is similar to the acute effect of AgNO<sub>3</sub> in juvenile and adult trout.

**Keywords**—Silver toxicity    Rainbow trout embryos    Development    Chorion    Ion regulation

### INTRODUCTION

A major source of silver in surface waters is natural leaching from sites of silver deposits. However, anthropogenic activities, such as silver mining and photoprocessing, may also cause elevated silver levels in aquatic environments [1]. To protect freshwater life from potential toxic effects of silver, both Canada and the United States have set guidelines for the total concentration of silver permissible in aquatic environments. The current Canadian Water Quality Guideline states that total silver concentrations in freshwater should not exceed 0.1 µg/L total silver, which represents both acute and chronic guidelines [2]. In the United States, the Environmental Protection Agency has set acute water quality criteria values based on water hardness (in mg/L of CaCO<sub>3</sub> equivalents) to protect against the acute toxicity of silver; e.g., in Lake Ontario water, with a hardness of 120 mg/L, the acute guideline is 4.7 µg/L total silver. There is no chronic national guideline in the United States. However, recently the U.S. hardness-based acute guideline has been brought into question based on experimental evidence that hardness itself offers little protection against acute toxicity and that the true protective agent is water chloride, which usually covaries with hardness [3–5].

In natural environments, silver is found most abundantly as a complex with an anionic ligand such as chloride, sulfide, or dissolved organic matter (DOM) [5]. Silver complexes have been reported to be much less toxic than the free silver ion (Ag<sup>+</sup>). For example, silver thiosulfate complexes, the main discharge from photographic processing, were at least four

orders of magnitude less toxic than the free silver ion to fathead minnows and rainbow trout [6].

Silver nitrate is used for most chronic and acute freshwater laboratory exposures because it is highly soluble and dissociates well to yield high levels of free ionic silver (Ag<sup>+</sup>), thereby representing a worst case scenario. The free silver ion is very toxic to freshwater fish, with 96-h 50% lethal concentrations (LC50 values) being between 6.5 and 65 µg/L total silver as AgNO<sub>3</sub> [4,6,7]. In freshwater, acute silver toxicity in rainbow trout arises from silver binding to specific sites on the fish gill [8], which results in noncompetitive inhibition of active Na<sup>+</sup> and Cl<sup>-</sup> uptake by blockade of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity [9–11]. As a result, blood plasma ion levels decrease, ultimately leading to circulatory collapse and death [5,12].

Although our understanding of the mechanisms of silver toxicity in juvenile and adult rainbow trout has greatly increased, there is nothing known about the mechanism of silver toxicity in early developmental stages of fish, which are often the most sensitive in the teleost life cycle [13]. Most studies of silver exposure on embryos have focused on survival, hatching, and growth success. Nebeker et al. [7] reported complete mortality at 1.3 µg/L total silver and significant mortality at concentrations as low as 0.5 µg/L when steelhead embryos were exposed to AgNO<sub>3</sub> from fertilization to post swim-up. Davies et al. [14] performed long-term toxicity tests initiated with rainbow trout embryos at the eyed stage of development (just after the halfway point between fertilization and hatching). A total silver concentration of 0.17 µg/L (as AgNO<sub>3</sub>) was reported to result in elevated embryonic mortality and premature hatching. Both of these studies were conducted in

\* To whom correspondence may be addressed  
(cbrauner@sunstroke.sdsu.edu).

relatively soft water (total hardness 20–40 mg/L CaCO<sub>3</sub>), likely with correspondingly low chloride levels. No published studies have examined the effect of chronic silver exposure in hard water with higher water chloride levels.

The four basic components of a fish embryo are the embryo, the yolk sac, the perivitelline fluid, and the chorion [6]. The yolk contains carbohydrates, lipids, and proteins, which the embryo metabolizes during development [15]. The perivitelline fluid, containing proteins, lipids, and carbohydrates, occupies the space between the chorion and the embryo [16]. The chorion is the outermost, acellular structure surrounding the embryo, which has a high protein content consisting predominantly of glycoproteins [16]. Rombough [17] demonstrated that negatively charged groups within the chorion bind cations with different affinities. For example, metal ions that act as Na<sup>+</sup> antagonists with a high affinity for sulfhydryl groups, such as silver, copper, and mercury, were bound tightly to the chorion. Rombough [17] demonstrated that steelhead trout embryos became more sensitive to static silver exposures >50 µg/L when the chorion was removed. In contrast, metal ions that act as Ca<sup>+</sup> antagonists with lower affinity for sulfhydryl groups, such as zinc, cadmium, and lead, bound less tightly to the chorion and penetrated the embryo rapidly. Although there have been numerous studies reporting adverse effects of metal exposure on freshwater fish embryos [14,18–21], few studies have focused on the uptake and distribution of the metal within the various compartments of the whole embryo throughout development.

The objectives of the present study were, first, to determine whether silver exposure during early life stages is associated with an ionoregulatory disturbance, as is observed in juvenile and adult fish; second, to determine whether whole embryo silver burden is correlated with mortality in moderately hard water with enough chloride and DOM levels to complex most of the free Ag<sup>+</sup>; and finally, to investigate the relative partitioning of silver within the embryo throughout development, with particular interest in the role of the chorion. Three nominal concentrations of total silver (0.1, 1, and 10 µg/L) added as AgNO<sub>3</sub> were used. The 0.1-µg/L concentration represents the present Canadian Water Quality Guideline [2] and was chosen to determine if this value is sufficient to protect the embryos from potential toxic effects of silver in Hamilton (Ontario, Canada) tap water (from Lake Ontario).

## MATERIALS AND METHODS

### Experimental animals

Experiments were performed on approximately 4,000 freshly fertilized rainbow trout eggs obtained from Rainbow Springs Trout farm (Thamesford, ON, Canada). They were maintained in flowing dechlorinated Hamilton (ON, Canada) tap water from Lake Ontario ([in mM] Na<sup>+</sup>, 0.60; Cl<sup>-</sup>, 0.70; Ca<sup>2+</sup>, 1.0; Mg<sup>2+</sup>, 0.2; K<sup>+</sup>, 0.05; HCO<sub>3</sub><sup>-</sup>, 1.9; DOM, 1.3 mg/L; alkalinity, 95 mg CaCO<sub>3</sub>; hardness, 120 mg CaCO<sub>3</sub>/L; pH, 7.5). A constant water temperature of 12.3 ± 0.1°C was maintained throughout the study. Because embryos are photosensitive early in development, they were kept in opaque chambers.

