J Comp Physiol B (2002) 172: 153–162
DOI 10.1007/s00360-001-0238-8

ORIGINAL PAPER

C.J. Brauner · C.M. Wood

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*)

Accepted: 14 September 2001 / Published online: 30 November 2001

Abstract Rainbow trout embryos and larvae were continuously exposed, in a flow-through system, to 0, 0.1 μg/l (measured = 0.098 ± 0.002 μg/l) or 1.0 μg/l (measured = 0.853 ± 0.022 μg/l) total silver (as $AgNO_3$) in moderately hard water (120 mg $CaCO_3$/l, 0.70 mM Cl^-, 1.3 mg/l dissolved organic matter and 13.7 ± 0.1 °C) from fertilization to 1 week post-hatch. The objectives of the study were to investigate the effects of chronic silver exposure on mortality, time to hatch and growth, and on sublethal physiological indicators of toxicity. Exposure to 1.0 μg/l total silver resulted in a small, but statistically significant, increase in mortality (16%) relative to controls (12%) but interestingly, resulted in an increased rate of growth (as indicated by larval weight, length and extractable protein) and ionoregulatory development over the duration of this study. Whole body unidirectional Na^+ uptake ($J_{in}Na^+$) increased with silver exposure concentration (both 0.1 μg/l and 1.0 μg/l total silver) just prior to and following hatch, with up to a three-fold elevation in $J_{in}Na^+$ in the 1.0 μg/l treatment relative to controls. Qualitatively similar changes in whole body Na^+,K^+-ATPase activity (per mg protein or per whole embryo or larvae) also occurred over this period. By 1 week post-hatch, there were no differences in $J_{in}Na^+$ among treatments and Na^+,K^+-ATPase activity levels in silver exposed groups were significantly reduced relative to controls. Within 2 days following hatch, there was an elevation in whole larval ammonia levels, while cortisol levels were elevated at 1 week post-hatch in the 1.0 μg/l treatment relative to controls. Ionoregulatory disturbance and elevations in both cortisol and ammonia have also been observed during acute silver exposure in adult rainbow trout, indicating that chronic and acute mechanisms of toxicity may be similar.

Keywords Rainbow trout · Silver · Toxicity · Ionoregulation · Development

Abbreviations *CC* chloride cells · *cpm* counts per minute · *DOM* dissolved organic matter · $J_{in}Na^+$ unidirectional Na^+ uptake · *SA* specific activity

Introduction

Although there are many natural sources that elevate silver levels in the environment, a large proportion comes from anthropogenic activities such as mining, silverware manufacturing and the photographic industry, the latter of which is the largest consumer of silver in the US (Purcell and Peters 1998). Silver is discharged from the photographic industry complexed with thiosulphate which has a high affinity for Ag^+ but is reduced to silver sulfide with an even higher binding affinity after passing through publicly owned treatment works (Eisler 1996; Shafer et al. 1998). Natural waters contain other ligands, such as Cl^- and dissolved organic matter (DOM) which can also bind Ag^+ (Janes and Playle 1995). In general, these bound forms of silver are much less acutely toxic to aquatic organisms than $AgNO_3$ (LeBlanc et al. 1984; McGeer and Wood 1998; Bury et al. 1999a, 1999b) the form which is frequently used in toxicity testing because it dissociates freely in solution to yield large amounts of the free ion Ag^+, and thus represents a worst case scenario.

The silver ion (Ag^+) is highly toxic to fish and during acute exposure results in an impairment of gill Na^+,K^+-ATPase activity, and a consequent reduction in active Na^+ and Cl^- uptake across the gills in freshwater fish

Communicated by L.C.-H. Wang

C.J. Brauner (✉)
Department of Biology, San Diego State University,
5500 Campanile Drive, San Diego, CA 92182–4614, USA
E-mail: cbrauner@sunstroke.sdsu.edu
Tel.: +1-619-5941081
Fax: +1-619-5945676

C.M. Wood
Department of Biology, McMaster University,
Hamilton, Ontario, Canada, L8S 4K1

(Wood et al. 1996; Morgan et al. 1997; McGeer and Wood 1998). Blood Na^+ and Cl^- levels decline, which initiates a suite of secondary effects including a blood acidosis, a generalized stress response manifested as increased plasma glucose and cortisol levels, increased blood ammonia levels, fluid volume disturbance and hemoconcentration, culminating in circulatory collapse and death (Wood et al. 1996; Hogstrand and Wood 1998; Webb and Wood 1998). Although a great deal is known about the physiological mechanisms and thresholds of acute silver toxicity, far less is known about the mechanisms and thresholds for chronic toxicity.

There have been relatively few studies conducted on the chronic toxicity of silver in fish (Davies et al. 1978; Nebeker et al. 1983; Galvez et al. 1998; Guadagnolo et al. 2001), however, the data that do exist indicate that early life stages are very sensitive to silver when it is presented as $AgNO_3$ (Davies et al. 1978; Nebeker et al. 1983). In steelhead trout, a total silver concentration of 0.51 μg/l total silver reduced percent hatch relative to controls and levels as low as 0.1 μg/l total silver reduced growth rate following 60 days exposure (Nebeker et al. 1983). In rainbow trout, exposure to 0.17 μg/l total silver (as $AgNO_3$) from the eyed stage caused premature hatching and ultimately reduced growth rates following 18-months exposure (Davies et al. 1978). A more recent study where rainbow trout embryos were exposed to silver (as $AgNO_3$) from fertilization to just following hatch, indicated that although there was silver accumulation at 0.12 μg/l and 1.22 μg/l total silver, mortality was not significantly different from controls. The next highest silver concentration tested, 13.5 μg/l, resulted in 33% mortality (Guadagnolo et al. 2001). The apparent discrepancy in silver toxicity between the studies of Guadagnolo et al. (2001) and the two former studies (Davies et al. 1978; Nebeker et al. 1983) is likely related to both the duration of silver exposure and differences in water chemistry. The exposure water used by Guadagnolo et al. (2001) had much higher levels of water Cl^- and DOM, ligands that are known to protect against acute silver toxicity in trout.

