Protective effects of water Cl^- on physiological responses to waterborne silver in rainbow trout

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Abstract: The importance of water Cl^- concentration in mitigating the effects of sublethal waterborne $AgNO_3$ exposure on rainbow trout was studied to test the relationship between physiological response and concentration of ionic silver (Ag^+). Trout were exposed to a total silver concentration of 30 nmol·L^{-1} (as $AgNO_3$) at a range of different water Cl^- concentrations from 20 to 1500 µmol·L^{-1}. These levels were chosen by speciation modelling with MINEQL+ to progressively reduce the concentration of Ag^+ largely by replacement with dissolved $AgCl_{aq}$. Fish exposed to $AgNO_3$ experienced a loss of Na^+; increasing the water Cl^- concentration reduced these losses. In vitro measurements after 50 h of exposure showed that Na^+ losses were related to Ag^+-induced inhibition of gill Na^+/K^+ ATPase activity; increasing the concentration of Cl^- in the water protected against the inhibition of Na^+/K^+ ATPase activity. The disruption of Na^+ regulation in $AgNO_3$-exposed fish was accompanied by increased plasma cortisol and total ammonia concentrations, but blood pH, plasma P_{CO_2} and HCO_3^-, and hematocrit were, in general, unaltered. Gill silver content increased in all $AgNO_3$-exposed trout and was not correlated with the disruption of Na^+ balance. We conclude that physiological measurements in combination with aquatic geochemical equilibrium modelling are useful in developing models to predict the acute toxicity of waterborne silver.

Résumé : Nous avons étudié dans quelle mesure la concentration de Cl^- dans l'eau atténue les effets de l'exposition hydrique de la truite arc-en-ciel à l'$AgNO_3$ pour examiner la relation entre la réaction physiologique et la concentration d'ions argent (Ag^+). Les truites ont été exposées à une concentration totale d'argent de 30 nmol·L^{-1} (sous forme d'$AgNO_3$), à une série de concentrations de Cl^- dans l'eau allant de 20 à 1 500 µmol·L^{-1}. Ces concentrations ont été choisies par modélisation de la différenciation à l'aide du MINEQL+ de façon à réduire progressivement la concentration d'Ag^+ en le remplaçant en bonne partie par de l'$AgCl_{aq}$ dissous dans l'eau. Les poissons exposés à l'$AgNO_3$ ont connu une baisse de Na^+; l'augmentation de la concentration de Cl^- a réduit cette déperdition. Les mesures in vitro effectuées après 50 h d'exposition ont montré que les déperditions de Na^+ étaient reliées à l'inhibition, induite par l'Ag^+, de l'activité branchiale de la Na^+/K^+ ATPase; l'augmentation de la concentration de Cl^- dans l'eau protégeait contre l'inhibition de l'activité de cette enzyme. La perturbation de la régulation du Na^+ chez les poissons exposés à l'$AgNO_3$ s'accompagnait d'une hausse des concentrations de cortisol plasmatique et d'ammoniac total, mais le pH sanguin, la P_{CO_2} et le HCO_3^- dans le plasma, de même que l'hématocrite, étaient globalement inchangés. La teneur en argent des branchies a augmenté chez tous les poissons exposés à l'$AgNO_3$, et n'était pas corrélée à la perturbation de l'équilibre du Na^+. Nous concluons que les mesures physiologiques, combinées à la modélisation de l'équilibre géochimique hydrique, sont utiles pour élaborer des modèles permettant de prévoir la toxicité aiguë de l'argent présent dans l'eau.