### Setup

Over a period of 32 d, from 2 to 3 h postfertilization to hatch, 1,000 embryos were exposed to each of three different nominal silver concentrations plus a control treatment (without added silver) in a chronic flow-through setup. Silver stock

Table 1. Measured water total silver concentrations (*n* = 16) during chronic exposures and silver speciation based on measured values calculated using *MINEQL* + [22] and the reported water chemistry in the Materials and Methods section

Nominal (µg/L)	Ag concentrations			
	Measured (µg/L)	Ag <sup>+</sup> (µg/L)	AgCl <sub>aq</sub> (µg/L)	Ag-DOM (µg/L)
0.1	0.117 ± 0.008	0.0021	0.0028	0.112
1	1.22 ± 0.16	0.026	0.033	1.159
10	13.51 ± 1.58	2.47	3.28	7.53

solutions of 0, 100, 1,000, and 10,000 µg/L were made using AgNO<sub>3</sub> in 0.5% nitric acid (trace metal grade, Fisher Scientific, Ottawa, ON, Canada) in deionized, distilled water and were kept in light-shielded containers. These stock solutions were delivered by a peristaltic pump into 150-ml header tanks (one header tank for each final silver concentration) at a flow rate of 0.14 ml/min and were mixed with dechlorinated Hamilton tap water at a flow rate of 140 ml/min. This resulted in 1,000-fold dilutions of the stock solutions, yielding continuously flowing exposure concentrations of 0, 0.1, 1, and 10 µg/L total silver (nominal values). The header tanks were vigorously aerated to facilitate mixing, and the overflow was directed to the embryo exposure chambers. To measure silver in the 0.1-µg/L treatment (which is very near the detection limit of the atomic absorption spectrophotometer), 10 µCi of <sup>110m</sup>Ag/L (Amersham International, Coustaboef, France) was added to the 100-µg/L silver stock solution so that final total silver concentration in the exposure chamber could be measured by radioisotopic dilution. Table 1 reports the mean measured silver concentration in each experimental treatment.

Polyethylene exposure chambers (32 × 19 × 9 cm) held water volumes of approximately 4 L each and were painted black to limit light penetration. Each exposure chamber received a flow rate of approximately 140 ml/min.

The aquatic geochemical modeling program *MINEQL* + [22] was used to calculate the amount of total free silver ion, silver chloride (aqueous), and silver-DOM in each experimental treatment based on the known chemistry of Hamilton tap water (from Lake Ontario) and corresponding silver-DOM constants from Janes and Playle [8] (Table 1).

### Experimental procedure

Starting on day 3 of exposure (also day 3 postfertilization), whole embryos from each chamber were sampled regularly at 3- to 8-d intervals for 32 d. Before sampling, mortalities in each treatment were recorded and dead embryos were removed. Embryos that had turned white were assumed to be dead, and when embryos were developed enough, the criteria for death was cessation of heartbeat. Eggs that had not been fertilized were identified by the lack of an embryonic axis and were not included in mortality calculations nor were they sampled. Embryos that were sampled were carefully checked to ensure that they were alive. In all treatments, whole embryos and chorions were sampled throughout the study. Dechorionated embryos with yolk sacs removed were sampled from day 18 to day 32 postfertilization. In addition, sampling of dechorionated embryos with intact yolk sacs began on day 23 postfertilization. By day 30, about 50% of embryos had hatched, and on day 32, hatched larvae were also collected in addition to intact embryos.

Dissection of the whole embryos involved dechoriation, which is a delicate procedure done by making a small, shallow puncture in the chorion with an 18-gauge needle. The chorion was then gently torn off using tweezers and was either retained for analysis or discarded when sufficient numbers of chorions had been obtained. Ten embryos were dechorionated, and the embryo was separated from the yolk sac and collected for analysis. Another 10 embryos were dechorionated and used to collect embryos with yolk sacs still attached.

All samples were rinsed with deionized, distilled water, blotted dry, weighed, and acidified with three times the sample weight of 1 N nitric acid (trace metal grade, Fisher Scientific, Canada). All acidified samples (in 1.5-ml polyethylene centrifuge tubes) were digested in an oven over a 24-h period at approximately 60°C. All samples were homogenized using a microcentrifuge sample pestle (Teflon fluorocarbon resin, Fisher Scientific, Canada) at a speed of approximately 5 revolutions/s. The digested, homogenized samples were spun down and a portion of the supernatant diluted with deionized water for total silver analysis.

Water samples were taken every second day from each exposure chamber, immediately acidified to 0.5% (v/v) with trace metal grade nitric acid (Fisher Scientific), and kept in the dark until total silver concentrations could be analyzed. Water and silver stock flow rates were measured every second day. The temperature of the water was recorded every day of the exposure period.

#### Analyses

Analysis of the silver concentration of the water from the 1- and 10- $\mu\text{g/L}$  total silver treatments and of diluted supernatant from digested samples was conducted using a graphite furnace, atomic absorption spectrophotometer (Varian 1275, Walnut Creek, CA, USA, fitted with a GTA-95 atomizer with operating parameters of 75°C for 5 s, 90°C for 30 s, 120°C for 10 s, 120°C for 2 s [no gas flow], 2,000°C for the next 3 s [no gas flow, absorption read], 2,000°C for 1 s). Analysis of silver concentration in the water from the 0.1- $\mu\text{g/L}$  total silver treatment was accomplished by radioisotopic dilution. Atomic absorption spectrophotometry was used to measure the silver concentration of the stock solution, and a gamma counter (Packard Instruments, Downers Grove, IL, USA) was used to measure radioactivity (total counts per minute; cpm) of  $^{110\text{m}}\text{Ag}$ . The specific activity of the stock solution was calculated (cpm/ $\mu\text{g}$ ) and the total silver concentration of the exposure chamber was calculated by dividing  $^{110\text{m}}\text{Ag}$  activity (cpm) of the water by the specific activity of the stock solution.

Standards used to measure silver in whole embryos and different embryo compartments were made by mixing known certified silver standards (Fisher Scientific, Canada) with digested rainbow trout tissue. This was done to correct for background and matrix effects.