Recently, a great deal of effort has been directed towards the development of geochemical type models that predict acute metal toxicity by relating it to predicted gill metal burden (Janes and Playle 1995; Playle 1998; MacRae et al. 1999). The models take into account all relevant water quality parameters that affect speciation of the metal, and its interaction with the gill surface, as well as the chemistry of the gill surface itself. Although this modeling approach works well for Cu, and to a lesser degree Cd, gill metal burden does not predict the acute toxicity of silver (McGeer and Wood 1998; Bury et al. 1999b; Wood et al. 1999). A firm understanding of the mechanism of acute silver toxicity (impairment of Na^+,K^+-ATPase activity) permitted conversion of a model from one that predicts gill silver accumulation (but not toxicity; Janes and Playle 1995) to a model that successfully predicts *acute* silver toxicity (McGeer et al. 2000). Similarly, an understanding of the mechanism of *chronic* silver exposure may permit the development of a model that can predict *chronic* toxicity on a site-specific basis. This would represent a considerable advancement over the present system where water quality parameters are rarely taken into account when generating chronic water quality guidelines.

The objectives of this study were to determine thresholds for toxicity and investigate whether the mechanism(s) of chronic toxicity in early life stages of rainbow trout are similar to that seen in juvenile and adult fish during acute silver exposure. Specifically, the effect of continuous exposure of freshly fertilized rainbow trout eggs to 0, 0.1 and 1.0 μg/l total silver (as $AgNO_3$) were investigated on mortality, time to hatch, growth (larval weight, length and extractable protein), ion regulation (unidirectional Na^+ uptake – $J_{in}Na^+$, Na^+,K^+-ATPase activity levels and whole body Na^+ and Cl^- levels) and whole body cortisol and ammonia levels. The concentrations of 0.1 μg/l and 1.0 μg/l total silver were chosen because the former represents the "Canadian Water Quality Guideline", representing both an acute and chronic value (Canadian Council of Ministers of the Environment 1995), and the latter represents the lowest concentration of $AgNO_3$ tested in a recent study that resulted in significant mortality and a reduction in whole embryo Na^+ levels following an acute silver challenge (Guadagnolo et al. 2000).

Materials and methods

Freshly fertilized rainbow trout eggs were obtained from Rainbow Springs Trout Hatchery and transported to McMaster University. Eggs were fertilized at 6 °C and warmed to 13.5 °C over a 3-h period and placed in a darkened 20-l holding tank supplied with dechlorinated Hamilton hard water (in mM: Na^+ 0.60, Cl^- 0.70, Ca^{2+} 1.0, Mg^{2+} 0.2, K^+ 0.05, HCO_3^- 1.9, dissolved organic carbon, 1.3 mg/l, alkalinity, 95 mg $CaCO_3$/l, hardness, 120 mg $CaCO_3$/l, pH 7.5). The water temperature was maintained at 13.7 ± 0.1 °C over the duration of the study.

Experimental protocol

Three hours following fertilization, embryos were randomly distributed to one of three flow-through silver exposure conditions, nominally of 0 (below the detection limit of 0.05 μg/l), 0.1 μg/l (measured = 0.098 ± 0.002 μg/l) and 1.0 μg/l (measured = 0.853 ± 0.022 μg/l) total silver, as AgNO3 (Fisher Scientific), in Hamilton hard water, with flow rates of 100 ml/min. Embryos were exposed to all silver concentrations in triplicate, with 300 embryos in two of the three replicates (0.5-l chambers) and 600 embryos in the third replicate (2-l chambers) which was the source for embryos 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 $AgNO_3$ in distilled water were made up in light-shielded, glass carboys at a concentration 1,000-times that of the desired final total silver concentration. Stock solutions were delivered by peristaltic pump to 150-ml header tanks at a flow rate of 0.1 ml/min which was mixed with dechlorinated Hamilton hard water flowing into the header tank at 100 ml/min. The header tanks were vigorously aerated to facilitate mixing and the overflow was introduced to the embryo exposure chamber, where each chamber had an independent flow-through source.

The stock solution for the 0.1 μg/l total silver treatment also contained 10 μCi ^{110m}Ag/l (Amersham International, Courtaboeuf, France) so that the final total silver concentration in the exposure chambers could be determined based upon radioisotope dilution as described below.

Mortalities were recorded daily and dead embryos were discarded. Early in development, the criterion for mortality was opaqueness of embryos while later in development it was cessation of heartbeat. Time to hatch was also recorded. On days 14, 19, 24, 30 and 37 post-fertilization, 50 living embryos from each treatment were collected, blotted dry, weighed and then frozen in liquid nitrogen for the determination of whole embryo/larval, Na^+,K^+-ATPase activity levels, extractable protein, Na^+, Cl^-, ammonia and cortisol concentrations as described below. An additional 20 embryos were removed at each sampling time for determination of whole embryo/larval J_{in} Na^+ as described below. Following hatch, larval weights and fork lengths were measured and recorded. Condition factor was calculated as:

$$wt/l^3 * 100$$

where *wt* refers to larval weight (with or without yolk as indicated; mg) and *l* refers to length (mm).

Water analysis

Water samples were collected bi-weekly from each exposure chamber, immediately acidified with 0.5% (v/v) trace metal grade HNO_3 (Fisher Scientific) and stored in the dark for future analysis. Samples were analyzed using an atomic absorption spectrophotometer (Varian AA 1275, Mississauga, Ont.) equipped with a graphite furnace atomizer (Varian GTA-95). The graphite furnace was programmed with the following temperature ramping profile: ambient temperature to 75 °C over 5 s, 75 °C to 90 °C over 12 s, 90 °C to 120 °C over 30 s with atomization occurring at 2,000 °C. The graphite tube was flushed with pre-purified N_2 gas between samples to eliminate contamination and memory effects. An automated sample injector was used to dispense 10 μl sample for each measurement, yielding a detection limit of about 0.05 μg/l total silver.

Total silver in the 0.1 μg/l exposure water was measured based upon radioisotopic dilution. The silver content of the stock solution was measured using atomic absorption spectrophotometry, radioactivity (total counts per minute; cpm) of ^{110m}Ag was measured on a gamma counter (Packard Instruments, Downers Grove, Ill., USA), and the specific activity (SA) of the stock solution was calculated (cpm/μg silver). The total silver concentration of the exposure tank water was determined by dividing ^{110m}Ag activity (cpm) of the water by the SA of the stock solution.

Water [Na^+] was measured by flame atomic absorption spectrophotometry (Varian AA 1275, Varian, Mississauga, Ont., Canada).