[Traduit par la Rédaction]

Introduction

Recently, silver has become a metal of toxicological interest and regulatory focus in North America and Europe (Hogstrand and Wood 1998). The presence of silver in the aquatic environment can arise as the result of anthropogenic inputs from many sources, including domestic and industrial discharges (Purcell and Peters 1998). In the environment, most silver is complexed to sulfide, chloride, and (or) dissolved organic carbon (DOC), depending on the water chemistry, and ionic silver (Ag^+) is usually <40% (usually substantially less) of total silver (Hogstrand and Wood 1998). $AgNO_3$ can be highly toxic to rainbow trout in freshwater with reported LC_{50} values ranging from about 60 to 120 nmol·L^{-1} or 6.5 to 13 µg·L^{-1} (Davies et al. 1978; Nebeker et al. 1983; Hogstrand et al. 1996). Although silver can be toxic at low concentrations, its acute toxicity is strongly influenced by chemical constituents in the water; Ag^+ is highly toxic whereas complexes of Ag^+ with sulphate, thiosulphate, Cl^-, and DOC are much less toxic (reviewed in Hogstrand and Wood 1998).

The varying acute toxicity of silver resulting from the complexation of Ag^+ to waterborne ligands conforms with the hypothesis proposed by Pagenkopf (1983) that the free ion form of a metal and its interaction with the fish gill are primarily responsible for acute toxicity (reviewed by Campbell 1995). For example, in rainbow trout (*Oncorhynchus mykiss*), the toxicity of $AgNO_3$, which readily dissociates to

Received March 27, 1998. Accepted August 26, 1998. J14517

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Table 1. Measured water Cl^- and total Ag concentration as well as calculated Ag speciation in the water Cl^- treatments used in exposures of rainbow trout to nominal concentrations of 30 nmol·L^{-1} $AgNO_3$. Prediction of Ag speciation was from the MINEQL+ software package and measured water quality variables. Only Ag species in which concentrations were altered by Cl^- content and >1% of the total Ag content are shown.

	Measured conc.		MINEQL+ predicted conc.			
Nominal water Cl^- treatment	Water [Cl] (μmol·L^{-1})	Total [Ag] (nmol·L^{-1})	$[Ag^+]$ (nmol·L^{-1})	$[AgCl_{aq}]$ (nmol·L^{-1})	$[AgCl_2^-]$ (nmol·L^{-1})	[AgDOC] (nmol·L^{-1})
20 (control)	13	0	0	0	0	0
20	14	27.8	24.1	0.7	0.0	2.9
120	115	28.1	20.0	5.1	0.0	2.9
300	292	28.1	15.1	9.8	0.3	2.9
500	538	28.0	11.1	13.2	0.7	2.9
1500	1440	28.3	5.4	17.4	2.7	2.8

form Ag^+, is more than 13 000 times that of $Ag(S_2O_3)_n^{-(2n-1)}$, a compound that does not readily dissociate (Hogstrand et al. 1996). Waterborne Cl^- reduces acute $AgNO_3$ toxicity (LeBlanc et al. 1984; Hogstrand et al. 1996; Galvez and Wood 1997) through the reduction of Ag^+ concentration in the water and its replacement largely by dissolved $AgCl_{aq}$. The protective effect of water Cl^- is dramatic: Galvez and Wood (1997) demonstrated that the 7-day LC_{50} for juvenile rainbow trout increased from 30 to 920 nmol·L^{-1} (3.2 to 99 μg·L^{-1}) total silver in waters with 50 and 2500 μmol·L^{-1} (2 and 89 mg·L^{-1}) Cl^-, respectively. The combined works of Galvez and Wood (1997) and Hogstrand et al. (1996) clearly demonstrated a strong correlation between Ag^+ and acute toxicity because, irrespective of total silver and water Cl^- concentration, the 7-day LC_{50} value, as calculated from the geochemical modelling program MINEQL+ (Schecher and McAvoy 1992), was consistently 28 to 32 nmol·L^{-1} (3.0 to 3.5 μg·L^{-1}) Ag^+.

The primary physiological disturbance, and the presumed mechanism of toxicity, arising from exposure to $AgNO_3$ is an inhibition of Na^+ and Cl^- uptake at the gill with consequent declines in plasma Na^+ and Cl^- concentrations (Wood et al. 1996; Webb and Wood 1998). In turn, this leads to a suite of secondary effects, including blood acidosis, a generalized stress response, increased plasma total ammonia concentration, fluid volume disturbance, and hemoconcentration, which if severe, will lead to death as the result of cardiovascular collapse (Wood et al. 1996; Webb and Wood 1998; reviewed by Hogstrand and Wood 1998). Morgan et al. (1997) showed that this disruption of ion uptake in rainbow trout was primarily due to an inhibition of gill Na^+/K^+ ATPase activity. Na^+/K^+ ATPase is considered to be the key enzyme powering active Na^+ and Cl^- transport in freshwater fish (Wood 1992; Perry 1997). A preliminary study has suggested that the inhibition is due to Ag^+ blocking the binding of Mg^{2+}, a cofactor required for ATP hydrolysis (Ferguson et al. 1997).