Analysis of the  $\text{Na}^+$  concentration in whole embryos and embryos was performed by atomic absorption spectrophotometry (Varian 1275), and that for  $\text{Cl}^-$  was measured using the calorimetric mercuric thiocyanate method [23].

#### Statistics

Mean values of silver concentrations were statistically compared between treatments (on respective days) or between days (within respective silver treatments) using a one-way analysis of variance. Mortality between treatments was tested for statistical significance using a one-way analysis of variance on

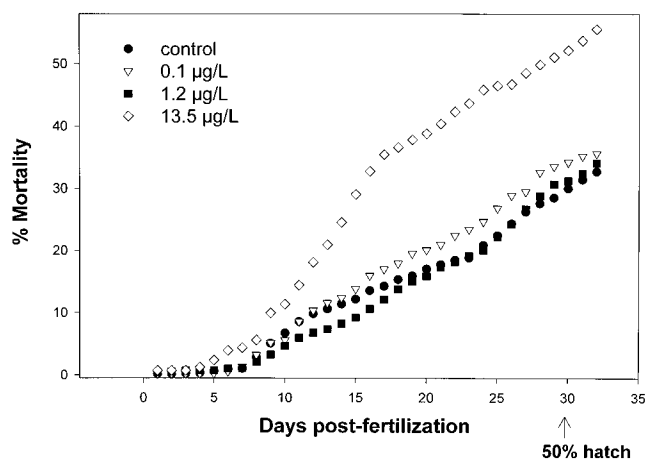


Fig. 1. Cumulative percent mortality in embryos of rainbow trout chronically exposed to 0 (closed circles), 0.1 (open triangles), 1.2 (closed squares), and 13.5  $\mu\text{g/L}$  (open diamonds) total Ag (as  $\text{AgNO}_3$ ) from fertilization through to hatch. At day 30, about 50% of the embryos had hatched. Mortality in the 13.5- $\mu\text{g/L}$  treatment was significantly different than controls ( $p < 0.05$ ).

the number of mortalities per day (i.e., on noncumulative mortality). A Dunnett's post hoc test was used to identify groups that differed significantly from the controls within each respective comparison. Mean values for whole embryo  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  in controls and the 13.5- $\mu\text{g/L}$  silver group were compared using a  $t$  test. The level of statistical significance for all analyses was  $p < 0.05$ .

## RESULTS

Measured silver levels in the water for each nominal silver concentration are shown in Table 1. Values in control water were below the detection limit of the atomic absorption spectrophotometer for total silver ( $<0.05 \mu\text{g/L}$ ) and are not reported. Results of silver speciation calculations using an equilibrium-based aquatic geochemical modeling program (MINELQ+) and known chemistry (see Methods) of Hamilton dechlorinated tap water (from Lake Ontario) are also summarized in Table 1.

#### Mortality and development

Mortality (as the number dead per day) was statistically greater from day 5 to day 33 in the 13.5- $\mu\text{g/L}$  group relative to controls, with the highest rate of mortality (as indicated by the change in slope) between days 8 and 15 postfertilization, reaching a maximum value of 56% by day 32 (Fig. 1). Mortalities in the 1.2- and 0.1- $\mu\text{g/L}$  groups and in the control group reached 34%, 36%, and 32%, respectively, by day 32, none of which differed significantly from one another. When the control mortality of 32% is taken into account [24], mortality in the 13.5- $\mu\text{g/L}$  group is 33% while those in the 1.2- and 0.1- $\mu\text{g/L}$  groups are just 2 and 4%, respectively. About 50% of surviving embryos had hatched by day 30 of the experiment, with no apparent difference between treatments. All samples reported throughout this study refer to intact embryos at the time of sampling with the exception of day 32, when hatched larvae were also collected.

#### Ag content

Background levels of Ag in the whole embryos of the control group were between 0.01 and 0.03  $\mu\text{g/g}$  throughout development (Fig. 2). The average total Ag concentration in the

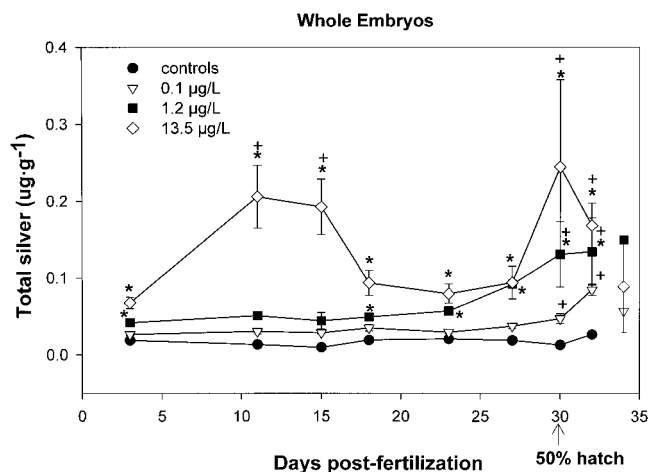


Fig. 2. Silver concentration in whole embryos chronically exposed to 0 (closed circles), 0.1 (open triangles), 1.2 (closed squares), and 13.5 µg/L (open diamonds) total Ag (as AgNO<sub>3</sub>) from fertilization through to hatch. At day 30 postfertilization, about 50% of the embryos had hatched. Values plotted to the right of the traces (i.e., at day 34) represent those for 32-d hatched larvae. Values are mean ± SEM (n = 8–20); \* indicates significant difference from controls at respective time (p < 0.05); + represents significant difference from day 3 values within each silver exposure group (p < 0.05).

whole embryos of the 0.1-µg/L Ag exposure group was low throughout development (0.03–0.08 µg/g), without any significant differences from control values. However, whole embryo silver concentrations were significantly greater on days 30 and 32 relative to that on day 3 in the 0.1-µg/L Ag exposure group. Silver concentration in whole embryos of the 1.2-µg/L Ag exposure group was between 0.04 and 0.13 µg/g throughout development, with significantly higher silver concentrations than whole embryos of the control group from days 18 to 32 postfertilization. Consistent with the 0.1-µg/L treatment, whole embryo silver concentrations in the 1.2-µg/L group were significantly greater on days 30 and 32 than on day 3. The average total Ag concentration in the embryos of the 13.5-µg/L group varied but was significantly greater than controls at all sampling times (Fig. 2). From day 3 to day 11, Ag concentration in the whole embryos increased significantly from 0.07 to 0.2 µg/g, but thereafter decreased to 0.08 µg/g by day 23. From day 23 to day 30, average total Ag concentration in the whole embryos again increased significantly from 0.08 to 0.24 µg/g.

