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Protection by two complexing agents, thiosulphate and dissolved organic matter, against the physiological effects of silver nitrate to rainbow trout (*Oncorhynchus mykiss*) in ion-poor water

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Abstract

Adult rainbow trout (*Oncorhynchus mykiss*) were exposed in ion-poor water (~ 50 μM Ca) to silver added as $AgNO_3$ or to $AgNO_3$ plus either thiosulphate ($Na_2S_2O_3$) or dissolved organic matter (DOM). The effects of these exposures were assessed through repetitive blood sampling over 4 days. Trout exposed to 0.1 μM $AgNO_3$ alone accumulated large amounts of Ag on their gills and in their plasma, showed progressive losses of plasma Na and Cl, and had elevated concentrations of plasma glucose. In one set of exposures trout exposed to $AgNO_3$ alone also had increased cough rates, slightly higher ventilation rates, somewhat lower arterial oxygen tensions, and increased blood lactate concentrations. In contrast, trout exposed to 0.1 μM $AgNO_3$ plus 5 μM thiosulphate or 35 mg C l^{-1} DOM accumulated less Ag on their gills and in their plasma, and showed no adverse ionoregulatory or respiratory effects due to Ag. These results demonstrate ionoregulatory and sometimes respiratory effects in fish exposed to ionic Ag^+ in ion-poor water, depending on water chemistry, and demonstrate the protective effects of synthetic and natural complexing agents through a reduction in the amount of ionic Ag^+ available to bind at the gills.

Keywords: Silver; Thiosulphate; Dissolved organic matter; Rainbow trout; Gills; Physiology

1. Introduction

Silver is released by human activity into the atmosphere through smelting and coal burning, and into the aquatic environment through mining, industrial discharges, and through sewage (see reviews by Eisler, 1996; Purcell and Peters, 1998; Ratte, 1999). Silver is an expensive metal so there is a worldwide trend to reclaim silver from wastes, particularly photographic wastes, but photographic manufacturing and film developing still contribute to the total potential release of silver into the environment. For example, photographic manufacturing accounts for about 50% of the

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industrial demand for silver in the US, and about 30% of silver waste to water and land is from the photographic industry (Purcell and Peters, 1998).

Silver from photoprocessing usually enters wastewater treatment plants in the form of silver-thiosulphate complexes, because thiosulphate is used as a fixer in film developing (Purcell and Peters, 1998). These and other forms of complexed silver, such as silver complexed by dissolved organic matter (DOM) and by chloride anions, are more commonly found in water than is uncomplexed ionic silver (Ag^+), and complexed silver species are less toxic to fish than is ionic Ag^+ (Nebeker et al., 1983; LeBlanc et al., 1984; Janes and Playle, 1995; Hogstrand et al., 1996; Wood et al., 1996a,b; Galvez and Wood, 1997; Erickson et al., 1998; Hogstrand and Wood, 1998; McGeer and Wood, 1998; Bury et al., 1999a,b,c; Karen et al., 1999; Wood et al., 1999).

Ionic Ag^+ interferes with sodium and chloride transport across the gills of freshwater fish (Wood et al., 1996a; Morgan et al., 1997; Galvez et al., 1998; Hogstrand and Wood, 1998; Webb and Wood, 1998), through the inhibition of basolateral Na^+, K^+-ATPase responsible for providing the energy to transport sodium into the fish (Morgan et al., 1997; Hogstrand and Wood, 1998; Bury et al., 1999a,b; Wood et al., 1999). The mechanism of acute silver toxicity to fish is similar to that of toxicity associated with low environmental pH, which is ion loss followed by decreased plasma volume, increased blood viscosity, and eventual cardiac failure (Wood et al., 1996a; Hogstrand and Wood, 1998; Webb and Wood, 1998; Wood et al., 1999). These ionoregulatory disruptions may be modified by water quality factors such as 'hardness' and alkalinity (Davies et al., 1978; Diamond et al., 1990; Erickson et al., 1998), acidity, salinity, and organic and inorganic complexing agents, conceptually through competition (by Ca^{2+}, Na^+, H^+) and complexation (by Cl^-, DOM, thiosulphate; Janes and Playle, 1995; Hogstrand et al., 1996; Galvez and Wood, 1997; Erickson et al., 1998; see also Playle, 1998; Wood et al., 1999).

With the goal of predicting metal toxicity, we can mathematically calculate the protective effect of complexing agents against binding of Ag^+ and other metal cations to the freshwater fish gill (Janes and Playle, 1995; Playle, 1998; Meyer et al., 1999), but a more mechanistic understanding of the protective effects of complexing agents is still needed. A previous study on the toxicity of $AgNO_3$ showed ionoregulatory disturbances in fish exposed to 0.1 μM $AgNO_3$ in moderately hard freshwater, as indicated by decreased plasma concentrations of Na^+ and Cl^-, and the fish showed increased concentrations of plasma glucose (Wood et al., 1996a). However, there was no evidence of respiratory toxicity of $AgNO_3$ alone (e.g. there were no increases in blood lactate concentrations), and in fact arterial oxygen tensions increased and arterial carbon dioxide tensions decreased in fish exposed to $AgNO_3$ as the fish hyperventilated to compensate a developing metabolic acidosis (Wood et al., 1996a). Very high concentrations of Ag complexed by thiosulphate (280 μM) had only minor effects on the fish, even though Ag accumulated in the plasma of the fish (Wood et al., 1996b). The physiological effects of $AgNO_3$ on fish in softer, ion-poor water have not yet been documented, but the toxicological effects of $AgNO_3$ would be expected to be greater than in moderately hard freshwater because of little competition by cations (Ca^{2+}, Na^+) and less complexation of Ag^+ by Cl^-.

The main purpose of the present study was to determine and compare the protective effects of the complexing agents thiosulphate and DOM against the physiological effects of ionic Ag^+ in ion-poor water, where the toxic effects of $AgNO_3$ to fish were expected to be large. We used concentrations of $AgNO_3$ (0.1 μM), thiosulphate (5 μM), and commercial DOM (35 mg C l^{-1}) similar to those used by us in work with small rainbow trout, where 2.5 μM thiosulphate was able to keep most Ag off trout gills when the fish were exposed to 0.06 μM $AgNO_3$ in ion-poor water for 6 days, and where 24 mg C l^{-1} DOM was needed to keep most of 0.17 μM $AgNO_3$ off trout gills for 2 to 3 h (Janes and Playle, 1995). The $AgNO_3$ plus DOM exposures were also intended to complement recent work which has documented the protective effects of natural and commercial DOM against the physiological and toxicological effects of a combined copper and cadmium exposure to

trout held in ion-poor water (Richards et al., 1999).

2. Materials and methods

2.1. Fish holding

Adult rainbow trout (*Oncorhynchus mykiss*, 200–300 g) were purchased from Rainbow Springs Hatchery, Thamesford, Ontario, and were held in aerated well water for at least 2 weeks. The composition of the well water was approximately 700 μM Na, 3300 μM Ca, pH 8.4, at 10°C. Fish were fed Purina Trout Chow until needed for an experiment. Fish were acclimated to ion-poor ('soft') water for at least 5 days before surgery (below). The ion-poor water was produced by a Culligan Series E reverse osmosis system, and flowed through the 90-l acclimation containers at a rate of about 100 ml min^{-1}. Water samples were taken periodically to determine the water composition of these containers, and was similar to the water composition used in the experiments outlined below.

2.2. General experimental procedures

Trout were anaesthetized for surgery with MS-222 (Sigma). During surgery each fish had a dorsal aortic cannula inserted to allow repetitive blood sampling (Soivio et al., 1972; PE-50 tubing, O.D. = 0.038 in., Intramedic). Once fish had revived in anaesthetic-free water they were transferred to opaque, 2.5-l fish boxes with aerated, cooled soft water flowing through at a rate of 50 ml min^{-1} box^{-1}. Fish were allowed to recover from surgery for 2 days. The day following cannulation all cannulae were infused with a small amount of heparinized saline (8 mg heparin Na salt (Sigma) to 25 ml Cortland saline; Wolf, 1963) to ensure that blood could be withdrawn from all fish.

During the 4-day exposures, water samples from near the mouths of the fish were collected with a 50-cc plastic syringe, emptied into scintillation vials, and acidified with one drop of 16 N HNO_3 (Ultrex II Ultrapure HNO_3 Baker analyzed reagent) for later analysis. Water pH was measured with a Radiometer PHM82 pH Meter (Bach-Simpson Limited, London, ON) with a Radiometer GK2401C combination electrode. Water PO_2 and PCO_2 were measured with a Blood/Gas Meter (Cameron Instrument Company, Port Aransas, TX) calibrated using analyzed gases (Praxair, Brampton, ON).