The objective of our work was to understand the physiological nature of the protective effect of waterborne Cl^- on the sublethal toxicity of silver to rainbow trout (Hogstrand et al. 1996; Galvez and Wood 1997). All trout were exposed to 30 nmol·L^{-1} (3.2 μg·L^{-1}) total silver (added as $AgNO_3$) and water Cl^- was manipulated to alter the concentrations of Ag^+ and $AgCl_{aq}$ (plus small amounts of $AgCl_2^-$) in different treatments. The relative proportions of the silver species in each treatment were predicted using the geochemical modelling software package MINEQL+ (Schecher and McAvoy 1992). A detailed physiological study was conducted with particular focus on Na^+ balance and gill Na^+/K^+ ATPase activity in light of present knowledge of the mechanism of toxicity (Wood et al. 1996; Morgan et al. 1997).

Material and methods

Fish

Rainbow trout (320 g, n = 39) were obtained from a local commercial trout farm (Humber Springs, Orangeville, Ont.) and reared for at least 1 month in dechlorinated Hamilton tap water (ionic concentration: Na^+, 0.6 mmol·L^{-1}; Ca^{2+}, 1.0 mmol·L^{-1}; Cl^-, 0.7 mmol·L^{-1}; pH 8.0; temperature 15°C) before transfer to, and 3 weeks (minimum) acclimation in, soft water (ionic concentration: Na^+, 0.04 mmol·L^{-1}; Ca^{2+}, 0.04 mmol·L^{-1}; Cl^-, 0.04 mmol·L^{-1}; DOC, 0.3 mg·L^{-1}; pH 7.0), which was made by mixing reverse osmosis (Anderson Water Systems, Dundas, Ont.) partially purified water with dechlorinated Hamilton tap water. Prior to experiments, fish were starved for 2 days, anaesthetised with a $NaHCO_3$-buffered solution of 100 mg·L^{-1} MS222 (tricaine methanesulphonate; Sigma), surgically fitted with chronic indwelling dorsal aorta catheters for repeated blood sampling (Soivio et al. 1975), and transferred to individual 3L black perspex boxes, each with flowing aerated soft water (0.15 L·min^{-1}). Recovery was for 2 days before experimental procedures began, and cannulae were flushed daily with heparinzed (10 U·mL^{-1}) Cortland salmonid saline (Wolf 1963).

Exposure protocol

Fish were exposed for 50 h to 30 nmol·L^{-1} (3.2 μg·L^{-1}) total Ag in combination with a range of Cl^- treatments via a flow-through system that included a head tank into which concentrated $AgNO_3$ and KCl solutions (for adjustment of water $[Cl^-]$) were metered and then mixed with reverse osmosis generated (Culligan Water Systems, Hamilton) soft water (ionic concentration: Na^+, 0.04 mmol·L^{-1}; Cl^-, 0.02 mmol·L^{-1}; Ca^{2+}, 0.04 mmol·L^{-1}; DOC, 0.3 mg·L^{-1}; pH 7.0) by vigorous aeration before distribution to fish boxes. Water Cl^- treatments were chosen to cover the range normally found in fresh water; nominal concentrations ranged from 20 to 1500 μmol·L^{-1} (0.7 to 53 mg·L^{-1}). The measured water Cl^- concentrations and predicted silver speciation of treatments are shown in Table 1. Along with the five treatment groups, a sixth group of trout was subjected to the sampling protocol but not exposed to $AgNO_3$ (controls).

