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Effect of long-term silver exposure on survival and ionoregulatory development in rainbow trout (*Oncorhynchus mykiss*) embryos and larvae, in the presence and absence of added dissolved organic matter☆

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Abstract

Developing rainbow trout were chronically exposed to silver (as $AgNO_3$) from fertilization to swim-up, in moderately hard water (120 mg $CaCO_3 l^{-1}$) in the presence and absence of an additional 12 mg C/L of dissolved organic carbon (DOC, as humic acid, Aldrich). Nominal silver concentrations were 0, 0.1 and 10 $\mu g\ l^{-1}$ total silver in a flow-through set-up maintained at 12 °C. The objectives of the study were to investigate the possible protective effects of DOC on growth, mortality, time to hatch and swim-up, and sublethal ionoregulatory disturbances during chronic exposure to ionic silver. Throughout development, there was a large increase in % daily mortality at 10 $\mu g \cdot l^{-1}$ total silver (in the absence of DOC), that was associated with an ionoregulatory disturbance, in particular a 35% reduction in whole body Na^+ just prior to hatch. At nominal 10 $\mu g \cdot l^{-1}$ total silver, the presence of additional DOC (reducing dissolved silver to 4.7 ± 0.3 $\mu g\ l^{-1}$) resulted in a significant reduction in % daily mortality up to hatch, demonstrating a protective effect of DOC. Interestingly, DOC did not appear to mitigate the ionoregulatory disturbance, with the exception of whole body [Cl^-] on day 44 of exposure. Exposure to 0.1 $\mu g \cdot l^{-1}$ total silver (in the absence of DOC) resulted in a statistically significant reduction in growth, and DOC did not prevent an ionoregulatory disturbance [based upon (J_{in} Na^+), whole body Na^+,K^+ ATPase activity and whole body (Na^+)] at this silver concentration relative to controls+DOC. DOC exerted a direct effect on growth and ionoregulatory development that complicates interpretation of the data, however, these data indicate that protective effects of DOC (in the form of Aldrich humic acid) during chronic silver exposure appear to be less than that observed during acute exposure. The ultimate goal of this and future studies is to develop a model that can predict chronic toxicity on a site-specific basis, taking into account protective effects of various ligands present in different waters, as is presently being employed for some metals during acute exposure.
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Keywords: Embryos; Larvae; Silver; Toxicity; Ionoregulation; Development; Dissolved organic matter (carbon); *Oncorhynchus mykiss*

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1. Introduction

Silver is released to the environment from natural and anthropogenic sources. Of the latter, mining, silverware manufacturing and the photographic industry are the greatest contributors in the US (Purcell and Peters, 1998). The photographic industry discharges silver complexed with thiosulphate that has a high affinity for ionic silver (Ag^+), but this is rapidly reduced to silver sulfide (sulfide has an even higher affinity for Ag^+) after passing through publicly owned treatment works (POTW's; Eisler, 1996; Shafer et al., 1998). Natural waters contain numerous ligands, such as dissolved organic matter (DOM), dissolved organic carbon (DOC), sulfide, and Cl^- that also bind Ag^+ (Janes and Playle, 1995), rendering silver much less acutely toxic to aquatic organisms than $AgNO_3$ (LeBlanc et al., 1984; Bury et al., 1999a,b; McGeer and Wood, 1998; Bianchini et al., 2002), the most frequently used form of silver in laboratory toxicity testing due to its rapid dissociation to the free silver ion, Ag^+.

Ag^+ is the form of silver causing toxicity to freshwater fish during acute exposure, and results in ionoregulatory impairment, through inhibition of gill Na^+,K^+ ATPase activity, leading to a dramatic reduction in plasma Na^+ and Cl^- levels (Wood et al., 1996; Morgan et al., 1997; McGeer and Wood, 1998). The decrease in plasma ions initiates a suite of secondary effects, including a generalized stress response, and most significantly, a cardiovascular fluid volume disturbance ultimately inducing circulatory collapse and death (Wood et al., 1996; Hogstrand and Wood, 1998; Webb and Wood, 1998). The physiological mechanisms and thresholds of acute silver toxicity (generally less than 96 h) in freshwater juvenile and adult fish have received considerable attention, however, much less is known about mechanisms of chronic silver toxicity (Galvez et al., 1998), especially in early life stages of fish which appear to be very sensitive to silver when presented as $AgNO_3$ (Davies et al., 1978; Nebeker et al., 1983).

In recent studies, rainbow trout embryos exposed to silver (as $AgNO_3$) from fertilization to just following hatch accumulated silver at measured exposure levels of 0.12 and 1.22 μg/l total silver, however, no significant effects on mortality were noted except at the next higher silver concentration tested (33% mortality at 13.5 μg/l total silver; Guadagnolo et al., 2001). Interestingly, exposure of rainbow trout embryos to measured silver levels of 0.10 and 0.85 μg/l total silver (as $AgNO_3$) from fertilization to hatch resulted in dose-dependent increase in unidirectional Na^+ uptake (J_{in} Na^+) and whole body Na^+,K^+ ATPase activity levels (Brauner and Wood, 2002) indicating that ionoregulatory development had been accelerated. Immediately following hatch however, mortality was statistically greater in the 0.85 μg/l total silver treatment (16%) than controls (12%), and there was a significant reduction in (J_{in} Na^+) and whole body Na^+,K^+ ATPase activity levels, and a significant increase in whole body cortisol and ammonia levels. These results indicate that the physiological mechanism(s) of chronic silver toxicity in developing embryos and larvae, may be similar to that in juvenile and adult fish during acute silver exposure (Wood et al., 1996; Webb and Wood, 1998).