Unidirectional Na^+ influx measurements

Whole embryo/larval J_{in} Na^+ measurements were conducted in 60-ml Nalgene bottles. Twenty embryos or larvae were added to each chamber containing 20 ml (embryos) or 40 ml (larvae) of water, to which the organisms had been exposed, and 7.2 μCi ^{22}Na was added to that chamber. During the flux measurement, the medium was continuously aerated and 2-ml water samples were taken in duplicate 10 min after the start of the flux, and again at the end of the flux period, for measurement of water radioactivity and [Na^+]. The flux period lasted a total of 6 h where ten embryos or larvae were removed at 2 h and the remaining ten embryos or larvae were removed at 6 h after the addition of the isotope. There is a large non-specific binding of ^{22}Na to the embryos which is complete within the 1st 1 h of the flux exposure. To ensure that active ^{22}Na uptake was measured, the difference in ^{22}Na accumulation between 2 h and 6 h was used in the flux study. This was less of a concern from day 24 post-fertilization and on where non-specific binding of ^{22}Na had very little or no affect on ^{22}Na uptake values. Embryos and larvae were anesthetized with a lethal dose of tricaine methanesulphonate (MS-222), rinsed three times in 5 mM NaCl to displace surface-bound ^{22}Na, and then deionized water, weighed and placed individually into scintillation vials for measurement of radioactivity using a gamma counter (Packard Instruments, Downers Grove, Ill., USA).

Whole embryo J_{in} Na^+ was calculated as follows:

$$J_{in}Na^+ = (q_6 - q_2)/(SA \cdot t \cdot wt)$$

where q_6 and q_2 represent cpm for the embryos or larvae at 6 and 2 h respectively, t is the time (h) between measurements (4 h), *wt* refers to the wet weight of the embryo and *SA* refers to the specific activity of the water calculated as:

$$SA = \left[\left(cpm_i/[ion]_i\right) + \left(cpm_f/[ion]_f\right)\right]/2$$

Where $_i$ refers to water samples taken 10 min following addition of the isotope and $_f$ refers to the water sample taken at the end of the flux period (time = 6 h).

Whole embryo/larval Na^+ and Cl^- concentration

Ten embryos were collected from each treatment at each sampling time, anesthetized with a lethal dose of MS-222, blotted dry, weighed individually, and then placed in 150 μl 1.0 N HNO_3 in individual 0.5-ml plastic "bullet" tubes. These tubes were left in an oven at 60–70 °C for 24 h and the chorion or tissue was crushed and vortexed to ensure that all tissue was thoroughly digested during this time. The tubes were then spun down at 10,000 *g* for 2 min and the supernatant was diluted for measurement of [Na^+] by flame atomic absorption spectrophotometry (Varian AA 1275, Varian, Mississauga, Ont., Canada) and for measurement of Cl^- by the colorimetric mercuric thiocyanate method (Zall et al. 1956).

Na^+,K^+-ATPase activity

Whole embryo/larval Na^+,K^+-ATPase activity was measured in crude homogenates according to McCormick (1993) using a plate reader (MRX, Dynex) and activity was expressed as the concentration of inorganic phosphate liberated per unit time and standardized to protein content or embryonic/larval mass. Extractable protein content of the homogenate was measured using Bradford reagent (Bio-rad, Richmond, Calif., USA) and bovine serum albumin as a standard.

Cortisol

Two embryos or larvae were pooled and homogenized in 2.5-times the volume of phosphosaline buffer (61.2 mM NaH_2PO_4; 39.1 mM $Na_2H_2PO_4$; 154 mM NaCl; 15.4 mM sodium azide; 1 g/l gelatin, pH 7.6) according to Stephens et al. (1997). Homogenates were extracted prior to radioimmunoassay in ether as described by De Jesus et al. (1991). Cortisol levels of the extracted homogenates were measured using a radioimmunoassay kit from Immunocorp (^{125}I-RIA kit, ICN Biomedical, Irvine, Calif., USA). Extraction efficiencies were determined by addition of radiolabeled cortisol prior to larval homogenization yielding a value of 57.3 ± 0.8%. Reported values are not corrected for extraction efficiency.

Statistics

Values are presented as mean ± 1 SE throughout. Calculation of time to 50% hatch was determined by log probit analysis. Mean values were statistically compared between treatments on respective days using a one-way analysis of variance (ANOVA). Mortality between treatments was tested for statistical significance using a one-way ANOVA on the number of mortalities per day (i.e., on

non-cumulative mortality). A Dunnett's post-hoc test was used to identify groups that differed significantly from the controls within each respective comparison. The level of statistical significance for all analyses was $P < 0.05$.

Results

Mortality and time to hatch

There was a statistically significant increase in the daily percent mortality from fertilization to hatch at 1.0 μg/l total silver relative to both the control and 0.1 μg/l treatments, although this difference was very small (Fig. 1). In all treatments mortality levels were low (<2%) up to about day 15 post-fertilization and then progressively increased through to hatch, reaching a maximum of 12% in controls and the 0.1 μg/l total silver treatment and 16% in the 1.0 μg/l total silver treatment. Although there was a trend toward an acceleration in time to hatch in the 0.1 μg/l total silver treatment, there were no statistically significant differences relative to control and 1.0 μg/l total silver treatments (Fig. 2).

Ionoregulation

There was a marked increase in J_{in} Na^+ with development in all treatments (Fig. 3). On day 14 post-fertilization, at which time there was some visible eye pigmentation, active Na^+ uptake was very low (between 13 nmol/g per h and 18 nmol/g per h) and remained low until day 24 post-fertilization (4 days prior to 50% hatch). In the absence of silver, J_{in} Na^+ increased dramatically by day 30 post-fertilization (1–2 days following hatch), where there was a ten-fold elevation in J_{in} Na^+ that continued to increase to day 37 post-fertilization.

A significant effect of silver exposure was observed on day 24 post-fertilization, where J_{in} Na^+ in the 1.0 μg/l treatment was more than two-fold greater than controls and by day 30 there was a significant difference among all three treatments, where J_{in} Na^+ increased with silver concentration. On day 30, J_{in} Na^+ values in the 1.0 μg/l treatment were three-fold that of controls. By day 37, however, there were no significant differences among any of the treatments, all exhibiting J_{in} Na^+ values of about 300 nmol/g per h.