The average measured total Ag concentration in the chorions of the control group remained between 0.07 and 0.13 µg/g throughout development (Fig. 3). The chorions of the 0.1-µg/L Ag exposure group contained low concentrations of Ag throughout development, ranging from 0.03 to 0.30 µg/g (not significantly different from controls). Similarly, the Ag concentration in the chorions of the 1.2-µg/L group (0.13–1.7 µg/g) was not statistically different from the control group, but there was a small but significant increase from day 8 onward relative to day 3 values within that group. From day 8 on, the average Ag concentration in the chorions of the 13.5-µg/L treatment was significantly greater than that of controls and increased from 0.4 to 3.9 µg/g from days 3 to 18 post-fertilization, fell slightly to 2.3 µg/g at day 23, and thereafter increased exponentially with time to 13.3 µg/g on day 32 (Fig. 3).

Embryos with yolk sacs attached were sampled from day 23 to day 32 postfertilization (Fig. 4). Interestingly, the only

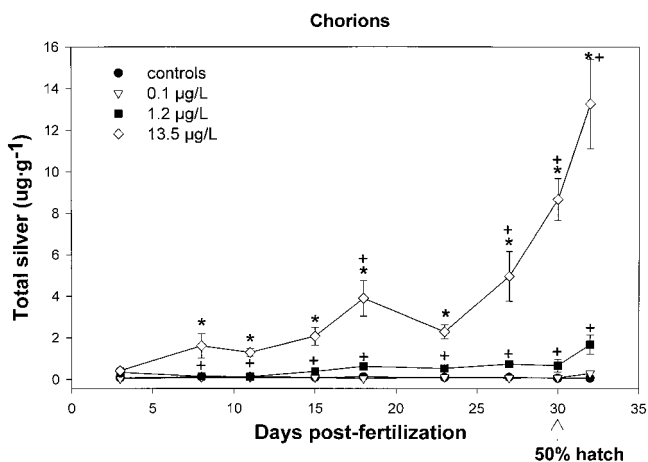


Fig. 3. Silver concentration in chorions from embryos chronically exposed to 0 (closed circles), 0.1 (open triangles), 1.2 (closed squares), and 13.5 µg/L (open diamonds) total Ag (as AgNO<sub>3</sub>) from fertilization through to hatch (n = 8–10). See caption of Figure 2 for further details.

treatment that resulted in a significant elevation in silver concentration at all sampling times was the 0.1-µg/L Ag exposed group. However, a significant elevation in silver concentration relative to controls was observed in the 13.5-µg/L Ag exposed group on days 30 and 32 and in the 1.2-µg/L Ag exposed group on day 32 postfertilization. In all treatments and controls, there was a significant increase in silver concentration by day 32 postfertilization relative to day 23 values, reaching a maximum of 0.02, 0.06, 0.05, and 0.05 µg/g total Ag in the controls and 0.1-, 1.2-, and 13.5-µg/L Ag exposed groups, respectively.

Samples of dechorionated embryos with yolk sacs removed were taken from day 18 to day 32 postfertilization and were analyzed for average total Ag concentration. Embryonic silver concentrations of the 0.1- and 1.2-µg/L Ag exposure treatments were not significantly different from embryonic silver concentrations of controls throughout development. From day 27 to day 32, embryos of the 13.5-µg/L group contained Ag concentrations from 0.065 to 0.083 µg/g, approximately three-

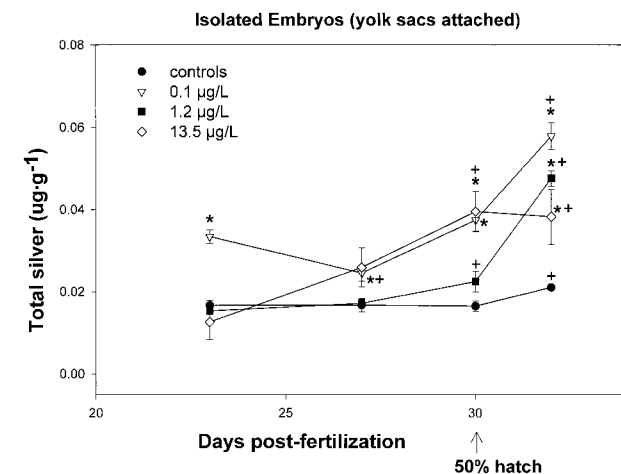


Fig. 4. Silver concentration in dissected embryos, with yolk sacs attached, from whole embryos chronically exposed to 0 (closed circles), 0.1 (open triangles), 1.2 (closed squares), and 13.5 µg/L (open diamonds) total Ag (as AgNO<sub>3</sub>) from fertilization through to hatch (n = 8–10). See caption of Figure 2 for further details.

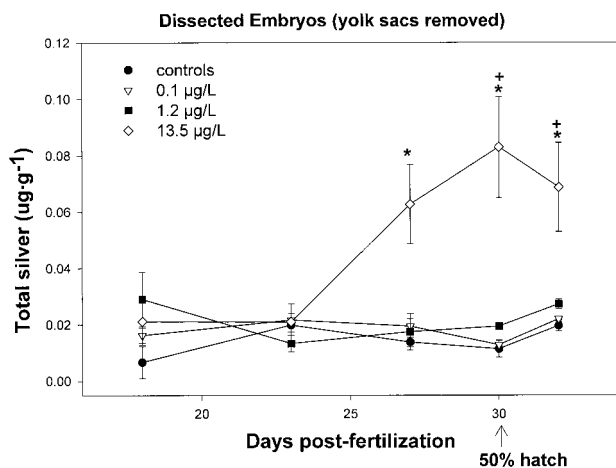


Fig. 5. Silver concentration in dissected embryos (with yolk sacs removed) from whole embryos chronically exposed to 0 (closed circles), 0.1 (open triangles), 1.2 (closed squares), and 13.5  $\mu\text{g/L}$  (open diamonds) total Ag (as  $\text{AgNO}_3$ ) from fertilization through to hatch ( $n = 8-10$ ). See caption of Figure 4 for further details.

fold greater than controls (Fig. 5). By day 30, the embryonic silver concentration (0.08  $\mu\text{g/g}$ ) in the 13.5- $\mu\text{g/L}$  treatment had significantly increased relative to that observed on day 18 (0.02  $\mu\text{g/g}$ ).