Previous studies have indicated greater sensitivity of early life stages of fish to chronic silver toxicity than those reported above (Brauner and Wood, 2002; Guadagnolo et al., 2001). For example, steelhead trout exhibited reduced % hatch relative to controls at a nominal silver concentration of 0.51 μg/l total silver and levels as low as 0.1 μg/l total silver reduced growth rate following 60 days of exposure (Nebeker et al., 1983). In rainbow trout, exposure to nominal levels of 0.17 μg/l total silver (as $AgNO_3$) from the eyed stage caused premature hatching and ultimately reduced growth rates following 18 months of exposure (Davies et al., 1978). The apparent discrepancy in silver toxicity between these groups of studies (Brauner and Wood, 2002; Guadagnolo et al., 2001 relative to Davies et al., 1978; Nebeker et al., 1983) may be related to the duration of exposure, developmental stage when exposure was initiated, the reporting of measured vs. nominal silver exposure levels, and/or differences in water chemistry. Exposure water used by Guadagnolo et al. (2001) and Brauner and Wood (2002) had much higher levels of water Cl^- and DOC than those of previous studies, and these ligands are known to protect against acute silver toxicity in trout (Bury et al., 1999a,b; Karen et al., 1999). Much less is known about the protective effects of these ligands during chronic silver exposure.

The objectives of the present study were to determine whether dissolved organic matter (as Aldrich humic acid) confers any protective effects during chronic silver exposure in early life stages

Table 1
Measured total silver concentration in filtered (0.45 μm) and unfiltered experimental water

Treatment (nominal total silver, DOC)	Measured total silver in water (unfiltered; μg/l)	Measured total silver in water (filtered; μg/l)
0 μg/l	<0.05	<0.05
0.1 μg/l	0.14±0.01	0.13±0.003
10 μg/l	10.7±0.3	10.16±0.14
0 μg/l+DOC	<0.05	<0.05
0.1 μg/l+DOC	0.11±0.03	0.02±0.004
10 μg/l+DOC	11.6±0.6	4.7±0.34

of rainbow trout. This DOC preparation has been shown to be highly protective against acute silver toxicity to juvenile trout (Bury et al., 1999a,b). Specifically, the effect of continuous exposure of silver to freshly fertilized rainbow trout embryos through to swim-up at 0, 0.1 and 10 μg/l total silver (as $AgNO_3$) were investigated in the presence and absence of an additional nominal elevation of 12 mg/l DOC (measured as dissolved organic carbon). End points of toxicity in this study were mortality, time to hatch, swim-up and yolk sac absorption, growth (larval weight, length and extractable protein) and ionoregulatory indicators (unidirectional Na^+ uptake, Na^+,K^+-ATPase activity levels and whole body Na^+ and Cl^- levels). Concentrations of 0.1 and 10 μg/l total silver were chosen because the former is the 'Canadian Water Quality Guideline' (CWQG), representing both an acute and chronic value (CCME, 1995), and the latter concentration of $AgNO_3$ is known to induce significant mortality and ionoregulatory disturbances in water of similar composition during acute and chronic exposure to developing rainbow trout embryos (Guadagnolo et al., 2000, 2001).

The ultimate goal of this and future studies is to develop a model that can predict chronic toxicity on a site-specific basis, taking into account protective effects of various ligands present in different waters. Such a model would be analogous to the Biotic Ligand Models (BLMs, Paquin et al., 1999; McGeer et al., 2000) that are currently being used to successfully predict acute toxicity for many types of surface waters. At present, water quality parameters are rarely taken into account when generating chronic water quality guidelines.

2. Materials and methods

Freshly fertilized rainbow trout embryos were purchased from Rainbow Springs Trout Hatchery in March, 1999 and transported to McMaster University. Eggs were fertilized at 6 °C (at the hatchery) and warmed to 12 °C over a 3 h duration 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, 2.86 mg/l, alkalinity, 95 mg $CaCO_3$/l, hardness, 120 mg $CaCO_3$/l, pH, 7.5–8.0). Water temperature was maintained at 12.2±0.1 °C over the duration of the study and the photoperiod was 12 h light, 12 h dark.

3. Experimental protocol

Within 3 h following fertilization, embryos were randomly distributed to one of three flow-through silver exposure concentrations (nominal of 0, 0.1 and 10 μg/l total silver, as $AgNO_3$; Fisher Scientific) in Hamilton hard water, in the presence and absence of dissolved organic carbon (as Aldrich humic acid, product #53680) at a nominal concentration of 12 mg/l (measured at 2.86±0.18 in the control and reference treatments, and 12.14±1.45 mg/l in the unfiltered water of experimental treatments, respectively). Consequently, there were a total of six treatments. Measured silver levels in filtered (Acrodisc 0.45 μm polyethersulfone inline filters; Gelman) and unfiltered water from each treatment were measured every 4 days and are presented in Table 1. Measured DOC in filtered water is not reported because filtration contaminated the filtrate with DOC. Embryos were exposed to all treatments in duplicate, with 200 embryos in the first replicate (0.5 l chambers) and 700 embryos in the second replicate (2 l chambers), the latter of which was the source for embryos and larvae terminally sampled throughout the experiment. In two treatments (control, and 0.1 μg/l total silver without added DOC), fungal outbreaks in the second replicate occurred and

these treatments were terminated preventing samples from being taken for some measurements. 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, 40 l glass carboys in the presence or absence of DOC. Stock concentrations of both silver and DOC were 1000 times that of the desired final concentrations and were aerated continuously to ensure mixing of solutions. Because metal–DOC complexation is not instantaneous (potentially, it may require several hours to days; Ma et al., 1999), stock solutions of silver and DOC were permitted to equilibrate for at least 1 week prior to dilution to increase the degree of complexation that would be achieved in the final diluted exposure waters. Stock solutions were delivered by peristaltic pump to 150 ml header tanks at a flow rate of 0.1 ml/min, and mixed with dechlorinated Hamilton hard water (100 ml/min). Header tanks were vigorously aerated to facilitate mixing, and the overflows were introduced to the embryo exposure chambers, where each chamber had an independent flow-through source.