At each sampling time, 1 ml of blood was withdrawn from the fish through the dorsal aortic cannula with an ice cold, 1 ml luerlock Hamilton syringe (Sigma) rinsed with heparinized saline. Fish were immediately infused with 750 μl Cortland saline and 250 μl heparinized saline to replace the removed blood volume. Twenty microliters of whole blood was dispensed into 5 ml Drabkins solution (Sigma), shaken, and stored in the dark for later hemoglobin analysis. Blood (~ 50 μl) was dispensed into two hematocrit tubes (lined with ammonium heparin) and spun for 5 min in a READACRIT centrifuge (Clay Adams, NJ). Hematocrit was determined by dividing the packed cell length by total length, expressed as a percent. All but 350 μl of the remaining blood was dispensed into a microcentrifuge tube and spun in an IECMicro-MB centrifuge (VWR Scientific, Canada) for 2 min. Plasma was removed from this tube, placed in a fresh microcentrifuge tube, and stored frozen at -40°C.

The remaining 350 μl of blood was passed through the blood/gas analyzer to measure arterial PO_2 and PCO_2. After gas measurement the blood was withdrawn from the analyzer and 100 μl was used for lactate analysis and dispensed into a microcentrifuge tube containing 200 μl of 8% perchloric acid. These tubes were refrigerated for at least 5 min then spun for 5 min and refrigerated until assayed.

At the end of the exposures fish were stunned with a blow to the head and a portion of gill tissue was removed using stainless steel forceps. Extracted gills were rinsed in 100 ml of soft water for 10 s and placed into a microcentrifuge tube and labelled. Gills were weighed and 1 N Ultrapure HNO_3 was added to each tube at five times the gill mass, which averaged 0.12 ± 0.01 g wet weight (± 1 S.E.M., $n = 19$). Gills were digested for 3 h at 80°C, and gill digests were further

diluted using 100 μl of digest added to 900 μl of ultrapure water in a fresh microcentrifuge tube, labelled, and refrigerated until analyzed.

Thawed plasma was diluted ten times with 1 N Ultrapure HNO_3 for Ag analysis. Analysis of plasma, gill, and water Ag concentrations was done using a graphite furnace atomic absorption spectrophotometer (Varian AA-600 with GTA-100 atomizer). Ten microliter samples were injected into the furnace, using argon gas, under the following operating conditions: 5 s at 85°C, 20 s at 95°C, 10 s at 120°C, 8 s at 400°C, and 4.9 s at 2000°C, during which Ag concentrations were read at 328.1 nm against standards prepared from Fisher certified standards.

Plasma glucose, blood lactate, whole blood hemoglobin, and plasma and water Cl were assayed using Sigma reagents and protocols (kits 16-20, 826-A, 525-A, and 461-M, respectively). Standards and samples were read using a SPECTRONIC 301 Spectrophotometer (Milton Roy Company, Rochester, NY) at the appropriate wavelengths. Twenty microliters of 10% $LaCl_3$ (VWR Scientific, West Chester, PA) was added to both water and plasma Ca samples before analysis by flame AAS to reduce Na interference; plasma and water Na and Ca samples were analyzed by flame atomic absorption spectrophotometer (Perkin Elmer 3100).

2.3. $AgNO_3$, $AgNO_3$ plus thiosulphate, and control exposures

Adult rainbow trout ($AgNO_3$ group: 211 ± 9 g (± 1 S.E.M.), $n = 9$; $AgNO_3$ plus thiosulphate group: 189 ± 8 g, $n = 11$) were exposed at the same time during two repeated experiments to a nominal Ag concentration of 0.15 μM (as $AgNO_3$; Sigma) or to nominal concentrations of 0.15 μM Ag and 5.0 μM thiosulphate (as $AgNO_3$ and $Na_2S_2O_3 \cdot H_2O$; Sigma). These concentrations (33 times more thiosulphate than Ag) were chosen from our previous work with small rainbow trout, where 28 times more thiosulphate than $AgNO_3$ was needed to keep Ag off trout gills in short, 2–3-h exposures, and where 42 times more thiosulphate than $AgNO_3$ kept most Ag off trout gills for 6 days (Janes and Playle, 1995). The $AgNO_3$ and thiosulphate solutions were delivered to the soft water flow lines by peristaltic pumps. Total Ag concentrations were measured (above) but thiosulphate concentrations were not.

These exposures lasted 4 days, with sampling occurring on the first day (initial samples before $AgNO_3$ was added) and at 21, 32 (plasma only, see below), 46, 68, and 94 h after the exposures started. Ventilation and cough rates were determined visually over 30 s. To reduce the total amount of blood removed from the fish, plasma samples only were taken at 33 h, when only 500 μl of blood was withdrawn: removed blood was replaced with 250 μl Cortland saline and 250 μl heparinized saline. Blood was dispensed into a microcentrifuge tube and spun to obtain plasma, as described earlier.

Control experiments with no added $AgNO_3$ or thiosulphate were run separately, when the reverse osmosis system was operating with less efficiency so that different water chemistry resulted (Section 3). These fish had a mean mass of 202 ± 32 g ($n = 8$) and were sampled as described above. For all these exposures, if a fish towards the end of an exposure lost its cannula and survived, all data were collected at subsequent sampling times except for blood data. At the conclusion of the exposure, blood was removed by caudal puncture from these fish to obtain blood for lactate analysis and plasma Ag, glucose, and ion concentrations (see results). Cannulated trout for which we only got one or two blood samples at the beginning of the exposures were excluded from the study, along with their associated water chemistry and breathing data.

2.4. $AgNO_3$ and $AgNO_3$ plus DOM exposures

Adult rainbow trout ($AgNO_3$ group: 263 ± 18 g, $n = 6$; $AgNO_3$ plus DOM group: 270 ± 16 g, $n = 6$) were exposed to a nominal concentration of 0.15 μM $AgNO_3$ (Sigma) or to nominal concentrations of 0.15 μM $AgNO_3$ plus 35 mg C l^{-1} DOM, added as Aldrich humic acid (Na salt). These concentrations were chosen from Janes and Playle (1995) and from preliminary experiments (Rose-Janes et al., 1997), in which greater than 24 mg C l^{-1} DOM was needed to keep ~ 0.1 μM

$AgNO_3$ off trout gills over 4 days. Peristaltic pumps delivered the $AgNO_3$ and DOM stock solutions to the ion-poor water. Dissolved organic matter samples stored in 5-ml quartz tubes were measured on a Shimadzu 5050A total organic carbon analyzer against total carbon and inorganic carbon standards from Nacalai Tesque Inc. (Kyoto, Japan).

These exposures also lasted four days, with sampling on the first day before $AgNO_3$ was added (initial samples) and at 17, 26, 42, 66, and 88 h after the exposures started. Ventilation and cough rates were not taken during these exposures, because the high DOM fish were not visible in their fish boxes due to the dark brown nature of the DOM.

2.5. Thiosulphate and DOM exposures alone

In our experiments outlined above, fish were exposed to $AgNO_3$ alone and to $AgNO_3$ plus a complexing agent at the same time, and therefore can be directly compared. Logistics made it impossible to run exposures to thiosulphate alone or to DOM alone at the same time as the $AgNO_3$ exposures, so these experiments were run separately close to the times the $AgNO_3$ experiments were run. Water chemistry, exposure conditions, and sampling protocols were generally similar to those given above (Section 3), and are described fully in Rose-Janes (1997), Richards et al. (1999), and in Richards and Playle (1999).

2.6. Computer modelling

Concentrations of ionic Ag^+ in the exposures were calculated using the aquatic chemistry program MINEQL$^+$ (version 4.0; Schecher and McAvoy, 1992) with the addition of the Ag-DOM conditional equilibrium binding constant (K) and number of binding sites per mg C previously determined by us (Janes and Playle, 1995). These values are log $K_{Ag\text{-}DOM} = 9.0$ with 35 nmol binding sites per mg C (e.g. 0.5 mg C l^{-1} DOM = 17.5 nmol l^{-1} binding sites). The system was modelled as open to the atmosphere at an exposure temperature, measured concentrations of ions and total Ag for each exposure were used, and ionic strength corrections were calculated by the computer program. The log K value for Ag to thiosulphate is 8.8, with either 0 or 5 μM thiosulphate entered into the program, and the log K value for Ag to Cl is 3.3.

Calculated gill Ag concentrations were obtained using the Ag-gill binding model of Janes and Playle (1995). This modelling approach considers the gill as a ligand with its own conditional equilibrium binding constant (K) and number of binding sites. When the gill as a ligand is incorporated into an aquatic chemistry program such as MINEQL$^+$, the amount of a metal expected to bind to the gills is calculated, taking into account metal concentration, cation competition for metal binding sites on the gills, and metal complexation in the water column by natural (e.g. DOM, Cl^-) and synthetic agents (e.g. thiosulphate; Playle, 1998).