On day 32, samples of larvae (i.e., from hatched embryos) from the 0.1-, 1.2-, and 13.5- $\mu\text{g/L}$  treatments were taken and analyzed for a total Ag concentration (Fig. 2). In all treatments, total Ag concentrations in the larvae were less than the Ag concentration of the whole embryos taken at day 32 (Fig. 2).

Figure 6 illustrates the silver content (in  $\mu\text{g}$ ) in the chorions, isolated embryos, and yolks of the dissected whole embryos from the three experimental treatments, partitioning concentrations according to the mass of the respective compartments. Data for the embryo and yolk was calculated from measured values, where yolk concentration is calculated by the difference between embryos with and without yolk sacs attached. Values for the chorion were determined as the difference in silver content between whole embryos and the dechorionated embryos with yolk sacs attached. The reason that chorionic silver load was not determined directly was that the error associated with measuring the mass of the chorion (3–5 mg) coupled with the very high level of silver contained in the chorion resulted in very large errors in the whole embryo silver budget. The method of deducing chorion silver content described above avoided this problem and was adopted in this analysis.

Of the total whole embryo silver content in both the 1.2- and 13.5- $\mu\text{g/L}$  treatments, 65 to 85% of the silver was bound by the chorion, 2 to 9% was found in the isolated embryos, and 10 to 28% was found in the yolk. In the 0.1- $\mu\text{g/L}$  treatment, the yolk contained the greatest amount of silver (50–70% of the total whole embryo silver content) compared with the isolated embryos (10%) and the chorions (20–40%). Interestingly, the yolks of the 0.1- $\mu\text{g/L}$  treatment contained more silver than the yolks of both the 1.2- and 13.5- $\mu\text{g/L}$  treatments.

#### Whole embryo and embryo ion content

From day 18 to day 32 postfertilization,  $\text{Na}^+$  concentration in the whole embryos of the 13.5- $\mu\text{g/L}$  treatment remained constant between 18 and 20.5 mM (Fig. 7a). In control whole embryos, there was a significant increase in  $\text{Na}^+$  concentration

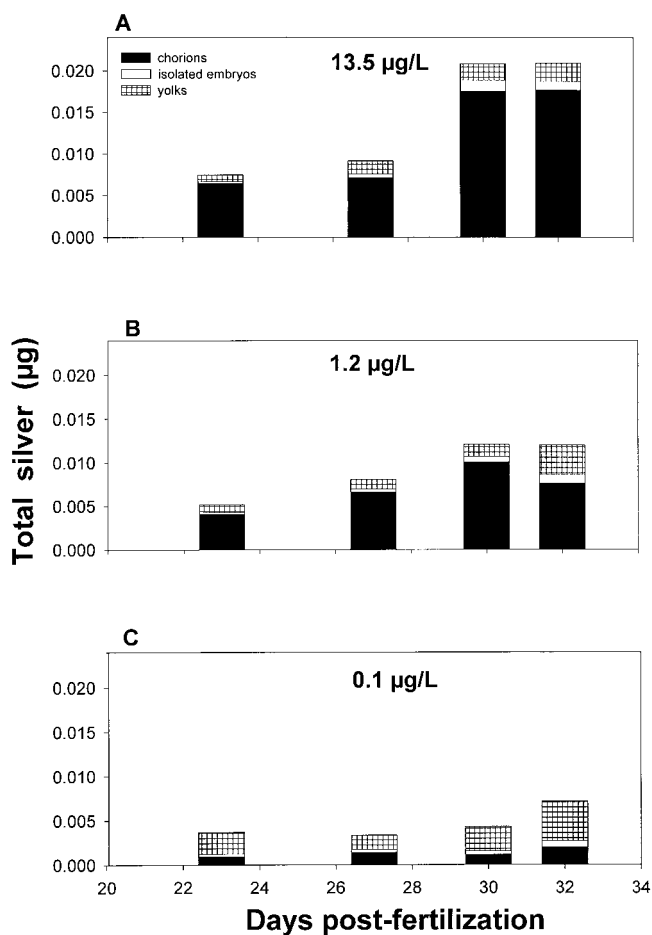


Fig. 6. Comparison of the total silver content in the compartments of the whole embryo (chorion, yolk, isolated embryo) of whole embryos exposed to total silver concentrations (as  $\text{AgNO}_3$ ) of 13.5 (A), 1.2 (B), and 0.1  $\mu\text{g/L}$  (C). Values presented for embryos were measured directly and those for yolks were calculated by the difference between measurements for embryos with yolk sacs attached and embryos with yolk sacs removed. Values for chorion silver content were calculated by subtracting the average total silver content in the yolk and embryos from the average total silver content in whole embryos.

from 18 to 24 mM and 31 mM on days 30 and 32 postfertilization, respectively. This resulted in a significantly lower whole embryo  $\text{Na}^+$  concentration in the 13.5- $\mu\text{g/L}$  treatment relative to control embryos from day 25 onward. Changes in whole embryo  $\text{Cl}^-$  levels were much more variable; however, there was a significant reduction in whole embryo  $\text{Cl}^-$  levels on day 27 of exposure in the 13.5- $\mu\text{g/L}$  treatment relative to controls (Fig. 7b). In dechorionated embryos with yolk sacs removed,  $\text{Na}^+$  concentration was not significantly different in the 13.5- $\mu\text{g/L}$  treatment relative to controls on day 30 ( $71.0 \pm 8.0$  mM vs  $86.2 \pm 7.1$  mM, respectively) but was 40% lower than controls on day 32 ( $61.9 \pm 5.6$  mM vs  $101.3 \pm 8.9$  mM, respectively). (Data not shown.)