To increase accuracy of detection, 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 using radioisotope dilution (cf. Guadagnolo et al., 2000, 2001) as described below.

Mortalities, % hatch, % swim-up were calculated, and degree of yolk sac absorption was visually estimated, daily. Dead embryos or larvae were removed immediately from the exposure chambers and discarded. When heartbeat could be visually observed, cessation of beating was the criterion for mortality. Prior to that it was opaqueness of embryos. On days 16, 23, 30, 37, 44 and 51 days post-fertilization, up to 50 live embryos or larvae from each treatment were collected, euthanized in tricaine methane–sulfonate (MS-222; 0.1 g/l buffered with $NaHCO_3$) blotted dry, weighed, placed individually into 1.5 ml microcentrifuge tubes, and then frozen in liquid nitrogen. These samples were used for the determination of whole embryo/larval Na^+,K^+-ATPase activity levels, extractable protein and Na^+, Cl^- concentrations (days 16, 23, 30 and 44) as described below. An additional 20 embryos were removed at each sampling time for determination of whole embryo/larval unidirectional Na^+ uptake (J_{in} Na^+; days 23, 30, 37, 44 and 51) 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 mass (with yolk attached) in mg and 'l' refers to length in mm.

3.1. Water analysis

Water and stock solution flow rates were checked daily and water samples were collected from each exposure chamber weekly. Samples used for DOC determination were stored in borosilicate glass vials and kept at 4 °C for future analysis. Samples used for silver and ion determinations were immediately acidified with 0.5% (v/v) trace metal grade HNO_3 (Fisher Scientific) and stored in the dark for up to one week prior to analysis. Atomic absorption spectrophotometry (AAS; Varian AA 1275, Mississauga, ON) was used to measure water [Na^+], and an associated graphite furnace atomizer (Varian GTA-95) was used for silver analyses as described by Brauner and Wood (2002) with a detection limit of approximately 0.05 μg/l total silver.

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

Dissolved organic carbon in water was measured using a Beckmann model 915B total carbon analyzer, following acidification and sparging to remove inorganic carbon. Values are reported in mg C/L.

3.2. Unidirectional Na^+ uptake measurements

Determinations of unidirectional Na^+ uptake (J_{in} Na^+) in whole embryos and larvae were conducted in 60 ml Nalgene® bottles according to

the method of Brauner and Wood (2002). Briefly, twenty embryos or larvae were transferred to each chamber containing 20 ml (for embryos) or 40 ml (for larvae) of water to which the organisms had been exposed, and 7.2 μCi ^{22}Na was added to that chamber. Any particulate DOC covering the eggs was removed during this procedure. The medium was continuously mildly aerated over the duration of the flux measurement, and 2 ml water samples were taken in duplicate, 10 min following the start of the flux, and again at the end of the flux period, for measurement of water radioactivity and [Na^+]. Especially in embryos, there is a large degree of non-specific binding of ^{22}Na that is complete within the first 1 h of the flux exposure (Brauner and Wood, 2002). To account for this, the difference in ^{22}Na accumulation between 2 h and 6 h following initiation of the flux was used to calculate J_{in} Na^+, during which time Na^+ uptake is linear with time (Brauner and Wood, 2002). Non-specific ^{22}Na binding is less of a concern from day 24 post-fertilization and on, although the same procedure was followed for consistency. At time 2 and 6 h, 10 embryos or larvae were anaesthetized with a lethal dose of tricaine methanesulfonate (MS-222), rinsed three times with 5 mM NaCl to displace surface bound ^{22}Na, and then with deionized water, weighed and placed individually into scintillation vials for measurement of radioactivity using a gamma counter (Packard Instruments, Downers Grove, IL, USA).

3.3. Embryo/larval Na^+ and Cl^- concentration

Embryos or larvae frozen in 1.5 ml microcentrifuge tubes were thawed, 150 μl of 1.0 N HNO_3 was added, and each sample was placed in an oven at 60–70 °C for 24 h. The chorion or tissue was crushed and/or vigorously 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 AAS, and Cl^-, by the colorimetric mercuric thiocyanate method (Zall et al., 1956).

3.4. Na^+,K^+-ATPase activity

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

3.5. Statistics

Calculation of time to 50% hatch and swim-up were determined by log-probit analysis. Values are presented as mean ± 1 standard error throughout, unless otherwise indicated. Statistical differences of mean values among fish in treatments (Wood, 2001) on respective days were compared using a one-way analysis of variance (ANOVA) followed by a Dunnett's post-hoc test to identify specific groups that differed significantly. Differences in mortality were tested among treatments up to hatch, and from hatch to the end of the experiment using a one-way ANOVA on the number of mortalities per day (i.e. on non-cumulative mortality). The level of statistical significance for all analyses was $P < 0.05$.