The important parameters for the Ag-gill model are: log $K_{Ag\text{-}gill} = 10.0$, log $K_{Ca\text{-}gill} = 3.3$, log $K_{Na\text{-}gill} = 4.7$, log $K_{H\text{-}gill} = 5.9$, log $K_{Ag\text{-}DOM} = 9.0$, log $K_{H\text{-}DOM} = 4.0$, log $K_{Ag\text{-}thiosulphate} = 8.8$, and log $K_{Ag\text{-}Cl} = 3.3$ (Janes and Playle, 1995). The number of Ag binding sites on the gills of the trout in their fish boxes was estimated from the maximum accumulation of Ag on the gills of about 15 nmol Ag/g wet tissue (see Table 2 column 2), approximately 3 g gill per fish, with each fish in a 2.5-l fish box (Richards et al., 1999). This number of Ag-gill sites (18 nmol l^{-1}) was entered into the program and the number of these sites filled by Ag was calculated using measured water chemistry, then was converted back to nmol Ag/g wet tissue for inclusion in Tables 2 and 4.

2.7. Statistical analysis

For all data in the figures and tables, error bars represent one standard error about the mean (S.E.M.). Statistical differences between treatments at a given time were determined by one-way analysis of variance (ANOVA) for the $AgNO_3$-thiosulphate exposures, and by Student's t-test for the $AgNO_3$-DOM exposures, and are indicated by asterisks. Crosses indicate significant differences within a treatment compared to initial values, as determined by ANOVA followed by the

Student–Newman–Keuls method of pairwise multiple comparisons (Sigmastat, version 1.02). When the normality test failed in the ANOVA analysis the non-parametric Kruskal–Wallis One Way ANOVA on Ranks was used, followed by the Dunnet's method of pairwise multiple comparisons. Differences in the data are given at the $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels of significance (one, two, and three symbols, respectively).

3. Results

3.1. $AgNO_3$, $AgNO_3$ plus thiosulphate, and control exposures

3.1.1. Water chemistry

Water Ag concentrations were 0.12 μM during the $AgNO_3$ alone and the $AgNO_3$ plus thiosulphate exposures (Table 1). Besides the addition of thiosulphate, water chemistry was nearly identical between the two $AgNO_3$ exposures, with only slight but significant differences in water PCO_2 and water temperature (Table 1). In the control experiments Ag concentrations were very low, essentially at our detection limit for Ag (Table 1). The control experiments were run separately from the $AgNO_3$ exposures, and water Cl and Na concentrations, plus water pH and PO_2, were significantly higher than in both $AgNO_3$ exposures ($P < 0.001$). Water PCO_2 was also higher in the control experiment compared to the $AgNO_3$ alone experiment ($P < 0.01$), and water temperature was lower than in the $AgNO_3$ plus thiosulphate exposure ($P < 0.001$).

Concentrations of ionic Ag^+ in the exposures were calculated using the concentrations of ions and total Ag for each exposure (Table 1) and using a background DOM concentration of 0.5 mg C l^{-1} (from Table 3; = 17.5 nmol l^{-1} binding sites). There was the same amount of total Ag in both $AgNO_3$ exposures, but calculated Ag^+ in solution was just 0.03% of total Ag in the presence of thiosulphate compared to 50% of total Ag in the exposure to $AgNO_3$ alone (Table 1). In the control exposures, 5.2% of the very low background Ag concentration was calculated to exist as Ag^+.

3.1.2. Mortalities and cannula failures

The number of fish sampled at each sampling time for each exposure are given in Fig. 1A. One trout exposed to $AgNO_3$ alone died at 68 h, possibly due to the $AgNO_3$ exposure (the fish had low arterial oxygen tension and low plasma ion concentrations; see results), another lost its cannula at 46 h but survived and was sampled by caudal puncture at 94 h, and another lost its cannula at 68 h but did not survive. Two fish in the $AgNO_3$ plus thiosulphate exposure lost their

Table 1
Chemical characteristics of exposure water from experiments with rainbow trout held in ion-poor water with added $AgNO_3$ alone, with added $AgNO_3$ plus 5 μM thiosulphate, or in water without added Ag (control)[a]

	$AgNO_3$ alone	$AgNO_3$ plus thiosulphate	Control
Ag	0.12 ± 0.01 (33)	0.12 ± 0.01 (38)	0.001 ± 0.000 (36)
Thiosulphate	0	5	0
Ca	59 ± 11 (41)	50 ± 9 (50)	252 ± 58 (35)
Cl	314 ± 17 (40)	346 ± 17 (53)	666 ± 42 (36)
Na	665 ± 45 (41)	712 ± 36 (52)	1687 ± 136 (36)
pH	6.9 ± 0.0 (41)	6.9 ± 0.0 (52)	7.4 ± 0.0 (35)
PO_2	120 ± 1 (41)	120 ± 1 (52)	130 ± 2 (35)
PCO_2	1.0 ± 0.0 (41)	1.1 ± 0.0 (52)*	1.2 ± 0.0 (35)
Temperature	11.0 ± 0.2 (41)	11.9 ± 0.2 (52)*	10.4 ± 0.2 (35)
Calculated Ag^+	50.0% of total	0.03% of total	5.2% of total

[a] Silver, thiosulphate (nominal), Ca, Cl, and Na are given in μM, PO_2 and PCO_2 are torr, and water temperature is degree Celsius. All values are the mean ± 1 S.E.M. (n). The two significant differences between the two $AgNO_3$ exposures are indicated by asterisks. See text for comparisons of the $AgNO_3$ exposures with the control exposure.

cannulae at 46 h; one survived and was sampled at 94 h by caudal puncture. A trout died in this exposure after 68 h, likely due to too low hematocrit, and another had a failed cannula at 68 h and was sampled by caudal puncture at 94 h. In the control exposures one trout died after 46 h, probably because of low hematocrit, one fish died at 69 h due to cannula failure, and two others died for no obvious reasons after 68 h.

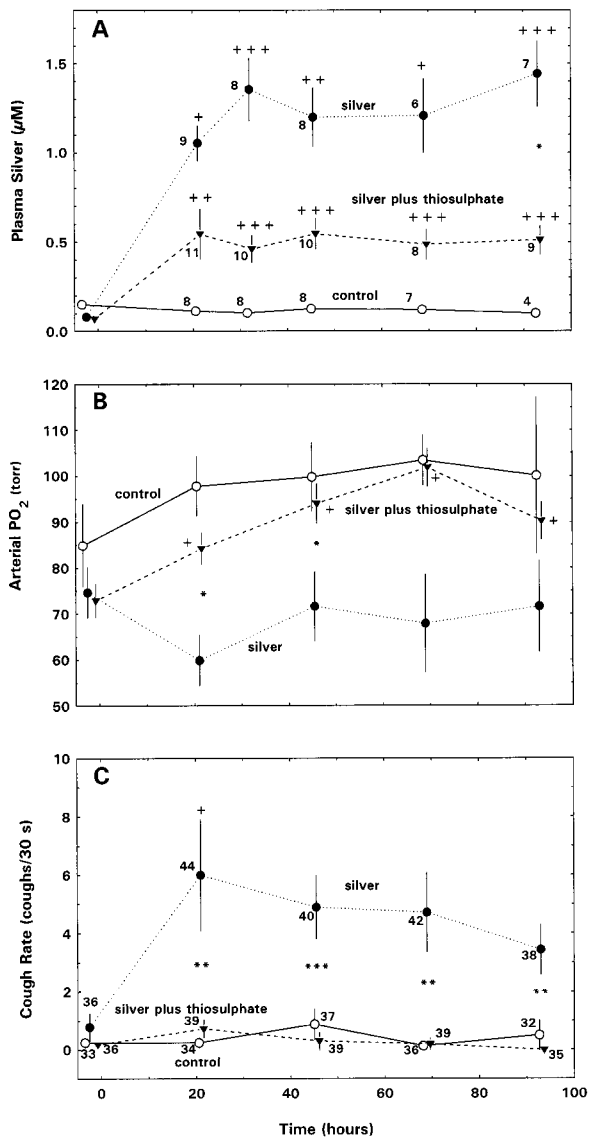

Fig. 1.

3.1.3. Physiological parameters

Trout exposed to 0.1 μM $AgNO_3$ alone had significant increases in plasma Ag from initial values at all sampling times, increasing from about 0.1 μM Ag to approximately 1.0 μM Ag by 21 h and to about 1.4 μM by 94 h (Fig. 1A); these values were highly significant compared to controls ($P < 0.001$). Trout exposed to 0.1 μM $AgNO_3$ plus 5 μM thiosulphate had smaller but still significant increases in plasma Ag over the initial value at all sampling times, but at 94 h were significantly lower than in the fish exposed to $AgNO_3$ alone (Fig. 1A). Plasma Ag concentrations in the $AgNO_3$ plus thiosulphate fish were higher compared to control fish at 21, 32, 46 and 68 h ($P < 0.05$). Note that even the initial increases in plasma Ag concentrations of about 1.0 and 0.4 μM Ag at 21 h (Fig. 1A) were higher than the concentrations of total Ag in the water of about 0.1 μM (Table 1).