Whole embryo and larval Na^+,K^+-ATPase levels increased gradually throughout development whether expressed relative to protein concentration (Fig. 4) or in absolute levels (per individual; data not shown but qualitatively identical to Fig. 4). This differed from J_{in}-Na^+ of control embryos, where an increase was not observed between day 14 and day 24 post-fertilization. In Fig. 4, there was a significant reduction in activity level in the 0.1 μg/l treatment relative to controls on day 19 post-fertilization, and on day 24 and day 30, there was a significant increase in activity levels in the 1.0 μg/l treatment relative to the 0.1 μg/l treatment and controls. In general, the changes in Na^+,K^+-ATPase levels associated with silver exposure were qualitatively

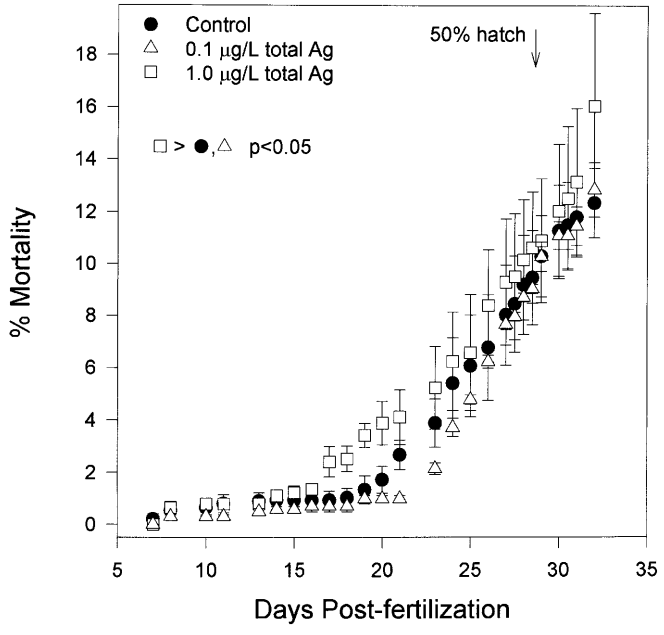

Fig. 1 Cumulative mortality (percentage) in rainbow trout embryos and larvae during continuous flow-through exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. *Closed circles* represent controls, *open triangles* represent exposure to 0.1 μg/l total silver, and *open squares* represent exposure to 1.0 μg/l total silver ($n = 3$). Mortality was significantly greater in the 1.0 μg/l total-silver-treatment group relative to the other treatments based upon mortalities per day (i.e., non-cumulative mortality)

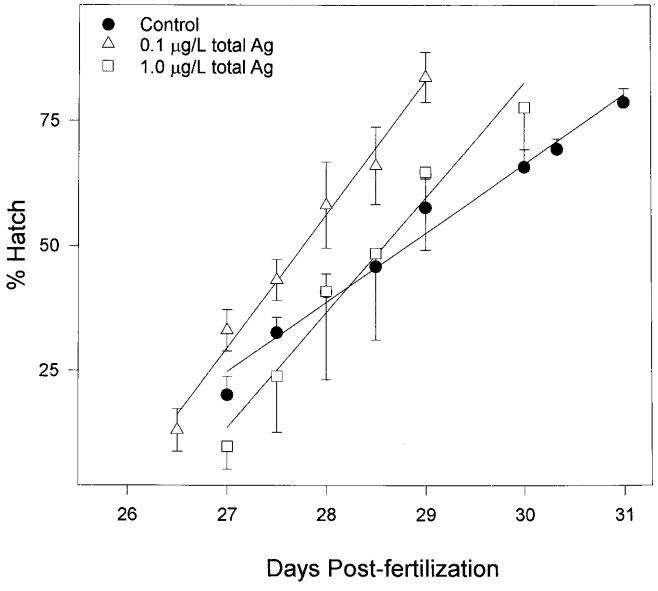

Fig. 2 Percent hatch in rainbow trout embryos during continuous flow-through exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. There were no significant differences in time to 50% hatch. See Fig. 1 legend for further details

similar to the data for $J_{in}Na^+$ described above. By day 37, however, there was a significant reduction in activity level in both silver groups relative to controls. Despite the increase in $J_{in}Na^+$ and Na^+,K^+-ATPase activity during silver exposure relative to controls, there were no significant effects of silver exposure on whole embryo or larval Na^+ (Fig. 5). There were also no significant differences among the treatments for whole embryo or larval Cl^- concentration, which was rather variable, from 18 m Eq/Kg to 68 m Eq/Kg over the rearing duration (data not shown).

Growth

In the first 20 embryos to hatch in each treatment, larvae that had been exposed to 0.1 μg/l and 1.0 μg/l total silver were significantly longer than control embryos, but only those from the 1.0 μg/l treatment group were significantly heavier, where the yolk made up a significantly lower proportion of the whole animal mass resulting in a lower condition factor (embryo and yolk sac; Table 1). In larvae taken randomly from each exposure tank on day 30 and day 37 post-fertilization, those exposed to 1.0 μg/l total silver were significantly longer and heavier (including yolk) with a significant reduction in larval condition factor, relative to controls (Table 2).

Extractable protein of the whole embryo and larvae increased with development and was significantly greater in the 1.0 μg/l silver treatment relative to 0.1 μg/l and controls on days 24 and 30 post-fertilization (Fig. 6).

Ammonia and cortisol

Whole embryo ammonia levels did not differ significantly during development in the absence of silver, but there was a trend toward an increase from 1.5 mM to