4. Results

4.1. Mortality and time to hatch and swim-up

Chronic exposure to 10 μg/l total silver (nominal), but not to 0.1 μg/l total silver, greatly increased mortalities relative to controls in treatments run in the absence of added DOC. There was a statistically greater daily % mortality from fertilization to hatch (up to day 32) during exposure to 10 μg/l total silver in the absence of DOC relative to the presence of DOC (Fig. 1), indicating that DOC was protective of silver induced mortality up to this point. Following hatch, however, daily % mortality was not statistically different between these treatments. Interestingly, daily % mortality was greater in the presence than absence of DOC during exposure to 0.1 μg/l total silver from fertilization to hatch (up to day 32).

Time to 50% hatch in controls without DOC was 29 days. This was delayed by two days in the groups exposed to 10 μg/l total silver, regardless of whether added DOC was present (31 days for 10 μg/l in the presence and absence of additional DOC, data not shown). Time to 50% hatch was also delayed by 2–3 days in the presence of added DOC at both 0 (31 days) and 0.1 μg/l total silver

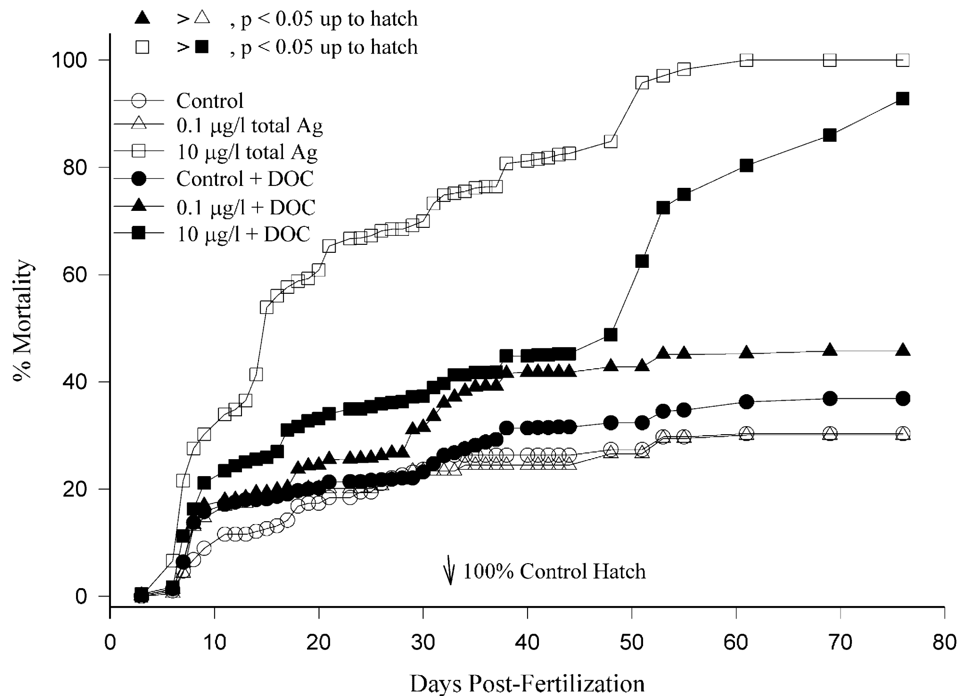

Fig. 1. Cumulative mortality (%) in rainbow trout embryos and larvae during continuous flow-through exposure to silver (added as $AgNO_3$) in the presence and absence of additional dissolved organic carbon (DOC) from fertilization to swim-up. Circles represent control conditions, open triangles represent 0.1 µg/l total silver and squares represent 10 µg/l total silver. Open symbols represent no additional DOC, while closed symbols represent an additional 12 mg/l DOC ($n=2$ except in control and 0.1 µg/l total silver chambers where $n=1$ due to fungal losses in one replicate). Up to hatch, % daily mortality was significantly greater in the 10 µg/l total silver treatment in the absence than presence of added DOC, indicating a protective effect of DOC. Percent daily mortality up to hatch was significantly greater in the presence of DOC than absence at 0.1 µg/l total silver. No other statistically significant differences were observed.

(32 days). Time to 50% hatch in 0.1 µg/l total silver without additional DOC was 30 days.

Time to 50% swim-up was 46 and 48 days in controls and 0.1 µg/l total silver without DOC, respectively (data not shown). This was delayed in the presence of added DOC by 7 and 6 days at 0 and 0.1 µg/l total silver (53 and 55 days respectively, data not shown).

4.2. Ionoregulation

Prior to hatch (days 23 and 30), there was a marked, significant reduction in J_{in} Na^+ in all embryos exposed to DOC (at 0, 0.1 and 10 µg/l total silver) and in embryos exposed to 10 µg/l total silver without DOC, relative to controls (Fig. 2). Therefore, added DOC at this concentration did not offer any protection against the inhibitory effect of silver on J_{in}, as DOC was inhibitory itself to Na^+ uptake. Embryos were not sampled from the 0.1 µg/l total silver treatment in the absence of DOC because of fungal related losses for that treatment.

Following hatch, J_{in} Na^+ was significantly reduced during exposure to 10 µg/l total silver (in the presence or absence of DOC), relative to all remaining treatments (control, control+DOC and 0.1 µg/l total silver+DOC; Fig. 2). Added DOC itself was again inhibitory, at least at day 37. On days 44 and 51, control J_{in} Na^+ had decreased so that there was no longer a significant difference with controls+DOC. There did not appear to be any significant protective effect of added DOC on J_{in} Na^+ during exposure to 10 µg/l total silver at any sampling time. By day 51, there was a significant reduction in J_{in} Na^+ in 0.1 µg/l total silver+DOC relative to the controls+DOC.

Whole embryo Na^+,K^+-ATPase levels were significantly elevated in the 10 µg/l total silver+DOC relative to the other DOC treatments (0 and 0.1 µg/l total silver) and relative to 10 µg/l total silver in the absence of DOC on day 23 only (Fig.