Control fish showed steady to slight but not significant increases in arterial oxygen tension (PO_2) over the initial value (Fig. 1B), likely a response to repetitive blood sampling. Fish exposed to $AgNO_3$ plus thiosulphate also showed

Fig. 1. A: Plasma Ag concentrations for rainbow trout exposed in ion-poor water to 0.1 μM $AgNO_3$ alone (filled circles), to 0.1 μM $AgNO_3$ plus 5 μM thiosulphate (filled triangles), and to ion-poor water alone (control fish, open circles). Means ± 1 S.E.M. The numbers beside the symbols indicate the number of fish at each sampling time. Crosses indicate significant differences from initial (pre-exposure) values within a treatment ($P < 0.05$, $P < 0.01$, $P < 0.001$). Asterisks represent significant differences between the two $AgNO_3$ treatments; see text for significant differences between the two $AgNO_3$ treatments and control fish. In this and other figures, symbols have been offset horizontally for clarity where necessary. B: Arterial oxygen tension for rainbow trout exposed in ion-poor water to $AgNO_3$ alone, to $AgNO_3$ plus thiosulphate, and to ion-poor water alone (control). Fish exposed to $AgNO_3$ alone had significantly lower arterial PO_2 than did the $AgNO_3$ plus thiosulphate fish at 21 and 46 h (asterisks). Fish exposed to $AgNO_3$ plus thiosulphate had significantly higher arterial PO_2 compared to their initial value (crosses). C: Cough rates for rainbow trout exposed in ion-poor water to $AgNO_3$ alone, to $AgNO_3$ plus thiosulphate, and to ion-poor water alone (control). Trout exposed to $AgNO_3$ alone had higher cough rates than trout exposed to $AgNO_3$ plus thiosulphate (asterisks). Numbers beside the symbols are ventilation rates, in breaths per 30 s.

Table 2
Summary of physiological parameters for rainbow trout from before and after 94 h exposures in ion-poor water to $AgNO_3$ alone, to $AgNO_3$ plus thiosulphate, or to no added Ag (control)[a]

	$AgNO_3$ alone		$AgNO_3$ plus thiosulphate		Control	
	Initial	94 h	Initial	94 h	Initial	94 h
$PaCO_2$ (torr)	1.8 ± 0.1 (9)	1.9 ± 0.2 (6)	2.0 ± 0.2 (11)	1.8 ± 0.2 (5)	2.0 ± 0.1 (8)	2.0 ± 0.1 (3)
Plasma Na (mM)	149 ± 2 (9)$^{+++}$	112 ± 7 (7)**	151 ± 3 (11)	152 ± 3 (9)	147 ± 3 (8)	137 ± 6.5 (4)
Plasma Ca (mM)	1.9 ± 0.1 (9)	1.8 ± 0.1 (7)	1.7 ± 0.1 (11)	1.7 ± 0.1 (9)	1.8 ± 0.1 (8)	1.4 ± 0.2 (4)
Hemoglobin (g dl^{-1})	6.5 ± 1.2 (9)	4.2 ± 0.5 (7)*	5.2 ± 0.9 (11)$^{+}$	2.4 ± 0.4 (8)	5.7 ± 0.9 (8)	2.8 ± 0.6 (3)
Hematocrit (%)	24.1 ± 3.5 (9)	18.6 ± 3.3 (7)*	19.2 ± 3.1 (11)$^{+}$	9.4 ± 1.2 (9)	20.7 ± 3.1 (8)	13.4 ± 2.0 (2)
MCHC (g ml^{-1})	0.2 ± 0.0 (9)	0.2 ± 0.0 (7)	0.3 ± 0.0 (11)	0.3 ± 0.0 (8)	0.3 ± 0.0 (7)	0.3 ± 0.0 (2)
Gill Ag	–	15.0 ± 2.1 (7)*	–	2.4 ± 0.4 (9)	–	0.4 ± 0.0 (5)
Calculated gill Ag	–	14.0	–	0.4	–	0.2

[a] Significant differences between initial and 94 h values within a treatment are indicated by crosses, and significant differences between $AgNO_3$ treatments at 94 h are indicated by asterisks. See text for comparisons of the $AgNO_3$ exposures with the control exposure. Measured and calculated gill silver units are nmol Ag/g wet tissue.

steady increases in arterial PO_2, which were significantly higher than the initial value at all sampling times. In contrast, trout exposed to $AgNO_3$ alone did not show increased arterial PO_2 and had lower arterial PO_2 compared to both the $AgNO_3$ plus thiosulphate and control fish at 21 and 46 h (Fig. 1B). Arterial carbon dioxide tensions were 1.8–2.0 torr in all groups of fish, with no significant differences within or between groups at 0 and 94 h (Table 2).

Cough rates were significantly higher in fish exposed to $AgNO_3$ alone compared to fish exposed to $AgNO_3$ plus thiosulphate, and at 21 h were significantly higher than the initial rate (Fig. 1C). Cough rates in the fish exposed to $AgNO_3$ alone were also significantly higher than control fish at 21 h ($P < 0.01$) and at 46 and 68 h ($P < 0.05$). At all sample times ventilation rates were slightly higher in the fish exposed to $AgNO_3$ alone (numbers in Fig. 1C), but the only significant difference in ventilation rates was at 21 h when trout exposed to $AgNO_3$ alone had 44 ± 3 (nine) breaths in 30 s compared to 34 ± 2 (eight) breaths in 30 s in the control fish ($P < 0.05$).

Reduced oxygen uptake through fish gills can lead to anaerobic respiration and subsequent production of lactate. Trout exposed to $AgNO_3$ plus thiosulphate had relatively low and constant blood lactate concentrations of about 0.5 mM, while trout exposed to $AgNO_3$ alone showed steady but slight increases in blood lactate which were significantly greater than those of the $AgNO_3$ plus thiosulphate fish at 94 h (Fig. 2A). Control fish had elevated but variable blood lactate at 46 h, but these values were not significantly different.

Control trout and trout exposed to $AgNO_3$ plus thiosulphate had relatively constant plasma Cl concentrations, although they were lower in the fish in the control experiments (Fig. 2B; significantly so at 21 and 32 h, $P < 0.05$). In contrast, plasma Cl concentrations decreased in trout exposed to $AgNO_3$ alone, were significantly lower than the initial value past 46 h, and were also significantly lower than in the trout exposed to $AgNO_3$ plus thiosulphate at these times (Fig. 2B). Changes in plasma Na concentrations were very similar to the changes in plasma Cl: control fish and trout exposed to $AgNO_3$ plus thiosulphate had relatively constant plasma Na concentrations throughout the exposures, although again the control fish generally had lower concentrations (Table 2). In contrast, plasma Na concentrations decreased significantly from initial values by 94 h in trout exposed to $AgNO_3$ alone (Table 2): plasma Na in these fish at 94 h was also signifi-

cantly lower compared to the $AgNO_3$ plus thiosulphate fish ($P < 0.01$) and to control fish ($P < 0.05$). Plasma Ca concentrations did not vary significantly between or within groups at different sampling times (Table 2).

Plasma glucose concentrations are an indication of overall stress in fish. Control trout and trout exposed to $AgNO_3$ plus thiosulphate had steady or slightly decreasing plasma glucose concentrations throughout the exposures (Fig. 2C). In contrast, plasma glucose increased steadily in trout exposed to $AgNO_3$ alone, and at 94 h there was significantly more glucose in plasma of these trout compared to the $AgNO_3$ plus thiosulphate fish (Fig. 2C). Plasma glucose was also higher in the $AgNO_3$ alone fish compared to control fish at 21 h ($P < 0.01$) and at 32 and 68 h ($P < 0.05$).

Whole blood hemoglobin concentrations decreased over the course of the exposures in all groups as a result of repetitive blood sampling, but the only significant decrease was in the $AgNO_3$ plus thiosulphate exposure, and hemoglobin concentration was higher in the $AgNO_3$ alone fish compared to the $AgNO_3$ plus thiosulphate fish (Table 2). Hematocrit also decreased significantly over time in the $AgNO_3$ plus thiosulphate fish due to repetitive blood sampling, and hematocrit was significantly higher in the $AgNO_3$ alone fish compared to the $AgNO_3$ plus thiosulphate fish at 94 h (Table 2). Mean corpuscular hemoglobin concentrations (MCHC; hemoglobin/hematocrit) remained constant at 0.2 to 0.3 g Hb per ml of red blood cells for all groups (Table 2).