## DISCUSSION

### Mortality

In the 13.5- $\mu\text{g/L}$  total Ag treatment, embryo mortality reached 56% by the end of the experiment, 2 d after 50% hatch. When control mortality (32%) is taken into account, mortality in the 13.5- $\mu\text{g/L}$  total Ag treatment was 33%. In contrast, mortalities in the 0.1- and 1.2- $\mu\text{g/L}$  total Ag treat-

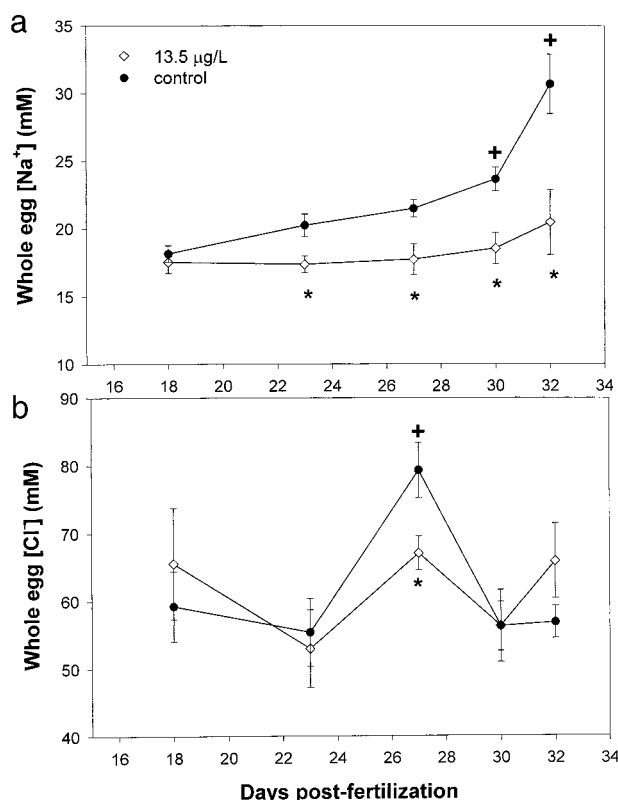


Fig. 7. Na<sup>+</sup> concentration (a) and Cl<sup>-</sup> concentration (b) from day 18 to day 32 postfertilization in whole embryos exposed to 0 (closed circles) and 13.5 µg/L (open diamonds) total silver (as AgNO<sub>3</sub>) (*n* = 6–8). See caption of Figure 2 for further details.

ments did not differ significantly from the control group. Previous studies have demonstrated 100% mortality in rainbow trout embryos that were exposed to 10 µg/L total silver from the eyed stage [14]. In the same study, however, Davies et al. [14] demonstrated that mortalities due to toxic silver effects were found at silver concentrations as low as 0.17 µg/L, a concentration that also caused premature hatching. The apparent discrepancy in sensitivity to chronic silver exposure in the present study (where there was no significant effect on mortality in the 1.2-µg/L total silver group relative to controls) and that of Davies et al. [14] may be due to differences in water chemistry between the two studies. In juvenile and adult rainbow trout, it has been clearly demonstrated that both water Cl<sup>-</sup> and DOM protect against silver toxicity [3,4]. While water chloride and DOM were not reported in the Davies et al. [14] study, the water hardness (20–40 mg/L CaCO<sub>3</sub>) was much lower than that in Hamilton (ON, Canada) tap water (120 mg/L CaCO<sub>3</sub>), and water chloride often tends to covary with hardness. Furthermore, in similar studies, Davies et al. reported DOM levels of about 0.8 mg/L (J. Gorsuch, personal communication). Thus, water Cl<sup>-</sup> and DOM, which are higher in the present study, may have protected against silver toxicity in trout embryos, as it does at the trout gill; however, this remains to be tested experimentally.

Due to the high water flow required to keep embryos at a constant temperature, equilibration time between the silver stock solution and the water was short. While interaction between silver and water chloride is very fast, the interaction with DOM is possibly considerably slower. Ma et al. [25] have demonstrated that increasing the reaction time between copper and humic acid reduces toxicity of the solution in bioassays.

At a humic acid concentration of 5 mg/L, the LC50 value increases by about 50% between 1 and 24 h. Thus, the speciation values reported in Table 1 may overestimate the proportion of silver as Ag-DOM, and the conditions of the present study may result in greater toxicity than if the silver solution was permitted to equilibrate for several hours.

The highest mortality rates in the 13.5-µg/L treatment occurred between days 8 and 15 postfertilization, indicating that embryos appear to be most sensitive in this developmental stage. Similar observations have been made in an acute silver challenge study [26].

#### Ag content

A significant increase in Ag concentration of whole embryos in the 13.5-µg/L treatment was observed by days 11 and 15 postfertilization (Fig. 2), a time at which the mortality rate was increasing (as indicated by the slope of the mortality curve in Fig. 1). Based on chorion levels at days 7 to 15 (Fig. 3), there was not nearly as much silver bound by the chorion relative to the whole embryo as was observed at later stages of development, and thus embryonic silver levels may have been relatively high, although they could not be measured at this time. Increases in Ag concentration in whole embryos were also observed at days 25 to 30 postfertilization in both the 1.2- and 13.5-µg/L treatments, without, however, the dramatic increase in mortality rate observed at days 11 to 15 (as indicated by the slope of the mortality curve in Fig. 1). Chorionic silver concentration increased in the 1.2-µg/L Ag treatment from day 30 to day 32 postfertilization and to a very large degree in the 13.5-µg/L treatment from day 23 to day 32 postfertilization. Thus, on days 25 to 30 postfertilization, silver levels in the chorion were higher and consequently embryonic levels were likely lower than on days 11 through 15 postfertilization, perhaps accounting for the reduced mortality rates despite similar silver whole embryo burdens. Thus, it may not be that embryos are especially sensitive to silver on days 11 to 15 postfertilization but that the relatively lower binding of silver to the chorion at this developmental stage results in higher embryonic silver levels, resulting in higher mortality.

Interestingly, Ag burden in whole embryos decreased dramatically after day 15 postfertilization (Fig. 2) while chorion silver concentration increased (Fig. 3). This stage, days 16 to 24 postfertilization, is also a period of decreased sensitivity (as indicated by mortality) in embryos acutely exposed to silver [26].