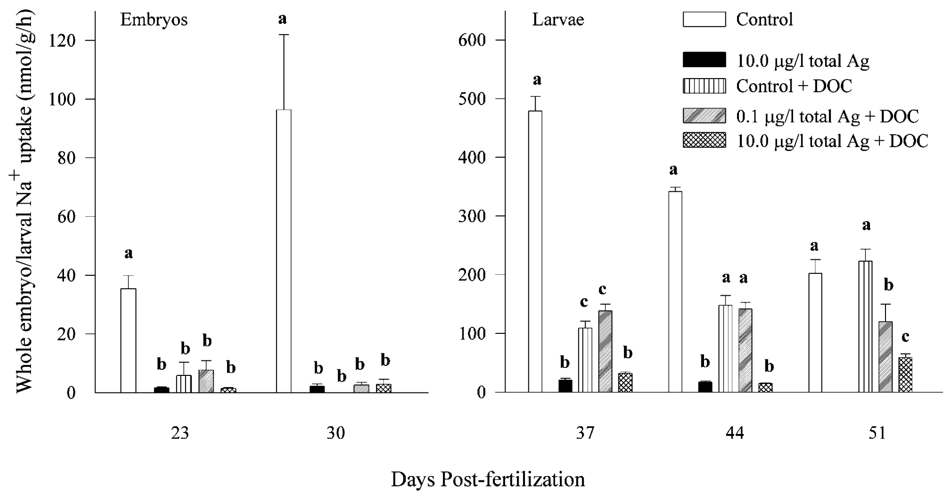

Fig. 2. Whole embryo/larval unidirectional Na^+ uptake in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water in the presence and absence of DOC, from fertilization to swim-up. No data are presented for the 0.1 μg/l total silver treatment due to fungal associated losses. Left panel data are pre-hatch, right panel data are post-hatch. Note the 5-fold expanded axis in the post-hatch group. Letters that differ within a given time period indicate statistically significant differences ($P<0.05$). $n=10$.

3). This was the only protective effect of DOC seen against the negative effects of silver on Na^+,K^+-ATPase activity. Larval Na^+,K^+-ATPase levels were qualitatively similar with the observed differences in J_{in} Na^+ on day 44. Na^+,K^+-ATPase activity was significantly reduced relative to DOC controls, in all remaining treatments (0.1 μg/l total silver+DOC, 10 μg/l total silver±DOC; day 44 only). Furthermore, there was a significant reduction in Na^+,K^+-ATPase levels in the presence of DOC in the 10 μg/l relative to the 0.1 μg/l total silver treatments. Due to a fungal outbreak, no data are presented for control or 0.1 μg/l total silver in the absence of DOC. In general, changes in Na^+,K^+-ATPase levels were qualitatively similar whether expressed relative to protein concentration or absolute levels (per individual; data not shown).

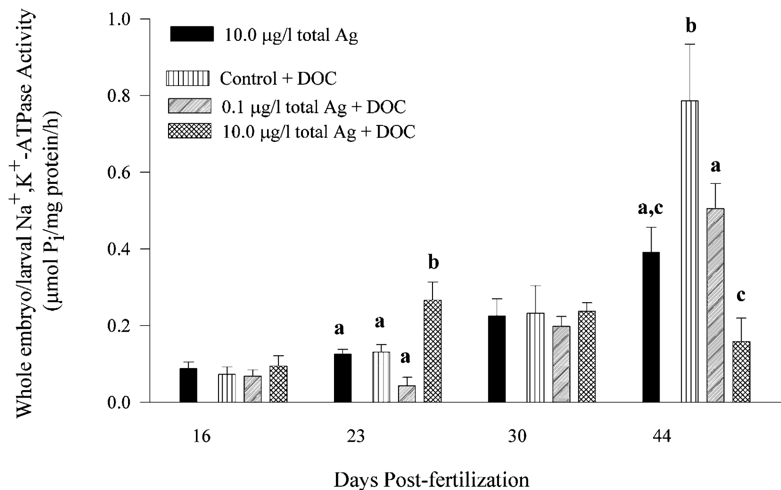

Fig. 3. Whole embryo (days 16, 23 and 30) and larval (day 44) Na^+,K^+-ATPase activity levels (standardized for protein concentration) in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water in the presence and absence of added DOC, from fertilization to swim-up. No data are presented for the 0 and 0.1 μg/l total silver treatment due to fungal associated losses. Letters that differ within a given time period indicate statistically significant differences ($P<0.05$). $n=10$.