3.1.4. Gill silver

By the end of the exposures there was six times more Ag on the gills of rainbow trout exposed to $AgNO_3$ alone compared to Ag on the gills of

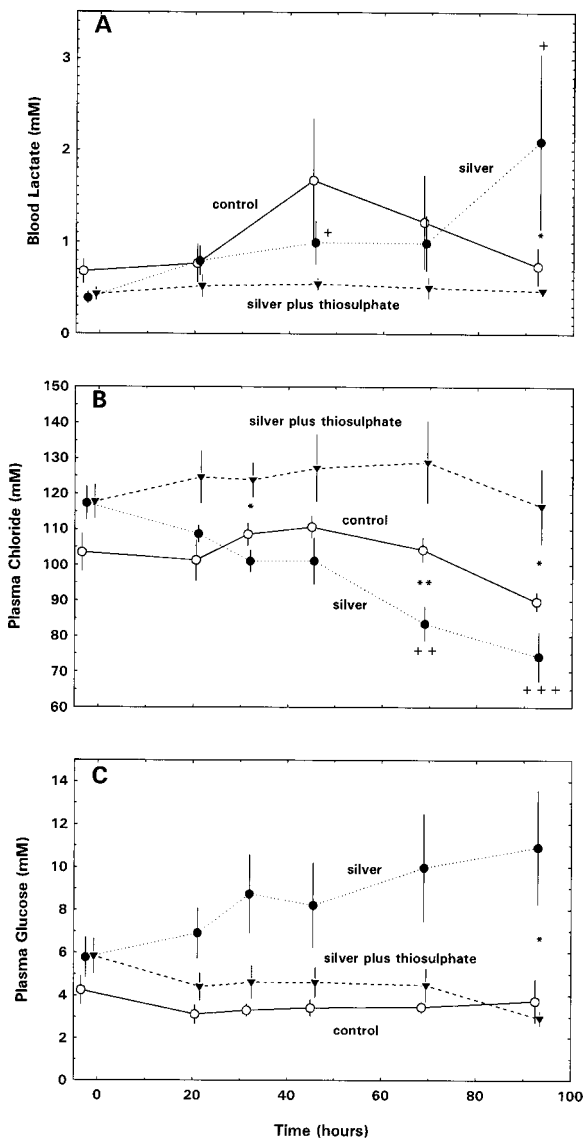

Fig. 2.

Fig. 2. A: Blood lactate concentrations for rainbow trout exposed in ion-poor water to $AgNO_3$ alone, $AgNO_3$ plus thiosulphate, and to ion-poor water alone (control). Means ± 1 S.E.M. Trout exposed to $AgNO_3$ alone had significantly higher blood lactate concentrations at 94 h compared to fish exposed to $AgNO_3$ plus thiosulphate (asterisk) and significantly higher blood lactate at 46 and 94 h compared to the initial value (crosses). B: Plasma Cl concentrations for trout exposed in ion-poor water to $AgNO_3$ alone, to $AgNO_3$ plus thiosulphate, and to ion-poor water alone (control). Fish exposed to $AgNO_3$ alone had significantly lower plasma Cl concentrations past 46 h compared to initial values (crosses), and were usually lower compared to trout exposed to $AgNO_3$ plus thiosulphate (asterisks). C: Plasma glucose concentrations for trout exposed in ion-poor water to $AgNO_3$ alone, to $AgNO_3$ plus thiosulphate, and to ion-poor water alone (control). A significant difference was seen at 94 h between trout exposed to $AgNO_3$ alone and those exposed to $AgNO_3$ plus thiosulphate (asterisk).

trout exposed to $AgNO_3$ plus thiosulphate (e.g. gill samples from fish still living at 94 h; Table 2). Trout exposed to $AgNO_3$ alone accumulated highly significant amounts of Ag on their gills compared to gills of control fish ($P < 0.001$), whereas the accumulation of Ag on the gills of the $AgNO_3$ plus thiosulphate fish was not significantly greater than in the control fish ($P > 0.05$).

Calculated gill Ag concentrations were obtained using the Ag-gill binding model of Janes and Playle (1995), 18 nmol l^{-1} gill binding sites, the water chemistry given in Table 1, and 0.5 mg C l^{-1} DOM (background DOM, from Table 3; = 17.5 nmol l^{-1} binding sites). Calculated gill Ag concentrations for the fish exposed to 0.12 μM $AgNO_3$ alone was 14.0 nmol Ag/g wet tissue (93.6% of the Ag-gill sites filled by Ag), close to the measured value (Table 2). In the presence of 0.12 μM $AgNO_3$ plus 5 μM thiosulphate the calculated gill Ag was 0.4 nmol Ag/g wet tissue (2.7% of the Ag-gill sites filled by Ag), lower than the measured value. Calculated Ag on the gills of control trout exposed to a background concentration of 0.001 μM Ag was 0.2 nmol Ag/g wet tissue, close to what was measured (Table 2).

Table 3
Chemical characteristics from experiments with rainbow exposed to ion-poor water with added $AgNO_3$ alone or with added $AgNO_3$ plus DOM[a]

	$AgNO_3$ alone	$AgNO_3$ plus DOM
Ag	0.07 ± 0.00 (30)	0.12 ± 0.01 (30)***
DOM	0.5 ± 0.1 (30)	34.6 ± 0.7 (30)***
Ca	28 ± 1 (29)	82 ± 5 (29)***
Cl	15 ± 15 (29)	1607 ± 80 (28)***
Na	1027 ± 35 (29)	1717 ± 96 (29)***
pH	7.3 ± 0.0 (30)	7.5 ± 0.0 (30)***
PO_2	133 ± 2 (29)	134 ± 1 (30)
PCO_2	0.9 ± 0.0 (29)	0.9 ± 0.0 (30)
Calculated Ag^+	73.1% of total	0.09% of total

[a] Water Ag, Ca, Cl, and Na concentrations are given in μM, DOM is mg C l^{-1}, and water PO_2 and PCO_2 are torr, means ± 1 S.E.M. (n) for each. Significant differences between the two $AgNO_3$ exposures are indicated by asterisks.

3.2. $AgNO_3$ and $AgNO_3$ plus DOM exposures

3.2.1. Water chemistry

Average Ag concentration in the $AgNO_3$ alone exposure was 0.07 μM, significantly lower than the 0.12 μM Ag in the $AgNO_3$ plus 35 mg C l^{-1} DOM exposure (Table 3). Water Ca, Cl, and Na concentrations were higher in the $AgNO_3$ plus DOM exposure compared to the exposure to $AgNO_3$ alone (Table 3), because the Aldrich humic acid used contained these ions; water pH was also slightly higher. Water PO_2, PCO_2, and water temperature (12.1 ± 0.1°C) were the same in each exposure.

Concentrations of ionic Ag^+ in the exposures were calculated using the input data given in Table 3, using 35 nmol binding sites per mg C of DOM (e.g. 17.5 and 1211 nmol l^{-1} binding sites for 0.5 and 34.6 mg C l^{-1} DOM, respectively). Calculated Ag^+ in the exposure to $AgNO_3$ alone was 73% of total Ag, and in the $AgNO_3$ plus DOM exposure was just 0.09% of the greater amount of total Ag (Table 3).

3.2.2. Mortalities and cannula failures

There were no mortalities or cannula failures in these $AgNO_3$ and $AgNO_3$ plus DOM exposures (six fish for each), although we did not always obtain a value from each fish for all parameters at each sampling time (see Figs. 1–4 captions and Table 4).

3.2.3. Physiological parameters

Rainbow trout exposed to $AgNO_3$ alone showed large increases in plasma Ag, from initial values of 0.5 μM to about 1.9 μM by 88 h (Fig. 3A). Trout exposed to $AgNO_3$ plus DOM showed slightly lower accumulations of Ag in the plasma (significantly lower at 66 h), increasing to about 1.5 μM Ag by 88 h. In both exposures even the increases in plasma Ag concentrations at 17 h were about ten times higher than the concentrations of Ag in the water.