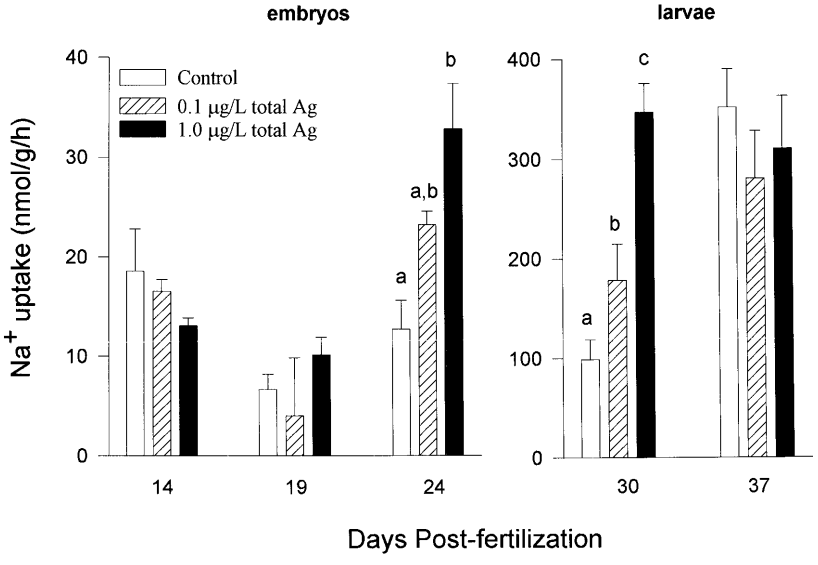

Fig. 3 Whole embryo/larval unidirectional Na^+ uptake in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. *Open bars* represent controls, *hatched bars* exposure to 0.1 μg/l total silver and *closed bars* exposure to 1.0 μg/l total silver. *Left panel* shows pre-hatch (*embryos*), *right panel* shows post-hatch (*larvae*). Note the ten-fold expanded axis in the post-hatch group. *Letters* that differ within a given time period indicate statistically significant differences ($P < 0.05$; $n = 10$)

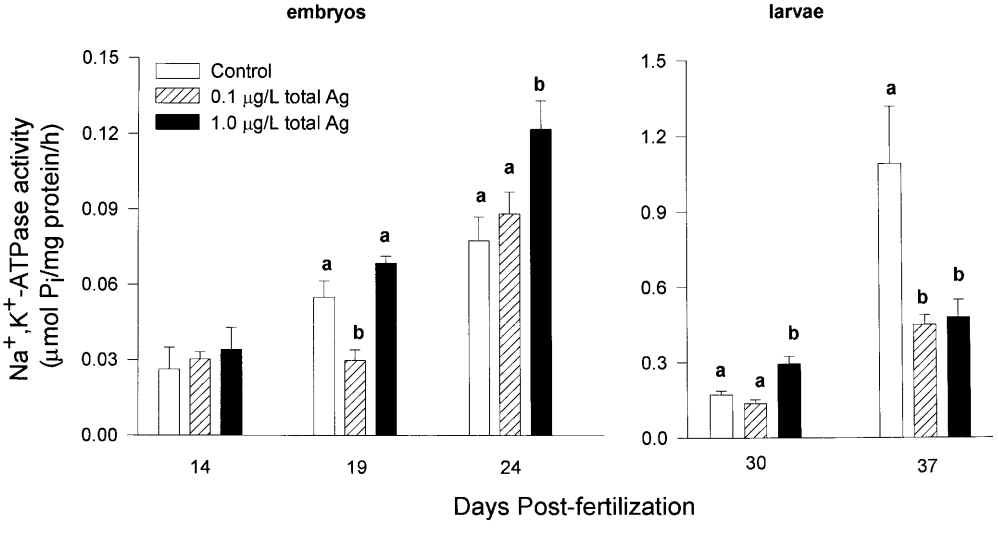

Fig. 4 Whole embryo/larval Na^+,K^+-ATPase activity levels (standardized for protein concentration) in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. See Fig. 3 legend for further information

2.5 mM from day 14 to day 37 post-fertilization (Fig. 7). By day 30 post-fertilization (2 days post-hatch), larval ammonia levels were significantly greater in the 1.0 μg/l total-silver-treatment group than the 0.1 μg/l treatment group and controls. On day 37 post-fertilization, there was a significant difference among all three groups, where ammonia concentration increased with silver exposure concentration. In the 1.0 μg/l total-silver-treatment group, ammonia levels were almost double that observed in the control group, reaching a value of 4.6 mM. There were no differences in whole embryo cortisol levels prior to hatch, but in larval rainbow trout (day 37), cortisol was significantly elevated in the 1.0 μg/l treatment group relative to the other groups. Thus, both cortisol and ammonia were elevated on day 37 in the 1.0 μg/l treatment group relative to controls. (Fig. 8).

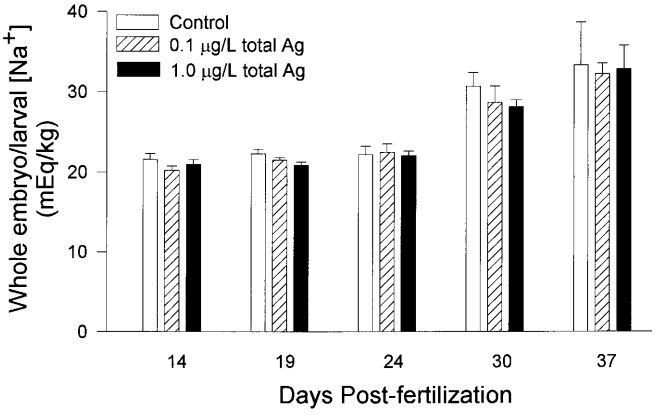

Fig. 5 Whole embryo/larval Na^+ concentration in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. There were no statistically significant differences between treatments at any given time but there is a significant increase in Na^+ concentration ($P < 0.05$) on day 30 and day 37 relative to the other time periods (n = 10)

Discussion

The highest concentration of silver tested in this study (1.0 μg/l total silver as $AgNO_3$), resulted in a small but statistically significant increase in mortality from fertilization to 1 week post-hatch. However, this treatment was also associated with an acceleration in ionoregulatory development up to hatch and an increase in growth over the duration of the study. Following hatch, at this exposure level, there was also an elevation in both whole body cortisol and ammonia levels indicative of sublethal effects of silver exposure, similar to those observed in acute silver exposure in both juvenile and adult fish (Webb and Wood 1998).