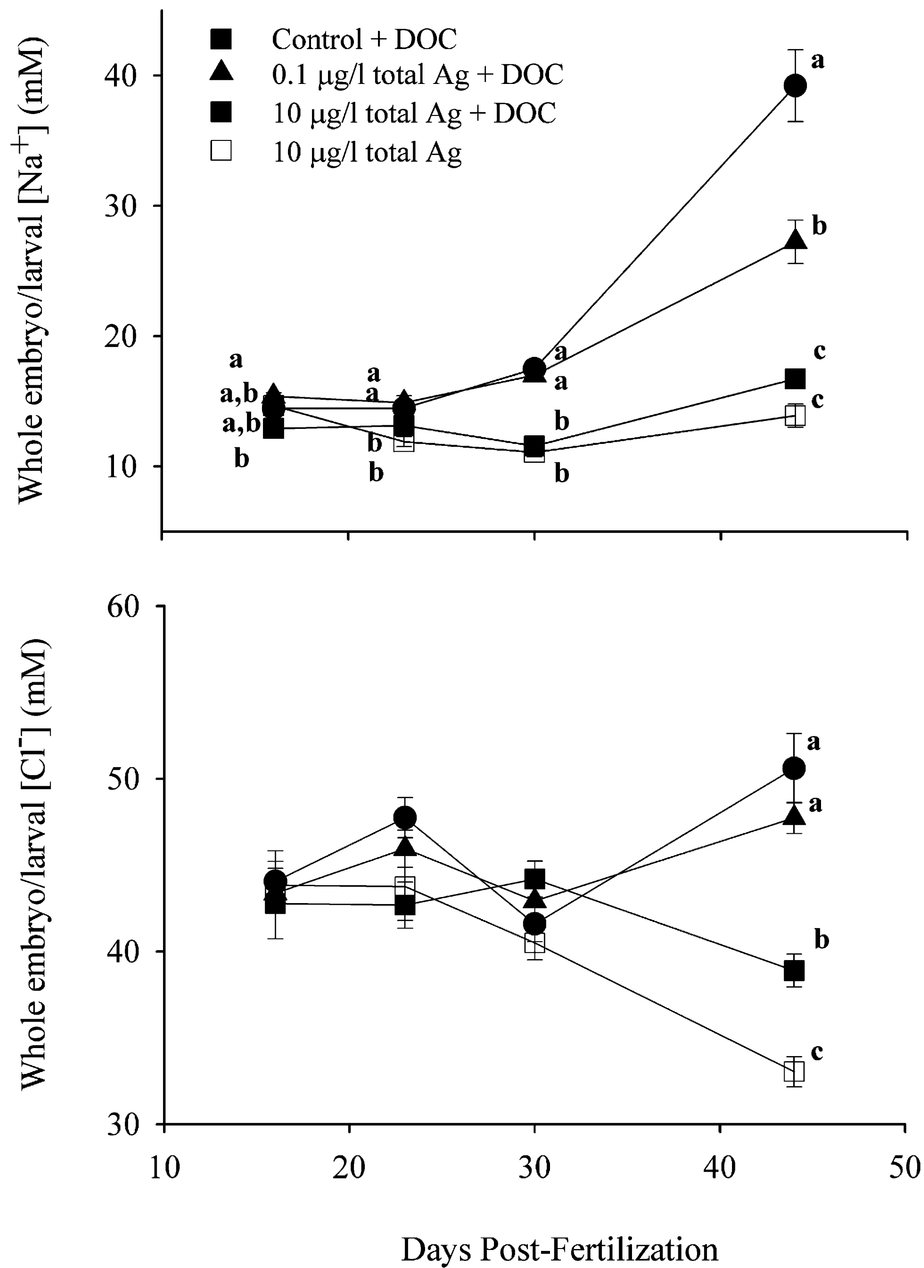

Fig. 4. Whole embryo (days 16, 23 and 30) and larval (day 44) Na^+ and Cl^- concentration in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water in the presence and absence of added DOC, from fertilization to swim-up. Letters that differ within a given time period indicate statistically significant differences ($P<0.05$). $n=10$.

Whole embryo and larval $[Na^+]$ was significantly reduced during exposure to 10 μg/l total silver in the presence and absence of DOC, relative to the control and 0.1 μg/l total silver DOC treatments from day 23 and on (Fig. 4). By day 44, there was a statistically significant reduction in larval $[Na^+]$ in the 0.1 μg/l total silver+DOC relative to the control+DOC treatments. Again, no data are presented for control or 0.1 μg/l total silver in the absence of DOC due to fungal related mortality. Changes in whole embryo and larvae $[Cl^-]$ were much less pronounced, and only on day 44 was a significant reduction observed in the 10 μg/l total silver treatments (in the presence

Table 2
Length, weight and condition factor for rainbow trout larvae on days 44 and 51 post-fertilization following chronic exposure to silver (as $AgNO_3$) in the presence and absence of additional DOC

Treatment	Day 44			Day 51			
	Length (mm)	Weight (mg)	CF	Length (mm)	Weight (mg)	CF	% Yolk sac abs.
Control	20.0 (0.3)	89.3 (2.4)	1.13 (0.04)	25.9 (0.2)	121.2 (2.0)	0.70 (0.02)	100
0.1 μg/l total silver	–	–	–	25.4[a] (0.2)	119.2[a] (3.0)	0.73 (0.02)	86.5 (4.7)
10 μg/l total silver	14.9[a] (02)	72.8[a] (1.6)	2.25[a] (0.11)	–	–	–	–
Control + DOC	13.9[a,b] (0.3)	65.1[a,b] (2.4)	2.49[a,b] (0.11)	22.7[a,b] (0.4)	103.4[a] (3.5)	0.89[a,b] (0.03)	64.0[a,b] (4.9)
0.1 μg/l total silver + DOC	13.9[a] (0.25)	67.6[a] (1.4)	2.62[a] (0.14)	24.0[a,b] (0.5)	113.1[b] (3.2)	0.83 (0.03)	66.0[a] (5.5)
10 μg/l total silver + DOC	13.8[a] (0.3)	67.3[a] (1.6)	2.61[a] (0.12)	17.4[a] (0.2)	82.8[a] (2.5)	1.57[a] (0.05)	0[a]

Length is reported in mm, weight in mg (consisting of larva and yolk), CF refers to condition factor (and includes yolk). Percent yolk sac absorption (% Yolk sac abs) is also reported for day 51. [a] refers to significant difference from control, and [b] refers to significant effect of DOC at a constant silver concentration.