Both groups of trout showed steady or slightly increasing arterial oxygen tensions during the course of the exposures, likely a response to repetitive blood sampling, and fish from the $AgNO_3$ plus DOM exposure tended to have higher arte-

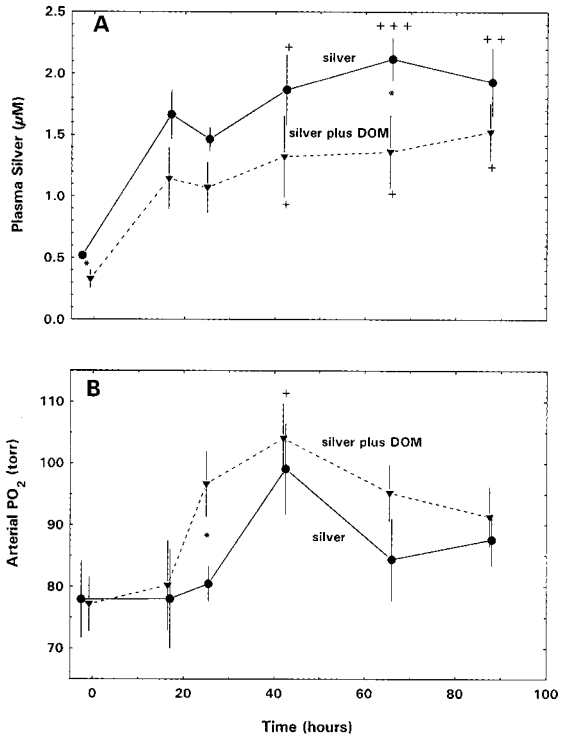

Fig. 3. A: Plasma Ag concentrations for rainbow trout exposed in ion-poor water to 0.1 μM $AgNO_3$ alone (filled circles; $n = 6$ except at 42 h, where $n = 5$), and to 0.1 μM $AgNO_3$ plus 35 mg C l^{-1} DOM (filled triangles; $n = 6$). Means ± 1 S.E.M. Crosses indicate significant differences from initial values within a treatment ($P < 0.05$, $P < 0.01$, $P < 0.001$). Asterisks indicate means significantly different between the two exposures at a given sample time. See Fig. 1A for control values. In this and other panels, symbols have been offset horizontally for clarity where necessary. B: Arterial oxygen tension for rainbow trout exposed in ion-poor water to $AgNO_3$ alone and to $AgNO_3$ plus DOM ($n = 6$ for all points). The cross indicates a significant difference from initial values within the treatment, and the asterisk indicates means significantly different between the two exposures. See Fig. 1B for control values.

rial PO_2 than the $AgNO_3$ alone group (significantly higher at 26 h; Fig. 3B). There were no significant differences in arterial PCO_2 (Table 4) except at 26 h, where the $AgNO_3$ group had a significantly higher arterial PCO_2 of 2.2 ± 0.1 (6) torr compared to the $AgNO_3$ plus DOM group with 1.7 ± 0.1 (6) torr ($P < 0.05$). Ventilation and cough rates were not taken during these exposures because the high DOM fish were not visible in their fish boxes.

Blood lactate concentrations were low but increased slightly during the exposures in both groups of fish, significantly so at 26 h in the $AgNO_3$ plus DOM fish (Fig. 4A). There were no significant differences in blood lactate concentrations between the two groups of fish, although they were usually slightly higher in the fish exposed to $AgNO_3$ alone.

Ionoregulatory effects of $AgNO_3$ alone included decreases, relative to the $AgNO_3$ plus DOM group, in plasma Cl concentrations by 66 h (Fig. 4B). Plasma Cl remained approximately constant in the $AgNO_3$ plus DOM fish. There was also a non-significant decrease in plasma Na in trout exposed to $AgNO_3$ alone, whereas plasma Na stayed roughly steady in the $AgNO_3$ plus DOM fish (Table 4). Plasma Ca concentrations did not vary significantly, except that plasma Ca was generally lower in the $AgNO_3$ plus DOM group (Table 4): this difference of about 0.1 mM was significant at 17 and 26 h ($P < 0.05$).

Plasma glucose, a general indicator of stress, was variable but tended to increase — but not significantly — in trout exposed to $AgNO_3$ alone (Fig. 4C). In contrast, plasma glucose concentrations remained low and steady in the fish exposed to $AgNO_3$ plus DOM.

Whole blood hemoglobin concentrations decreased significantly due to repetitive blood sampling during the course of both exposures, but less so for the $AgNO_3$ alone fish so that there was a significant difference between the two groups at 88 h (Table 4). Hematocrit also decreased in both groups due to repetitive blood sampling, but was not significantly different at 88 h. These trends in hemoglobin and hematocrit resulted in a significant difference in MCHC in the two exposures at 88 h (Table 4), an indication of hemoconcentration in the fish exposed to $AgNO_3$ alone.

3.2.4. Gill silver

At the end of the exposures, fish exposed to $AgNO_3$ alone accumulated much more Ag on their gills compared to the trout exposed to $AgNO_3$ plus DOM (Table 4). Calculated gill Ag concentrations were obtained using the water chemistry given in Table 3. Trout exposed to $AgNO_3$ alone had calculated gill Ag concentra-

tions of 13.1 nmol Ag/g wet tissue (87.3% of the Ag-gill sites filled by Ag), higher than the measured value (Table 4). Trout exposed to $AgNO_3$ in the presence of 34.6 mg C l^{-1} DOM (= 1211 nmol l^{-1} binding sites) had calculated gill Ag concentrations of just 0.2 nmol Ag/g wet tissue (1.1% of the Ag-gill sites filled by Ag), lower than the measured gill Ag for these fish. The addition of DOM increased the concentrations of Ca, Cl, and Na in the exposure water: if lower concentrations of these ions are used in the simulation (e.g. ion concentrations from the $AgNO_3$ alone exposure are used, but still with high DOM), the calculated amount of Ag on the gills increases slightly to 0.3 nmol Ag g^{-1}. That is, the additional Na^+ and Ca^{2+} competition at the gill binding sites for Ag^+ and the added complexation of Ag^+ by Cl^- have negligible effects on calculated Ag^+ binding to the gills, in comparison to the large effect of the 35 mg C l^{-1} DOM.

3.3. Softwater, thiosulphate, and DOM exposures alone

Rainbow trout exposed to ion-poor (soft) water alone for 94 h showed low plasma Ag concentrations throughout the exposure, steady or increasing arterial PO_2, steady cough and ventilation rates, steady (but variable) blood lactate, constant plasma ion concentrations, steady plasma glucose, and, due to our repetitive blood sampling regime, decreasing blood hemoglobin content and hematocrit but constant MCHC (Figs. 1 and 2; Table 2). These are broadly similar results to those obtained in our laboratory using similar softwater exposures and sampling protocols (Richards and Playle, 1999; Richards et al., 1999). Thus, although we did not run softwater controls during the $AgNO_3$ plus DOM set of exposures, results from these exposures can be compared to the softwater control results from the $AgNO_3$ plus thiosulphate exposures.

Similarly, although we did not run controls for the effects of DOM alone, previously we showed that rainbow trout exposed to Aldrich humic acid in metal-free water showed no deleterious effects of 31 mg C l^{-1} DOM alone (Richards et al., 1999). That is, there were no effects of DOM on respiratory gas transfer, blood lactate concentrations, or plasma Na or glucose concentrations compared to fish exposed to softwater alone. The only influence of the high DOM exposure was an increase in plasma Cl concentrations by 94 h, probably an effect of the unavoidable increase in aqueous Cl concentration from adding the Aldrich humic acid (Richards et al., 1999), also seen in our present experiments (Table 3).

For thiosulphate alone, preliminary work of ours exposing cannulated rainbow trout to silver-free softwater plus 5 μM thiosulphate in water containing similar concentrations of ions to those given in Table 1 (82 μM Ca, 481 μM Cl, 1540 μM

Table 4
Summary of physiological parameters for rainbow trout from before and after 88 h exposures in ion-poor water with added $AgNO_3$ alone or with added $AgNO_3$ plus DOM[a]

	$AgNO_3$ alone		$AgNO_3$ plus DOM	
	Initial	88 h	Initial	88 h
$PaCO_2$ (torr)	2.2 ± 0.1 (6)	1.7 ± 0.1 (6)	1.8 ± 0.2 (6)	1.7 ± 0.1 (6)
Plasma Na (mM)	145 ± 4 (6)	130 ± 7 (6)	134 ± 4 (6)	137 ± 6 (5)
Plasma Ca (mM)	1.8 ± 0.0 (5)	1.7 ± 0.1 (6)	1.7 ± 0.0 (6)	1.6 ± 0.1 (5)
Hemoglobin (g dl^{-1})	3.1 ± 0.4 (6)++	1.7 ± 0.2 (6)**	2.6 ± 0.2 (6)+++	0.7 ± 0.2 (6)
Hematocrit (%)	14.3 ± 1.4 (6)++	7.8 ± 0.8 (6)	14.1 ± 0.9 (6)+++	5.6 ± 0.6 (6)
MCHC (g ml^{-1})	0.2 ± 0.0 (6)	0.2 ± 0.0 (6)*	0.2 ± 0.0 (6)	0.1 ± 0.0 (6)
Gill Ag	–	9.0 ± 0.6 (6)***	–	3.5 ± 0.4 (6)
Calculated gill Ag	–	13.1	–	0.2

[a] Significant differences between initial and 88 h values within a treatment are indicated by crosses, and significant differences between the $AgNO_3$ treatments at 88 h are indicated by asterisks. Measured and calculated gill Ag units are nmol Ag/g wet tissue.