Serial blood samples (600 μL) from the dorsal aorta were taken before treatment (pre-exposure) and after 1.5, 6, 24, and 48 h of exposure. Blood was centrifuged, plasma was saved, and red blood

cells were reconstituted in Cortland saline and returned to the fish via the catheter. After each blood sample, water flow to boxes was suspended for a period of 1.5 to 2 h and the volume was set to 2.7 L. Water samples (15 mL) were collected at the beginning and end of the period of box closure for measurements of net total ammonia and Na^+ fluxes as well as to confirm total silver and Cl^- concentrations. The flux measurements were performed before exposure began (pre-exposure) and then at 1.5–3, 6–8, 24–26, and 48–50 h of exposure.

After the 50-h of exposure, a lethal dose of anaesthetic (250 mg·L^{-1} MS222) was added to the box and the fish were quickly euthanized. Gill samples were collected, rinsed briefly (10 s) in exposure water without silver, immediately freeze clamped in liquid N_2, and then stored at –70°C.

Sample analysis, calculation, and statistics

Whole-blood pH was measured with a glass capillary electrode thermostated to the experimental temperature and connected to an acid–base analyser (PHM 71, Radiometer). Hematocrit was measured after centrifugation (5000 × *g* for 5 min). Plasma total CO_2 content was measured using a Capnicon 5 (Cameron Instruments). Partial pressure of CO_2 (P_{CO_2}) and HCO_3^- concentration in plasma were calculated using the measured blood pH and total CO_2 values together with the pK´ and solubility coefficients for trout plasma from Boutilier et al. (1984). Plasma total ammonia (T_{amm}) concentration was measured with a commercial kit (Sigma 171 UV), water T_{amm} was measured by the salicylate hypochlorite method (Verdouw et al. 1978), and plasma cortisol was measured via radioimmunoassay (Immunocorp).

Water and plasma Na^+ concentrations were determined by atomic absorption spectrophotometry (AA1275 Varian) and water Cl^- was determined by the mercuric thiocyanate method of Zall et al. (1956). Gill filament tissue was digested in 5 volumes of 1 N HNO_3 (Janes and Playle 1995) and the total Ag concentrations of digested tissue and water were measured by graphite furnace atomic absorption spectrophotometry (AA1275 and GTA-95, Varian) as described by Galvez and Wood (1997).

The increases in water T_{amm} concentration that occurred in fish boxes of known volume during intervals when water flow was stopped were used to calculate T_{amm} excretion rates (Wright and Wood 1985). Changes in water Na^+ concentration were used to calculate net flux (Morgan et al. 1997).

Gill filament samples were assayed for Na^+/K^+ ATPase activity using the methods of Holliday (1985), described in detail by Morgan et al. (1997). Na^+/K^+ ATPase activity was expressed as the concentration of inorganic phosphate liberated per unit time and adjusted for the protein content of the sample (μmol PO_4·mg $protein^{-1}\cdot h^{-1}$), the latter was determined using the dye binding assay of Bradford (1976). All spectrophotometric assays were modified for use with 96-well microtitre plates and microplate reader (MRX, Dynex), except for inorganic phosphate measurements of gill sample homogenates, which were measured in a cuvette-style spectrophotometer (LKB).

Results of 2-day exposures of trout to $AgNO_3$ were compared over time for each treatment or among treatments at 48 h by ANOVA and, when appropriate, Duncans New Multiple Range test. In all cases, $\alpha \leq 0.05$ was the accepted level of significance and data are shown as the mean ± 1 SEM.

Results

Control fish exhibited net Na^+ fluxes that did not differ significantly from zero throughout the 50-h experimental period (Fig. 1A). Additionally, plasma ions, acid–base status, and T_{amm} as well as T_{amm} excretion rate and hematocrit were stable in control fish (Table 2). Cortisol declined slightly with time (Fig. 1B). Thus the experimental procedures had minimal influence on the parameters measured.