#### Na<sup>+</sup> content

The increase in whole embryo silver content from day 25 onward is likely related to the development of ion regulatory capacity of the embryo. Previous studies have indicated that, from the eyed stage of development and on, there is a significant uptake of Na<sup>+</sup> from the water [27,28], which increases exponentially through to hatch (C.J. Brauner and C.M. Wood, unpublished data). In juvenile rainbow trout, Bury and Wood [29] demonstrated that both Na<sup>+</sup> uptake and Ag<sup>+</sup> uptake at the fish gills were reduced by specific blockers of the Na<sup>+</sup> channel (phenamil) and H<sup>+</sup>-ATPase (bafilomycin A<sub>1</sub>), indicating that uptake of Ag<sup>+</sup> across the apical membrane of the fish gill occurs via a Na<sup>+</sup> channel coupled to an H<sup>+</sup>-ATPase. Thus, the increased silver burden observed in whole embryos at later stages of development may reflect development of the Na<sup>+</sup> uptake system, which becomes more significant for silver up-

take as the ion regulatory ability of the developing embryo increases.

In juvenile and adult rainbow trout, Ag<sup>+</sup> binds to specific sites on the fish gill, resulting in blockade of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity, and noncompetitive inhibition of Na<sup>+</sup> and Cl<sup>-</sup> uptake [8–10]. This leads to a decrease in the levels of ions in blood plasma and ultimately results in circulatory collapse and death [5]. In early life stages of rainbow trout, chronic exposure to a total measured silver concentration of 10.7 µg/L in water with identical characteristics to the present study reduced Na<sup>+</sup> uptake, relative to controls, at all stages of development through to hatch (C.J. Brauner and C.M. Wood, unpublished data). In the present study, Na<sup>+</sup> content in whole embryos exposed to 13.5 µg/L total silver was reduced relative to controls from day 23 through to hatch. Similarly, on day 32 post-fertilization, Na<sup>+</sup> concentration in embryos of the 13.5-µg/L treatment was 40% lower than that of controls. Thus, the mechanism of silver toxicity in developing embryos is at least partly associated with ion regulatory impairment, as has been shown for juvenile and adult rainbow trout [5].

#### The role of the chorion

The chorion is generally viewed as a protective barrier for the embryo from environmental stress [30]. In the present study, the chorion accumulated the highest Ag concentration relative to other compartments of the whole embryo, comprising between 60 and 85% of the total whole embryo burden in the 1.2- and 13.5-µg/L Ag treatments (Fig. 6). Embryonic Ag concentrations were substantially less than chorionic concentrations in all experimental groups. Previous studies demonstrated that, in rainbow trout embryos exposed to cadmium, most of the metal was found associated with the chorion [18,31].

The chorion of a rainbow trout embryo possesses about 650 to 700 µg total protein [32], corresponding to 13 to 14% of the chorion, assuming a chorion mass of about 5 mg (present study). Of the total chorion protein, about 80% consists of four major components of asparagine-linked glycoproteins [32]. The predominant amino acids in the chorion appear to be proline (10–20%) and glutamic acid (10–17%), but there are also significant amounts of cysteine (1–10%) [33,34], which possesses sulfhydryl groups. Silver ions likely enter the intact embryo by initially binding to these sulfhydryl groups, which possess a very high binding affinity for silver [35], as well as to other anionic sites such as glutamic acid. As suggested by Rombough [17], because silver binds to the chorion with high affinity, the movement of silver into the embryo was probably impeded by the chorion. Thus, by accumulating a large burden of silver, the chorion protects the embryo from potential toxic effects of silver. In all experimental groups, the larvae sampled at day 32 postfertilization had less silver concentration than whole embryo samples taken at that time (Fig. 2), likely due to the shedding of the chorion and thus a significant portion of the whole embryo silver burden.

The concentration of silver in the chorion increased throughout development, but interestingly, the ratio between chorion and whole embryo silver concentration increased, most noticeably a few days before hatch (Figs. 2 and 3), indicating that the composition of the chorion may be changing with development, exposing new silver binding sites. Shortly following fertilization, the enzyme transglutaminase, which is found in the chorion, catalyzes the formation of disulfide cross-links between cysteine residues and cross-links between other

constitutive peptides, thereby greatly increasing the integrity of the chorion during the process of water hardening [36,37]. Cross-linked cysteine residues do not possess sulfhydryl groups available to bind silver. However, as the embryo approaches hatch, a number of proteolytic hatching enzymes are secreted by the embryo that break down the various peptide cross-links within the chorion [38]. In brine shrimp cysts, over 90% of the protease activity is cysteine protease [39], and thus, cysteine disulfide cross-links are broken, reducing the integrity of the chorion and creating many available sulfhydryl groups. Assuming the same is true in trout embryos, this could explain the increase in silver concentration of the chorion in the days before hatch (Fig. 3).

#### Implications for water quality criteria

Under the current national guideline for freshwater life protection in Canada, total silver concentrations of up to 0.1 µg/L (acute and chronic value) are acceptable [2]. Mortality rates of embryos in the 0.1- and 1.2-µg/L total silver (as AgNO<sub>3</sub>) treatments did not differ significantly from controls throughout embryonic development. The general lack of significant Ag uptake in the internal whole embryo compartments was correlated with low mortality rates. Although silver accumulated in the whole embryos in the 0.1- and 1.2-µg/L total silver chronic exposures, the chorion appeared to protect the embryo from any toxic effects of silver. Therefore, according to the chronic data presented, the current Canadian Water Quality Guideline is sufficient in preventing mortality due to toxic effects of silver during the embryonic development of rainbow trout in Hamilton tap water (from Lake Ontario).

*Acknowledgement*—This study was supported by a Natural Sciences and Engineering Research Council of Canada Industrial Oriented Research grant in conjunction with Kodak Canada Incorporated. We thank Joe Gorsuch, Tom Purcell, and Ken Robillard for many helpful comments and two anonymous referees for valuable suggestions.