Table 1 Lengths, weights and percent body mass comprised of yolk (% yolk) and condition factor in the first 20 larvae to hatch during chronic exposure to silver as $AgNO_3$. Larval weight includes the yolk. *CF* condition factor; *values in brackets* refer to 1 SE of the mean

Treatment	Length (mm)	Weight		CF (including yolk)	% Yolk
		Larvae + yolk (mg)	Larvae (mg)		
Control	13.9a (0.3)	77.7 (1.4)	14.7a (0.9)	3.02a (0.19)	80.9a (1.4)
0.1 μg/l total silver	14.5b (0.4)	79.6 (1.7)	16.0a (0.6)	3.67b (1.27)	79.7a (0.8)
1.0 μg/l total silver	15.2b (0.3)	78.6 (1.2)	18.6b (0.9)	2.34b (0.54)	76.3b (1.1)

[a,b]Letters that differ indicate a statistically significant difference between treatments

Table 2 Length, weight and condition factor of larvae on days 30 and 37 post fertilization during chronic exposure to silver as $AgNO_3$. See Table 1 legend for further information

Treatment	Length (mm)	Weight (mg) larva + yolk	CF (including yolk)
Day 30			
Control	14.8a (0.3)	81.6a (0.9)	2.62a (0.13)
0.1 μg/l total silver	15.2a (0.3)	81.5a (1.15)	2.44a (0.13)
1.0 μg/l total silver	16.5b (0.2)	83.8b (1.06)	1.89b (0.05)
Day 37			
Control	19.8a (0.3)	95.2a (1.7)	1.27a (0.05)
0.1 μg/l total silver	20.2a (0.3)	97.3a (1.9)	1.21a (0.04)
1.0 μg/l total silver	21.4b (0.2)	100.5b (1.4)	1.04b (0.02)

[a,b]Letters that differ indicate a statistically significant difference between treatments

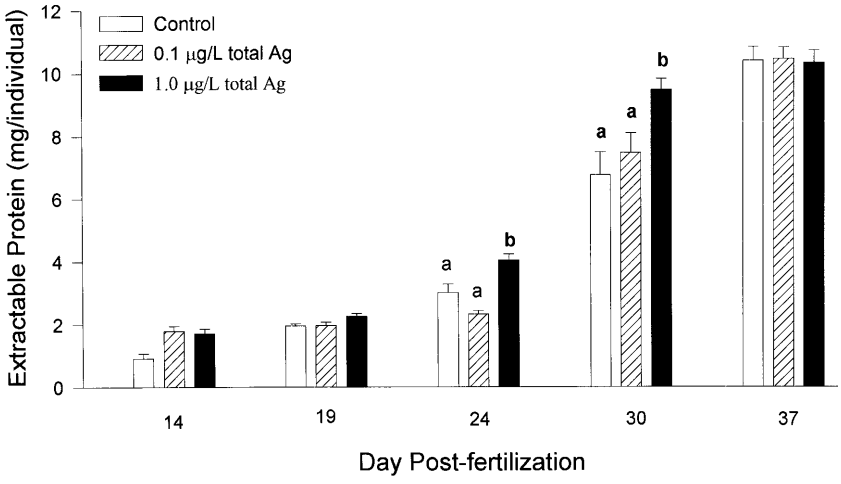

Fig. 6 Whole embryo/larval extractable protein in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. Data for 30 days and 37 days post-fertilization are for post-hatch fish. See Fig. 3 caption for further information

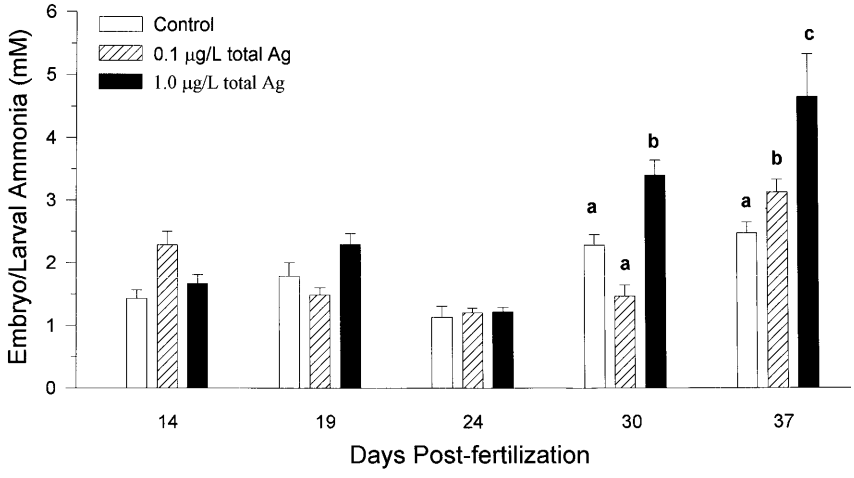

Fig. 7 Whole embryo/larval ammonia in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. Data for 30 days and 37 days post-fertilization are for post-hatch fish. See Fig. 3 caption for further information

Ionoregulation

Due to the relatively undeveloped condition of the gills and kidneys in developing embryos, the skin and yolk sac are generally assumed to be the predominant site of active uptake of ions from freshwater based upon the high density of mitochondria-rich cells in these regions (see: Hwang and Hirano 1985; Alderdice 1988 for reviews). In the present study, very low levels of unidirectional Na^+ uptake occurred in rainbow trout prior to the development of eye pigmentation, an observation that correlates well with studies on Atlantic salmon embryos (Rudy and Potts 1969; Eddy and Talbot 1985). However, few studies have examined the pattern of J_{in} Na^+ during development. From day 14 to day 24 in the present study, there was very little change in J_{in} Na^+ despite a ten-fold increase in embryonic Na^+,K^+-ATPase activity. The lack of change in J_{in} Na^+ correlates well with a fairly constant number of chloride cells (CCs) predominantly in the yolk sac epithelium, over this duration of development (Rombough 1999). Whether the lack of correlation between J_{in} Na^+ and Na^+,K^+-ATPase activity is a reflection of the chorion acting as a barrier to ion uptake, or that the majority of Na^+,K^+-ATPase is associated with tissue development and growth rather than to drive active ion uptake from the water is unknown. A few days following hatch, there is a dramatic increase in the CC numbers of the gills, at which time they surpass that of the yolk sac epithelium (Rombough 1999). This expansion of gill CC density correlates with the approximately ten-fold increase in J_{in} Na^+ and doubling of Na^+,K^+-ATPase activity per individual in the control embryos and larvae of the present study on day 30 (2 days post-hatch) relative to day 24 post-fertilization.