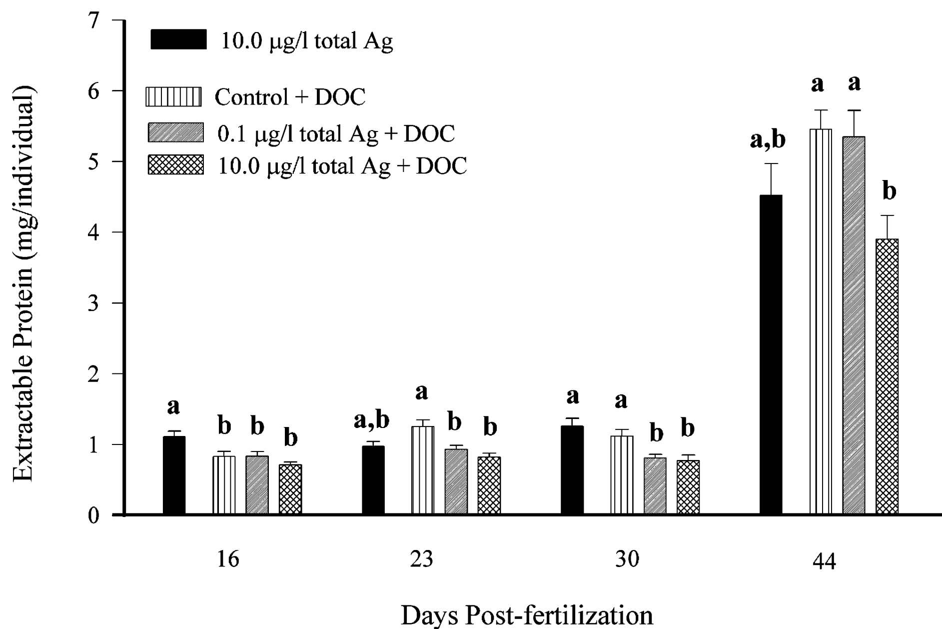

Fig. 5. Whole embryo (days 16, 23 and 30) and larval (day 44) extractable protein in rainbow trout during chronic exposure to silver (as $AgNO_3$) in dechlorinated tap water in the presence and absence of added DOC. Letters that differ within a given time period indicate statistically significant differences ($P<0.05$). $n=10$.

and absence of DOC) relative to the control and 0.1 μg/l total silver DOC treatments. On day 44, larval [Cl^-] was significantly reduced at 10 μg/l total silver in the absence relative to presence of DOC.

4.3. Growth

On day 44, we noticed significantly reduced length and weight, and increased condition factor in larvae in all experimental treatments (except

0.1 μg/l total silver which was not measured at this time so as to conserve numbers for later sampling) relative to controls (without DOC; Table 2). On day 51, length remained significantly lower in all treatments relative to controls (without DOC) and the same was true for weight, with the exception of 0.1 μg/l total silver + DOC (which was not significantly different). Those exposed to 10 μg/l total silver did not survive to this stage. Condition factor was significantly greater, and in accord with this result, percent yolk sac absorption was significantly lower, in all DOC treatments relative to controls at this time.

Extractable protein content was significantly lower in all DOC treatments (0, 0.1 and 10 μg/l total silver) relative to the 10 μg/l total silver (without DOC) on day 16 (Fig. 5). On days 23, 30 and 44, extractable protein was lower in the larvae exposed to 0.1 and 10 μg/l total silver treatments + DOC, relative to control + DOC treatments (with the exception of day 44 when the 0.1 μg/l total silver + DOC did not differ significantly from control + DOC).

5. Discussion

Complexation of metals with DOC is not instantaneous and can take hours to days to approach completion (Ma et al., 1999). In this study, silver and DOC were maintained together in the stock solutions for several weeks to permit complexation, but then diluted 1000-fold in header tanks supplying the experimental chambers. The time between dilution of the respective stock solutions to achieve the final experimental water, and exposure of embryos and larvae to the water was short (< 1 min) and the degree of silver–DOC complexation in this diluted water is not known. Clearly there was protection of added DOC in the 10 μg l^{-1} total silver treatment against induced mortality up to hatch, and against Na^+,K^+-ATPase inhibition on day 23 indicating that at least moderate complexation between silver–DOC had been achieved. A confounding factor in this study was that the added DOC (humic acid) may not have been completely 'dissolved' as evidenced by the reduction in water silver concentrations in the DOC treatments in filtered relative to unfiltered water (Table 1). Because developing embryos and pre-swim-up larvae remain on the bottom of the chambers, undissolved DOC was problematic because it resulted in a light dusting of DOC, likely responsible for some of the direct impairment of DOC on growth and ionoregulatory development. Whether this DOC induced impairment is associated with smothering affecting oxygen transfer or a direct effect of DOC remains to be tested.

5.1. Ionoregulation

Unidirectional Na^+ uptake in salmonids remains low throughout embryonic development until eye pigmentation is readily visible (Rudy and Potts, 1969; Eddy and Talbot, 1985), which in rainbow trout reared at 12–13 °C occurs at approximately 23–24 days post-fertilization (Brauner and Wood, 2002). At this time, J_{in} Na^+ was significantly greater in the control treatment (in the absence of DOC) relative to all other treatments measured, up until day 37. These data indicate that there was a direct impairment of J_{in} Na^+ due to the presence of DOC (at 0, 0.1 and 10 μg/l total silver) as well as silver at 10 μg/l in the absence of DOC.