#### REFERENCES

1. Purcell TW, Peters JJ. 1998. Sources of silver in the environment. *Environ Toxicol Chem* 17:539–546.
2. Canadian Council of Ministers of the Environment. 1995. Canadian water quality guidelines: Silver. In *Canadian Council of Ministers of the Environment 1995*. Winnipeg, Manitoba, pp 3–22.
3. Bury NR, Galvez F, Wood CM. 1999. Effects of chloride, calcium, and dissolved organic carbon on silver toxicity: Comparison between rainbow trout and fathead minnows. *Environ Toxicol Chem* 18:56–62.
4. Galvez F, Wood CM. 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.
5. Hogstrand C, Wood CM. 1998. Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: Implications for water quality criteria. *Environ Toxicol Chem* 17: 547–561.
6. Le Blanc GA, Masone JD, Paradice AP, Wilson B, Lockhart B, Robillard KA. 1984. The influence of speciation on the toxicity of silver to fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 3:37–46.
7. Nebeker AV, Mc Auliffe CK, Mshar R, Stevens DG. 1983. Toxicity of silver to steelhead and rainbow trout, fathead minnows and *Daphnia magna*. *Environ Toxicol Chem* 2:95–104.
8. Janes N, Playle RC. 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 14: 1847–1858.
9. McGeer JM, Wood CM. 1998. Protective effects of water Cl<sup>-</sup> on physiological responses to waterborne silver in rainbow trout. *Can J Fish Aquat Sci* 55:2447–2454.

10. Morgan IJ, Henry RP, Wood CM. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na<sup>+</sup> and Cl<sup>-</sup> transport. *Aquat Toxicol* 38:145–163.
11. Webb NA, Wood CM. 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.
12. Wood CM, Hogstrand C, Galvez F, Munger RS. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. Effects of ionic Ag<sup>+</sup>. *Aquat Toxicol* 35:93–109.
13. von Westernhagen H. 1988. Sublethal effects of pollutants on fish eggs and larvae. In Hoar WS, Randall DJ, eds, *Fish Physiology*, Vol 11A. Academic, San Diego, CA, USA, pp 253–346.
14. Davies PH, Goettl JP, Sinley JR. 1978. Toxicity of silver to rainbow trout (*Salmo gairdneri*). *Water Res* 12:113–117.
15. Heming TA, Buddington RK. 1988. Yolk absorption in embryonic and larval fishes. In Hoar WS, Randall DJ, eds, *Fish Physiology*, Vol 11A. Academic, San Diego, CA, USA, pp 408–446.
16. Blaxter JHS. 1988. Pattern and variety in development. In Hoar WS, Randall DJ, eds, *Fish Physiology*, Vol 11A. Academic, San Diego, CA, USA, pp 1–58.
17. Rombough PJ. 1985. The influence of the zona radiata on the toxicities of zinc, lead, mercury, copper, and silver ions to embryos of steelhead trout *Salmo gairdneri*. *Comp Biochem Physiol C* 82:115–117.
18. Rombough PJ, Garside ET. 1982. Cadmium toxicity and accumulation in eggs and alevins of Atlantic salmon *Salmo salar*. *Can J Zool* 60:2006–2014.
19. Shazili NAM, Pascoe D. 1986. Variable sensitivity of rainbow trout (*Salmo gairdneri*) eggs and alevins to heavy metals. *Bull Environ Contam Toxicol* 36:468–474.
20. Weis JS, Weis P. 1989. Effects of environmental pollutants on early fish development. *Aquat Sci* 1:45–73.
21. Woodworth J, Pascoe D. 1982. Cadmium toxicity to rainbow trout, *Salmo gairdneri*, Richardson: A study of eggs and alevins. *J Fish Biol* 21:47–57.
22. Schecher WD, Mc Avoy DC. 1994. *MINEQL+*. User's Manual, Ver 3.01. Hallowell, Maine, USA.
23. Zall DM, Fisher D, Garner MD. 1956. Photometric determination of chlorides in water. *Anal Chem* 28:1665–1678.
24. Sprague JB. 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. *Water Res* 3:793–821.
25. Ma H, Kim SD, Cha DK, Allen HE. 1999. Effect of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. *Environ Toxicol Chem* 18:828–837.
26. Guadagnolo CM, Brauner CJ, Wood CM. 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.
27. Eddy FB, Talbot C. 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 C* 81:259–266.
28. Rudy PP, Potts WTW. 1969. Sodium balance in the eggs of the Atlantic salmon, *Salmo salar*. *J Exp Biol* 50:239–246.
29. Bury NR, Wood CM. 1999. The mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na<sup>+</sup>-channel. *Am J Physiol* 277:R1385–R1391.
30. Rombough PJ. 1988. Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In Hoar WS, Randall DJ, eds, *Fish Physiology*, Vol 11A. Academic, San Diego, CA, USA, pp 59–161.
31. Beattie JH, Pascoe O. 1978. Cadmium uptake by rainbow trout, *Salmo gairdneri* Richardson, eggs and alevins. *J Fish Biol* 13:631–637.
32. Brivio MF, Bassi R, Cotelli F. 1991. Identification and characterization of the major components of the *Oncorhynchus mykiss* egg chorion. *Mol Rep Dev* 28:85–93.
33. Reiger JC, Kafatos FC, Goodfliesch R, Hood L. 1978. Silkmoth chorion proteins: Sequence analysis of the products of a multigene family. *Proc Natl Acad Sci* 75:390–394.
34. Sugiyama H, Murata K, Iuchi I, Yamagami K. 1996. Evaluation of solubilizing methods of the egg envelope of the fish, *Oryzias latipes*, and partial determination of amino acid sequence of its subunit protein, ZI-3. *Comp Biochem Physiol B* 114:27–33.
35. Bell RB, Kramer JR. 1999. Structural chemistry and geochemistry of silver-sulfur compounds: Critical review. *Environ Toxicol Chem* 18:9–22.
36. Ha C, Iuchi I. 1998. Enzyme responsible for egg envelope (chorion) hardening in fish: Purification and partial characterization of two transglutaminases associated with their substrate, unfertilized egg chorion, of the rainbow trout, *Oncorhynchus mykiss*. *J Biochem* 124:917–926.
37. Yamagami K, Hamazaki TS, Yasumasu S, Masuda K, Iuchi I. 1992. Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. *Int Rev Cytol* 136:51–92.
38. Yamagami K. 1988. Mechanisms of hatching in fish. In Hoar WS, Randall DJ, eds, *Fish Physiology*, Vol 11A. Academic, San Diego, CA, USA, pp 447–499.
39. Warner AH, Perz MJ, Osahan JK, Zielinski BS. 1995. Potential role in development of the major cysteine protease in larvae of the brine shrimp *Artemia franciscana*. *Cell Tissue Res* 282:21–31.