Exposure to 30 nmol·L^{-1} $AgNO_3$ resulted in a significant disruption in Na^+ balance in rainbow trout, the effects of which were reduced with increasing water Cl^- concentration. At the low Cl^- concentration of 20 μmol·L^{-1}, plasma Na^+ declined continuously and at 48 h differed significantly from pre-exposure measurements (Table 2). In general, the declines in plasma Na^+ concentration by 48 h were less as water Cl^- concentrations increased (Table 2). Changes in plasma Cl^- tended to parallel those in the plasma Na^+, but none of the Cl^- alterations was significant (Table 2). In conjunction with the loss of plasma Na^+, all fish exposed to silver (except those at the highest Cl^- level tested) showed a significant net loss of Na^+, particularly in the first day of exposure (Fig. 1A). In general, the net loss of Na^+ was reduced with increasing water Cl^- level, particularly at 48 h (Fig. 1A). Cortisol was significantly increased in most trout exposed to Ag^+ (Fig. 1B), and during the first day, fish with a significant loss of Na^+ also showed elevated cortisol levels (Figs. 1A and 1B).

Gill Na^+/K^+ATPase activity complemented the pattern of Na^+ loss from $AgNO_3$ exposed trout. In water of low Cl^- concentration, Na^+/K^+ATPase activity was severely inhibited (80%) relative to control values (Fig. 2A), but as Cl^- increased, this inhibition decreased (Fig. 2A). When expressed as a function of the Ag^+ concentration, as calculated by MINEQL+, the inhibition of Na^+/K^+ATPase was highly correlated with Ag^+ ($r^2 = 0.98$, $n = 5$; Fig. 2B).

In spite of the protective effect that increasing Cl^- had on Na^+ loss (Fig. 1, Table 2) and gill Na^+/K^+ ATPase activity (Fig. 2), the accumulation of silver in the gill did not vary. Branchial total Ag content was elevated relative to unexposed fish in all silver-exposed trout and there were no significant differences among Cl^- treatments (Fig. 3).

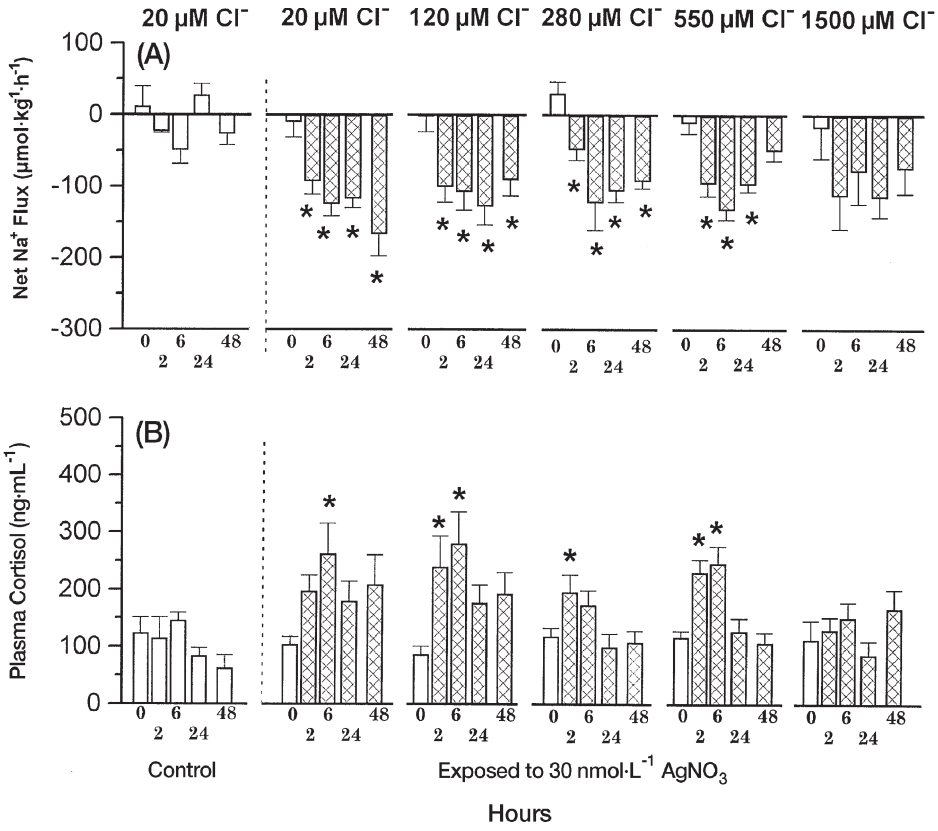
Exposure to waterborne $AgNO_3$ did not result in any consistent acid–base disturbances; but plasma T_{amm} accumulated in all exposed fish and this accumulation was similar among Cl^- treatments (Table 2). In spite of increased plasma T_{amm} content, there was no increase in T_{amm} excretion (Table 2).