Silver accumulation in embryos was not measured in this study, but significant silver accumulation was observed in embryos chronically exposed to 0.12 μg/l and 1.22 μg/l total silver under identical conditions to the present study (Guadagnolo et al. 2001). Although up to 85% of the silver burden was contained within the chorion (illustrating its protective role during silver exposure), there was measurable silver accumulation in the embryo and yolk sac. Although the route(s) of silver uptake into the ionoregulatory cells of embryos and larvae is unknown, it is likely similar to that observed in

the fish gill, given the similarity between gill and larval skin CC structure (Hwang 1989, 1990) and biochemistry (Hwang et al. 1999). In the gills of fish, both Na^+ and Ag^+ uptake are inhibited by phenamil and bafilomycin A_1, indicating that silver crosses the apical membrane of the gill by a proton-coupled Na^+ channel (Bury and Wood 1999). Once within the gill cells, silver binds to the Mg^{2+}-binding site of the basolateral Na^+,K^+ ATPase enzyme (Hogstrand and Wood 1998) in a dose-dependent manner (Bury et al. 1999c) leading to a non-competitive inhibition of Na^+ and Cl^- uptake in freshwater fish (Morgan et al. 1997; McGeer and Wood 1998). As a result, plasma Na^+ and Cl^- levels fall, illustrating the primary toxic mechanism of acute silver toxicity.

In the present study, silver exposure at 0.1 μg/l and 1.0 μg/l did not impair J_{in} Na^+ or Na^+,K^+-ATPase activity up to day 30, despite a small but statistically significant elevation in mortality at 1.0 μg/l total silver. Interestingly, just following hatch (day 30 post-fertilization), there was a dose-dependent increase in unidirectional ion fluxes, and on days 19, 24 and 30, there was an elevation in Na^+,K^+-ATPase activity in the 1.0 μg/l-exposed group. Whether this represents hormesis, acclimation or acceleration of development (see below) is not known, but regardless, the effect was short-lived. By day 37 post-fertilization, J_{in} Na^+ levels were similar in all treatments and Na^+,K^+-ATPase activity was reduced relative to controls at both silver levels. Despite the increases in J_{in} Na^+ and Na^+,K^+-ATPase activity with silver exposure at certain stages, there were no differences in whole body [Na^+] or [Cl^-]. Chronic exposure to 10.7 μg/l total silver under otherwise identical conditions to the present study, resulted in complete elimination of J_{in} Na^+ and a reduction in Na^+,K^+-ATPase activity throughout development to hatch (C.J. Brauner and C.M. Wood unpublished observations), and a significant reduction in whole embryo Na^+ by day 23 post-fertilization (Guadagnolo et al. 2001). Thus, exposure to both 0.1 μg/l and 1.0 μg/l total silver in the moderately hard water of Lake Ontario does have significant sublethal effects on ionoregulatory development despite relatively minor effects on mortality.

Growth

In addition to a stimulation of ionoregulatory development during chronic exposure to silver, there also appeared to be a general stimulation of growth. The slight acceleration in time to hatch in the 0.1 μg/l total-silver group was associated with a significant increase in larval length relative to controls and those exposed to 1.0 μg/l total silver were significantly longer and heavier than controls at hatch. This trend was maintained in the 1.0 μg/l-exposed group up to day 37 post-hatch when the experiment was terminated.

Extractable protein of the whole embryo and larvae was also significantly greater in the 1.0 μg/l silver-treatment group relative to the 0.1 μg/l group and controls on days 24 and 30 post-fertilization. The increase in Na^+,K^+-ATPase activity in embryos and larvae is not simply a function of increased size in the silver-exposed groups because there is an elevation in Na^+,K^+-ATPase activity levels whether expressed per individual or standardized for protein content. Thus, although a component of the silver-exposure-induced elevation in Na^+,K^+-ATPase activity is related to size, there must also be either more copies or a higher activity level of the transporter per milligram of protein.

Cortisol and ammonia

In adult rainbow trout acutely exposed to silver at 9.2 μg/l (total silver as $AgNO_3$) in identical water to the present study, there was a significant increase in both plasma cortisol and ammonia levels by 96 h (Webb and Wood 1998). Ammonia efflux was not impaired in that study and it was concluded that the elevated ammonia levels were a result of increased metabolic production brought about by the proteolytic and gluconeogenic effects of cortisol. In fish early life stages, there is an increase in whole embryo ammonia levels during development due to yolk protein metabolism (Smith 1947) despite the ability to excrete ammonia (Wright et al. 1995). Around the time of hatch in the present study,

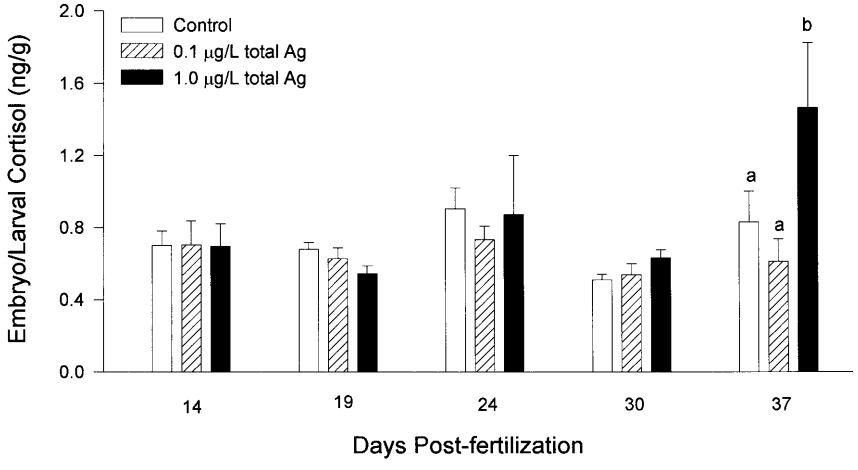

Fig. 8 Whole embryo/larval cortisol in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water from fertilization to post-hatch. Data for 30 days and 37 days post-fertilization are for post-hatch larvae. See Fig. 3 caption for further information

there was a trend toward an increase in embryo or larval ammonia levels relative to day 14 values. The increase in ammonia levels associated with hatch has been observed by others and is thought to be associated with the exhaustive muscular activity required during the hatching process (Wright et al. 1995). Because urea levels increase in concert with ammonia levels around hatch, it has been concluded that urea synthesis is important in preventing ammonia toxicity at this developmental stage in fish (Wright et al. 1995). In the 1.0 μg/l total-silver-exposed group, there was a large increase in whole larval ammonia levels to 4.6 mM following hatch, almost double that observed in the control group. This dramatic increase in ammonia level coincided with a significant elevation in whole body cortisol concentration. Elevations in whole-body cortisol have also been observed in turbot within 2 days following hatch during exposure to the water-soluble fraction of crude oil (Stephens et al. 1997). Whether silver exposure impairs urea synthesis or the elevated cortisol promotes ammoniagenesis is unknown, but these high levels of ammonia and cortisol are likely indicative of sublethal toxicity of silver at 1.0 μg/l. Thus, during chronic silver exposure in early life stages of trout, there may be a generalized stress response as is observed during acute silver exposure in both juvenile and adult fish (Webb and Wood 1998).