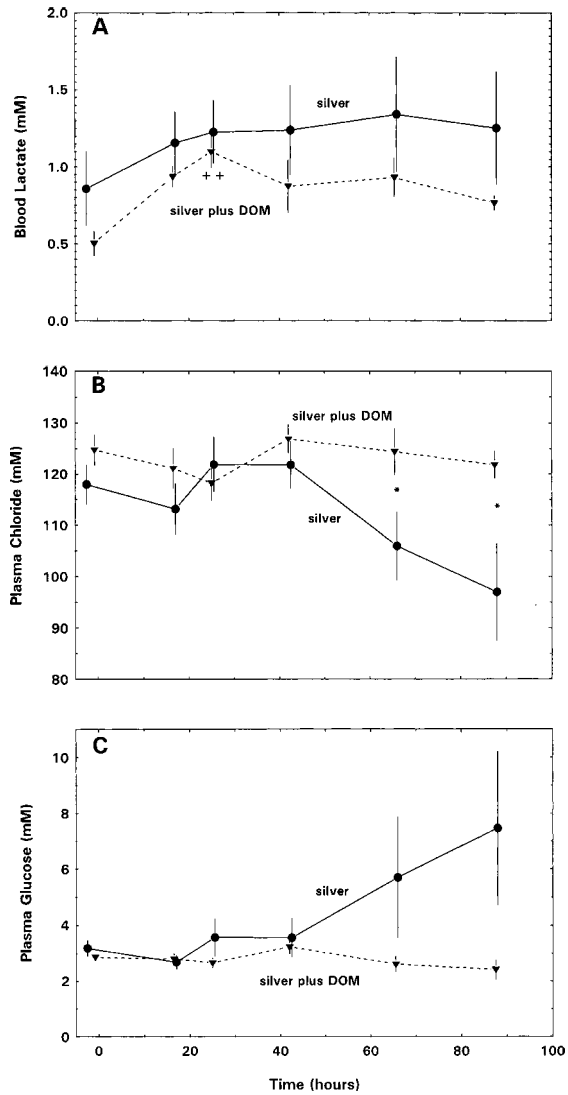

Fig. 4. A: Blood lactate concentrations for rainbow trout exposed in ion-poor water to $AgNO_3$ alone and to $AgNO_3$ plus DOM. Means ± 1 S.E.M. Crosses at 26 h indicate a significant difference from the initial value. See Fig. 2A for control values. B: Plasma Cl concentrations for trout exposed in ion-poor water to $AgNO_3$ alone ($n = 6$, except $n = 5$ at 42 h) and to $AgNO_3$ plus DOM ($n = 6$, except $n = 5$ at 88 h). After 66 h, trout exposed to $AgNO_3$ alone had significantly lower plasma Cl compared to trout exposed to $AgNO_3$ plus DOM (asterisks). See Fig. 2B for control values. C: Plasma glucose concentrations for trout exposed in ion-poor water to $AgNO_3$ alone and to $AgNO_3$ plus DOM. See Fig. 2C for control values.

Na, pH 7.5, water PO_2 132 torr, water PCO_2 1.2 torr) yielded similar results as repetitive blood sampling in softwater alone (described above; Rose-Janes 1997). That is, there were no ionoregulatory or respiratory effects of 5 μM thiosulphate. In silver-free water of lower ion content than in the present experiments (24 μM Ca, 284 μM Cl, 119 μM Na, pH 7.0, water PO_2 106 torr, water PCO_2 1.3 torr) there were no respiratory effects of 5 μM thiosulphate, but there were significant decreases in plasma Na and Cl from about 140 to 109 mM Na and from 109 to 92 mM Cl in 48 h (Rose-Janes, 1997). In our present thiosulphate exposures with water ion concentrations bracketed by these exposures, 5 μM thiosulphate did not exacerbate the effects of the low ion concentrations in the water, and in fact protected against the ionoregulatory effects of 0.1 μM $AgNO_3$ alone (Fig. 2B, Table 2).

4. Discussion

Adult rainbow trout were exposed to $AgNO_3$ in ion-poor water in the presence and absence of the complexing agents thiosulphate and dissolved organic matter (DOM), and the physiological effects of these exposures were assessed through repetitive blood sampling. Trout exposed to 0.1 μM $AgNO_3$ alone accumulated Ag on their gills and in their plasma, lost plasma Cl and Na, and had increased plasma glucose concentrations. Trout in the first set of exposures to $AgNO_3$ alone also experienced respiratory distress, indicated by increased cough rates, lower arterial PO_2, and elevated blood lactate concentrations. In contrast, fish exposed to 0.1 μM $AgNO_3$ plus 5 μM thiosulphate or 35 mg C l^{-1} DOM accumulated less Ag on their gills and in their plasma, did not lose plasma ions or have increased plasma glucose concentrations, and did not show respiratory distress, and responded in a very similar manner as did control fish.

The ionoregulatory effects of $AgNO_3$ plus its effects on plasma glucose have been reported before (Janes and Playle, 1995; Wood et al., 1996a; Webb and Wood, 1998), but here is the first time respiratory toxicity of $AgNO_3$ has been reported for fish exposed at the low end of the

LC_{50} values for $AgNO_3$ (e.g. 0.06–0.12 μM $AgNO_3$, McGeer and Wood, 1998; 0.05–0.65 μM $AgNO_3$, Hogstrand and Wood, 1998). The most likely reason for the respiratory toxicity of $AgNO_3$ seen in our first set of exposures, in addition to the ionoregulatory effects of $AgNO_3$, was the ion-poor water used in the exposures plus the slightly higher concentration of total Ag in that experiment: 0.12 versus 0.07 μM Ag in the second $AgNO_3$ alone experiment. Indeed, not only were the respiratory effects of $AgNO_3$ alone more evident in the first set of exposures, the ionoregulatory effects of $AgNO_3$ were greater (e.g. cf. Fig. 2B, Fig. 4B; Tables 2 and 4).

Exposure to a metal in soft water generally results in greater toxic effects of the metal (Spry and Wiener, 1991). The relative paucity of Ca^{2+} in our soft water (<90 μM), which normally would stabilize the gill membrane (McDonald, 1983) and, along with Na^+, would compete for Ag^+ binding sites on the gills (Janes and Playle, 1995), plus the low concentrations of Cl^- which would otherwise partially complex ionic Ag^+, would result in greater interactions of Ag^+ at the gills so that the trout in our first exposure to $AgNO_3$ alone showed some respiratory distress as well as the ionoregulatory effects of $AgNO_3$.

The respiratory effects of $AgNO_3$ probably resulted from branchial inflammation and excess mucus production, interfering with gas transfer by increasing the diffusion distance for O_2 and CO_2. Thus $AgNO_3$ can cause respiratory distress secondary to ionoregulatory effects at elevated but not extreme concentrations of ionic Ag^+, depending on the moderating effects of water chemistry (e.g. Fig. 1 in Wood et al., 1999). In similar work of ours in ion-poor water, a combined exposure to 0.2 μM Cd and 0.8 μM Cu caused respiratory effects which included decreased arterial PO_2, increased arterial PCO_2, increased blood lactate concentrations, and increased ventilation rates (Richards and Playle, 1999). Increasing the water Ca concentrations from 40 to 910 μM Ca eliminated the respiratory effects and the acute toxicity of the Cd and Cu solution, but did not protect fully against the longer-term ionoregulatory effects of the metal mixture (Richards and Playle, 1999).

Trout exposed to 0.1 μM $AgNO_3$ in the presence of 5 μM thiosulphate accumulated about six times less Ag on their gills compared to trout exposed to $AgNO_3$ alone (Table 2), and accumulated about one third the amount of Ag in their plasma compared to trout exposed to $AgNO_3$ alone (Fig. 1A). Thiosulphate eliminated the ionoregulatory and respiratory toxicity of $AgNO_3$ alone by complexing ionic Ag^+ and reducing the amount of Ag^+ available to bind to the gills, the toxic site of action of ionic Ag^+. Using the Ag-gill model of Janes and Playle (1995), the chemical equilibrium program MINEQL$^+$ (Schecher and McAvoy, 1992), and water chemistry given in Table 1, the concentration of Ag on the gills of trout exposed to $AgNO_3$ alone was calculated to be 14.0 nmol Ag/g wet tissue, whereas the Ag concentration on the gills was calculated to be just 0.4 nmol Ag g^{-1} in the presence of thiosulphate (Table 2). The ability of thiosulphate to keep Ag^+ off the gills was overestimated (e.g. calculated gill Ag was lower than the measured concentration), but considering that the Janes and Playle (1995) model was based on 2–3 h exposures of trout to $AgNO_3$, the discrepancy between measured and calculated gill Ag might be expected. Slow accumulation of Ag by the gills was seen before in small rainbow trout exposed to 0.07 μM $AgNO_3$ plus 2.5 μM thiosulphate (significant accumulation by 6 days; Janes and Playle, 1995), and we suggested then that slow accumulation of Ag over days could be due to the slow reaction of the very low concentration of Ag^+ with the gills or the diffusion of Ag complexed by thiosulphate into the gills during longer exposures.