Just prior to hatch, whole body Na^+ levels were significantly reduced by 35% during chronic exposure to 10 μg/l total silver, in the presence and absence of added DOC, relative to the other DOC treatments (control and 0.1 μg/l total silver). A reduction in whole embryo Na^+ during silver exposure was not observed early in development (i.e. day 15) as has been demonstrated previously (Guadagnolo et al., 2001), however, this is expected because J_{in} Na^+ is so low at this stage and the yolk acts as a major store for both Na^+ and Cl^-. Thus, whole body measurements of Na^+ and Cl^- at this stage would not permit detection of an ionoregulatory disturbance.

The large reduction in whole body Na^+ just prior to hatch has also been observed by Guadagnolo et al. (2001) and indicates that the mechanism of toxicity is likely ionoregulatory, however, there were no significant differences in J_{in} Na^+ or Na^+,K^+-ATPase activities among these treatments. Thus, the large loss of whole body Na^+ may be associated with an increased Na^+ efflux (J_{out}), although this will require further study. During acute exposure to silver in adult rainbow trout, however, J_{out} does not increase (Webb and Wood, 1998). It should be noted that whole body Na^+,K^+-ATPase activity and not gill activity level was measured in this study. Because it has been demonstrated that the primary mechanism of silver toxicity in juvenile and adult rainbow trout is impairment of gill Na^+,K^+-ATPase activity

(Wood et al., 1996), measurement of whole body levels may underestimate the magnitude of ionoregulatory impairment.

The presence of DOC at 10 μg/l total silver was protective against mortality up to hatch, however, the mechanism is not clear because whole body Na^+, J_{in} Na^+ and Na^+,K^+-ATPase activities did not differ at 10 μg/l total silver in the presence or absence of DOC with the exception of day 23 when there was greater Na^+,K^+-ATPase activity at 10 μg/l total silver in the presence of added DOC compared with the absence of added DOC.

Following hatch, greater changes in ionoregulatory variables were observed among treatments. J_{in} Na^+ remained low at 10 μg/l total silver in the presence or absence of DOC, relative to all other treatments, and Na^+,K^+ ATPase and whole body Na^+ and Cl^- were significantly reduced relative to the control and 0.1 μg/l total silver treatments in the presence of DOC. These data indicate that the mechanism of silver induced mortality is likely ionoregulatory in origin, as has previously been concluded during chronic silver exposure in early life stages of rainbow trout (Brauner and Wood 2002; Guadagnolo et al., 2001). With the exception of whole body Cl^-, however, none of these parameters differed during exposure to 10 μg/l total silver in the presence or absence of DOC. DOC likely protects through a reduction in silver induced ionoregulatory impairment, however, at this silver concentration, it offers only partial protection, extending the duration over which mortality would otherwise be observed. None of the larvae exposed to 10 μg/l total silver in the presence or absence of DOC reached swim-up or were capable of any yolk–sac absorption over the duration of this experiment. This differs from acute silver toxicity studies with DOC where a pronounced reduction in toxicity is consistently observed (Bury et al., 1999a,b; Ma et al., 1999). Whether the difference in protection conferred by DOC in acute and chronic studies is simply related to the duration of exposure or is associated with the procedures used in this study remains to be determined.

5.2. Growth

Following 51 days exposure to 0.1 μg/l total silver (in the absence of DOC), a small but statistically significant reduction in larval length and weight was observed relative to controls. This is consistent with the data of Nebeker et al. (1983) where a reduction in growth rate was also observed at 0.1 μg/l total silver following 60 days of continuous exposure.

While exposure to DOC in controls appeared to slow ionoregulatory development in general, there was also a reduction in growth rate as indicated by significant reductions in length, weight and extractable protein, and a significant increase in condition factor on days 44 and 51. While the basis for the general impairment of growth and development is unknown, it may be a direct effect of the particulate DOC that resulted in a light dusting of embryos and larvae, although larvae would be less affected as they become increasingly mobile. The observation that there is a rather large, direct effect of DOC on growth and ionoregulatory development complicates interpretation of the magnitude of any protective effects of DOC on silver toxicity. There appeared to be little if any protection of DOC at this concentration on larval length, weight and extractable protein during exposure to silver (0.1 or 10 μg/l total silver) in the present study.

5.3. Regulatory implications

Chronic exposure of rainbow trout early life stages to silver leads to sublethal impairment of Na^+,K^+-ATPase activity and an elevation in whole body cortisol and ammonia (Brauner and Wood, 2002). Consequently, it was concluded that the mechanism(s) of chronic toxicity are similar (at least in part) to that of acute toxicity (Brauner and Wood, 2002). Assuming that the mechanism(s) are similar, it may be possible to extend Biotic Ligand Models that predict acute silver toxicity as a function of water quality (Paquin et al., 1999; McGeer et al., 2000), to one that predicts chronic toxicity. The data presented in this study further confirm that long-term silver exposure is associated with an ionoregulatory disturbance, however, the anticipated protective effects of DOC on silver toxicity appear to be much less during chronic than acute silver exposure (Bury et al., 1999a; McGeer et al., 2000), at least in the present study. Furthermore, the mechanism through which silver induced mortality is reduced by DOC (at 10 μg/l total silver) is unclear. Whether DOC offers only limited protection during chronic silver exposure or whether the means through which DOC was introduced to the embryos/larvae in this study

was problematic (i.e. pre-incubated with silver and then diluted 1000-fold just prior to exposure) requires further attention.

During acute exposure to silver, it is well documented that water Cl^- offers substantial protection against silver toxicity, while Ca^{2+} offers only very modest protection (LeBlanc et al., 1984; Bury et al., 1999a,b; McGeer and Wood, 1998; McGeer et al., 2000). Studies investigating the possible protective effect of these water quality variables during chronic silver exposure are presently underway to incorporate into a chronic silver toxicity model.

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