Environmental and modeling implications

Chronic toxicity can be manifested as developmental defects, later reductions in fecundity, reductions in growth rate, changes in time to hatch in early life stages and mortality over the exposure duration. During such responses, the site and mechanism of toxicity may be similar to that observed during *acute* silver exposure but occur at a slower rate. The results of this study indicate that this may, at least partly, be the case, as chronic silver exposure of trout early life stages resulted in sublethal effects including an elevation in whole-body cortisol and ammonia levels as well as a reduction in Na^+,K^+-ATPase activity level on day 37 post-fertilization as is seen during acute silver exposure in adult trout (Hogstrand and Wood 1998; Webb and Wood 1998). Assuming that the mechanism(s) of chronic toxicity are similar to those of acute toxicity, it may be possible to extend a physiologically based model which predicts acute silver toxicity as a function of water quality (McGeer et al. 2000), to one that will predict chronic toxicity. The effects of chronic silver exposure in this study, however, were not always negative and thus further research is required to further elucidate the mechanisms of chronic silver toxicity and the extent of any potential adverse effects.

It is well established that water ligands such as Cl^-, Na^+, Ca^{2+} and DOM provide protection during acute silver exposure (LeBlanc et al. 1984; Bury et al. 1999a, 1999b; McGeer and Wood 1998; McGeer et al. 2000), although very little is known about these effects during chronic silver toxicity. The relatively low mortality rates observed in this study relative to those in others (Davies et al. 1978; Nebeker et al. 1983) that were conducted in relatively soft water (total hardness 20–40 mg/l $CaCO_3$) likely with correspondingly low chloride levels, indicate that chloride, at least, may be protective during chronic silver exposure. Thus, the next step is to assess the potential protective effects of natural ligands on chronic silver toxicity in order to develop a chronic model. Some of these experiments are presently underway in our laboratory.

Acknowledgements 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 Erin Fitzgerald for valuable technical assistance. These experiments were conducted in compliance with local animal care guidelines.

References

Alderdice, DF (1988) Osmotic and ionic regulation in teleost eggs and larvae. In: Hoar WS, Randall DJ (eds) Fish physiology. Vol XIA. Academic Press, New York, pp 163–251

Bury NR, Wood CM (1999) Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. Am J Physiol 277:R1385–R1391

Bury NR, Galvez F, Wood CM (1999a) 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 NR, McGeer JC, Wood CM (1999b) Effects of altering freshwater chemistry on the physiological responses of rainbow trout to silver exposure. Environ Toxicol Chem 18:49–55

Bury NR, Grosell M, Grover AK, Wood CM (1999c) ATP-dependent silver transport across the basolateral membrane of trout gills. Toxicol Appl Pharmacol 159:1–8

Canadian Council of Ministers of the Environment (1995) Canadian water quality guidelines: silver. In: Canadian Council of Ministers of the Environment. Winnipeg, MB, pp 3–22

Davies PH, Goettl JP, Sinley JR (1978) Toxicity of silver to rainbow trout (*Salmo gairdneri*). Water Res 12:113–117

De Jesus EG, Hirano T, Inui Y (1991) Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. Gen Comp Endocrinol 82:369–376

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

Eisler R (1996) Silver hazards for fish, wildlife, and invertebrates: synoptic review. Natl Biol Serv Biol Rep 32:1–44

Galvez F, Hogstrand C, Wood CM (1998) Physiological responses of juvenile rainbow trout to chronic low level exposures of waterborne silver. Comp Biochem Physiol C 119:131–137

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

Guadagnolo CM, Brauner CJ, Wood CM (2001) Chronic effects of silver exposure on ion levels, survival and silver distribution within developing rainbow trout embryos (*Oncorhynchus mykiss*). Environ Toxicol Chem 20:53–560

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

Hwang PP (1989) Distribution of chloride cells in teleost larvae. J Morph 200:1–8

Hwang PP (1990) Salinity effects on development of chloride cells in the larvae of ayu (*Plecoglossus altivelis*). Marine Biology 107:1–7

Hwang PP, Hirano R (1985) Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleost development. J Exp Zool 236:115–126

Hwang P, Lee TH, Weng CF, Fang MJ, Cho GY (1999) Presence of Na^+,K^+-ATPase in mitochondria-rich cells in the yolk-sac epithelium of larvae of the teleost *Oreochromis mossambicus*. Physiol Zool 72:138–144

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

LeBlanc GA, Masone JD, Paradice AP, Wilson B, Lockhart HB, Robillard KA (1984) The influence of speciation on the toxicity of silver to fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 3:37–46

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

MacRae RK, Smith DE, Swoboda-Colberg N, Meyer JS, Bergman HL (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

McGeer JM, Wood CM (1998) Protective effects of water Cl^- on physiological responses to waterborne silver in rainbow trout. Can J Fish Aquat Sci 55:2447–2454

McGeer JC, Playle RC, Wood CM, Galvez F (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

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^+ and Cl^- transport. Aquat Toxicol 38:145–163

Nebeker AV, McAuliffe 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

Playle RC (1998) Modelling metal interactions at fish gills. Sci Total Environ 219:147–163

Purcell TW, Peters JJ (1998) Sources of silver in the environment. Environ Toxicol Chem 17:539–546

Rudy PP, Potts WTW (1969) Sodium balance in the eggs of the Atlantic salmon, *Salmo salar*. J Exp Biol 50:239–246

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

Shafer MM, Overdier JT, Armstrong DE (1998) Removal, partitioning, and fate of silver in a wastewater treatment plant. Environ Toxicol Chem 17:630–641

Smith S (1947) Studies in the development of the rainbow trout (*Salmo irideus*). J Exp Biol 23:357–378

Stephens SM, Alkindi AYA, Waring CP, Brown JA (1997) Corticosteroid and thyroid reponses of larval and juvenile turbot exposed to the water-soluble fraction of crude oil. J Fish Biol 50:953–964

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

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^+. Aquat Toxicol 35:93–109

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

Wright PA, Felskie A, Anderson PM (1995) Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. J Exp Biol 198:127–135

Zall DM, Fisher D, Garner MD (1956) Photometric determination of chlorides in water. Anal Chem 28:1665–1678