Although the ionoregulatory effects of $AgNO_3$ and the increase in plasma glucose due to $AgNO_3$ were eliminated by thiosulphate in the work of Wood et al. (1996a,b), at extremely high exposures of ~ 280 μM Ag-thiosulphate more Ag actually entered the fish gills and plasma compared to trout exposed to 0.1 μM $AgNO_3$ alone (Wood et al., 1996b). This entry of Ag was presumably by diffusion of Ag-thiosulphate down its concentration gradient into the fish (Wood et al., 1999). The accumulated Ag in the plasma and particularly in the gills was non-toxic, because the ionoregulatory effects of $AgNO_3$ were eliminated

(Wood et al., 1996b). Wood et al. (1996b) concluded that ionic Ag^+ is the toxic form of Ag and acts at the gill surface, and that internalized Ag is not toxic. In our present work the Ag accumulated by the gills and in plasma of the $AgNO_3$ plus thiosulphate fish also appears to be non-toxic, either by virtue of the lower concentrations of accumulated Ag compared to the fish exposed to $AgNO_3$ alone or the form of Ag that was accumulated. Thiosulphate itself has no major physiological effects aside from a slight anaesthetic effect (Wood et al., 1996b) and causing slight decreases in plasma glucose concentrations (Fig. 2C; Wood et al., 1996b), and perhaps adding to ion losses of fish held in very ion-poor water (Rose-Janes, 1997).

Our $AgNO_3$ plus DOM exposures demonstrate the physiological mechanisms behind the protective effects of DOM against Ag^+ toxicity. Less Ag entered the fish, and the ionoregulatory effects evident in the fish exposed to $AgNO_3$ alone were eliminated (Figs. 3 and 4; Table 4). The respiratory effects of $AgNO_3$ alone seen in the first experiment were not very evident in this set of exposures because, as mentioned earlier, measured total Ag was lower plus there was more Na^+ in the water to compete with Ag^+ binding at the gills. In addition, we did not record ventilation and cough rates in this set of exposures because the fish with added DOM were not visible in their boxes, so we would have missed these respiratory effects if they occurred. In these exposures there were unavoidable increases in water Ca, Cl, and Na concentrations when the DOM was added (Table 3), but computer simulations indicated that these ions would exert negligible protection against Ag^+ accumulation at the gills compared to the large influence of Ag^+ complexation by the DOM (Section 3.2.4). The increase in these ions in the water would also reduce the gradient for loss of these ions from inside to outside the fish, yielding additional protection against the ionoregulatory effects of Ag^+ alone, but the similarities between the $AgNO_3$ plus DOM and the $AgNO_3$ plus thiosulphate results also suggest that complexation of Ag^+ was the main effect of the added DOM.

Our $AgNO_3$ plus DOM results generally agree with those from similar experiments run in our laboratory with cannulated trout exposed in ion-poor water to 0.2 μM Cd and 0.8 μM Cu in the presence and absence of natural and commercial DOM (Richards et al., 1999). In those experiments the respiratory, ionoregulatory, and hemoconcentration effects of the combined Cd and Cu exposure were eliminated by the addition of DOM, as was seen with our present results (Figs. 3 and 4; Table 4). Furthermore, there were no adverse effects of 31 mg C l^{-1} DOM on its own in those experiments (Richards et al., 1999).

The main difference between our $AgNO_3$ experiments and those Cd and Cu experiments was the ability of just 7 mg C l^{-1} Aldrich humic acid to keep 0.2 μM Cd and 0.8 μM Cu out of trout plasma (although the metals still accumulated on the gills; Richards et al., 1999), whereas 35 mg C l^{-1} Aldrich humic acid, a relatively high concentration of DOM in the environment, reduced but was unable to eliminate Ag accumulation in plasma of our fish exposed to 0.1 μM $AgNO_3$ (Fig. 3A). Our $AgNO_3$ results highlight the strong binding of Ag^+ to trout gills and the relatively weaker binding of Ag^+ to DOM. Indeed, our modelling efforts suggest that a greater difference may exist between the log $K_{Ag\text{-}gill}$ and log $K_{Ag\text{-}DOM}$ values, with a difference of more than the one log unit difference in the current Ag-gill model of Janes and Playle (1995), because we overpredicted the ability of DOM to keep Ag off the gills (Table 4). A higher Ag-gill log K value would also reduce the overprediction of the ability of thiosulphate to keep Ag^+ off the gills (Table 2), and a lower log $K_{Ag\text{-}DOM}$ value could account for differences in Ag^+ binding between the natural Luther Marsh DOM, for which the model was developed, and the Aldrich humic acid used here. However, as discussed earlier, the discrepancy between calculated and measured Ag accumulations may be the result of applying a model based on fast Ag accumulation by the gills to longer term exposures where diffusion of complexed Ag into the gills would be occurring, along with more time for the small amount of Ag^+ in solution to react at the gills. However, diffusion of large molecules like DOM across the gills is less likely than diffusion

of small Ag-thiosulphate complexes. More work is certainly needed to apply acute metal-gill models to longer-term metal exposures (Playle, 1998; Richards and Playle, 1999; Wood et al., 1999).

The mechanism of acute toxicity from ionic Ag^+ is fish death due to circulatory failure (Wood et al., 1996a). Circulatory failure occurs following decreases in plasma Na and Cl, resulting in a stress response by fish which includes increases in plasma glucose and cortisol (Webb and Wood, 1998). Ionoregulatory disturbances result in hemoconcentration and increased blood viscosity, which, along with increased blood pressure and heart rate due to adrenaline release, eventually kills the fish (Wood et al., 1996a; Hogstrand and Wood, 1998). We also observed mild hemoconcentration in fish exposed to $AgNO_3$ alone, as indicated by their slightly higher hematocrit values (Tables 2 and 4), but our exposures were one day shorter than in Wood et al. (1996a,b), and our repetitive blood sampling regime reduced the degree of hemoconcentration.

In our exposures the Ag accumulation in trout plasma was greater than the total concentration of Ag in the water, an indication of active uptake of ionic Ag^+ at the gills. Using the Nernst equation and a transepithelial potential for freshwater fish of between -5 and -7 mV (Stormer et al., 1996; Bijveld et al., 1998), the increase in plasma Ag concentrations due to passive diffusion of Ag^+ would only be expected to be 0.1–0.2 μM Ag. The observed increases in plasma Ag concentrations were between about 0.4 and 1.5 μM Ag (Fig. 1A, Fig. 3A), which would require a transepithelial potential of between -33 and -68 mV to explain these interior Ag concentrations through passive uptake of Ag^+. These are large differences in transepithelial potentials, even if plasma proteins act as a sink for Ag^+.

That is, the uptake of Ag from low concentrations of $AgNO_3$ outside the fish to the observed higher concentrations inside the fish was probably through active transport and not through Ag^+ diffusion alone, because the slightly negative transepithelial potential would not support this degree of passive Ag^+ uptake (e.g. similar calculations made by Bijveld et al. (1998) to propose active Mg^{2+} uptake at freshwater fish gills). This high concentration of Ag in trout plasma compared to the concentration in the water suggests that Ag^+ is bound first by the gills then is transported actively across the gill epithelium into the plasma, possibly by incorporation into an active transport pump such as the basolateral Na^+/K^+-ATPase (Wood et al., 1999). Indeed, it appears that Ag^+ enters freshwater rainbow trout via Na^+ channels on the apical gill membrane (Bury and Wood, 1999) and is actively extruded from the gills by an ATP-dependent process (but not necessarily Na^+/K^+-ATPase) on the basolateral membrane of the gills, as demonstrated using basolateral membrane vesicles prepared from trout gills (Bury et al., 1999c). In this respect, the reduced supply of ionic Ag^+ available for binding to the gills in our $AgNO_3$ plus thiosulphate and DOM exposures (Tables 1 and 3) would be expected to result in less Ag^+ entering the plasma, as we observed (Fig. 1A, Fig. 3A), even if some diffusion of complexed Ag was ongoing over the longer term.

In conclusion, rainbow trout exposed to 0.1 μM $AgNO_3$ alone in ion-poor water experienced ionoregulatory disturbances, and some respiratory distress depending on water chemistry and total Ag concentration, which were eliminated by the addition of 5 μM thiosulphate or 35 mg C l^{-1} DOM. These results further illustrate the importance of water chemistry on metal toxicity to aquatic organisms, because the secondary respiratory effects of $AgNO_3$ occurred in ion-poor water where fewer competitive (Na^+, Ca^{2+}) and complexing (Cl^-) ions were available to reduce binding of ionic Ag^+ at the gills, and because the complexing agents thiosulphate and DOM eliminated the toxic effects of $AgNO_3$ by reducing, through complexation, the availability of ionic Ag^+ to bind at the gills.

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