Discussion

These experiments support previous reports that water Cl^- has a protective effect on $AgNO_3$ toxicity (Galvez and Wood 1997; Hogstrand et al. 1996) and suggest that at least part of the mitigation of toxicity is via a reduction of the inhibitory effect that silver has on gill Na^+/K^+ ATPase activity. As waterborne Ag^+ and Cl^- form complexes, free Ag^+ concentration is reduced, inhibition of branchial Na^+/K^+ ATPase is reduced, and the disruption of Na^+ balance in the fish is minimized. These results may provide a physiological explanation for the protective effect of water Cl^- on the acute toxicity of $AgNO_3$ (Galvez and Wood 1997; Hogstrand et al. 1996) and provide a model for studying the effects of water chemistry on sublethal silver exposure.

The concept that geochemical characteristics of the water influence the toxicity of waterborne heavy metals has been recognized previously (Babich and Stotzky 1983; Campbell and Stokes 1985; Newman and Jagoe 1994; Allen and Hansen 1996). However, the protective effect that water Cl^- ex-

Fig. 1. (A) The net flux of Na^+ and (B) plasma cortisol concentration in groups of rainbow trout exposed to 30 nmol·L^{-1} total Ag (as $AgNO_3$, cross-hatched bars) over 50 h in water with different water Cl^- treatments ranging from 20 to 1500 μmol·L^{-1}. The net flux was measured over 1.5 to 2 h with positive bars indicating a net movement of Na^+ into fish and negative bars indicating a net loss of Na^+ from fish. Within each water Cl^- treatment, Na^+ flux and plasma cortisol were measured before exposure began (0 h) and at 2, 6, 24, and 48 h and in a group of trout similarly sampled but not exposed to $AgNO_3$ (control, open bars). Mean (± SEM) from at least six fish are shown, except for controls where $n = 4$. * indicates a significant difference from the pre-exposure measurement for that treatment.



erts on the acute toxicity of silver is not specifically recognized in the water quality guidelines–criteria of regulatory bodies such as Environment Canada or the United States Environmental Protection Agency (U.S. EPA). The latter agency does recognize a protective role for one water chemistry variable only, specifically water hardness (U.S. EPA 1980). However, Hogstrand et al. (1996), Galvez and Wood (1997), and Hogstrand and Wood (1998) argued that this protective role for hardness was based on a misinterpretation of original data. Galvez and Wood (1997) suggested that the most important protective agent in the original data set used to derive the U.S. EPA (1980) "hardness equation" for acute Ag toxicity was Cl^- and not hardness, although the latter did exert a relatively minor protective influence. While we did not measure acute toxicity directly, our study supports the conclusion that water Cl^- protects against the deleterious effects of waterborne Ag^+. DOC, a naturally occurring anionic ligand that strongly binds Ag^+ (Janes and Playle 1995), is another geochemical factor that might influence silver toxicity in a similar manner.

Our study also provides more evidence that complexed silver causes significantly less sublethal physiological effect than does Ag^+ and demonstrates that predictions of Ag^+ concentration derived from relatively easy-to-use computer packages correlate well with one of the suggested mechanism of toxicity (Morgan et al. 1997). The combination of a sensitive physiological measure, such as inhibition of gill Na^+/K^+ ATPase activity, with geochemical speciation modelling software packages, such as MINEQL+ (Schecher and McAvoy 1992), may provide a method for developing models that will predict the acute toxicity of silver on a site-specific basis. New approaches based on speciation modelling, such as the gill receptor loading model, have recently been endorsed as possible tools for predicting acute metal toxicity (Bergman and Dorward-King 1997).

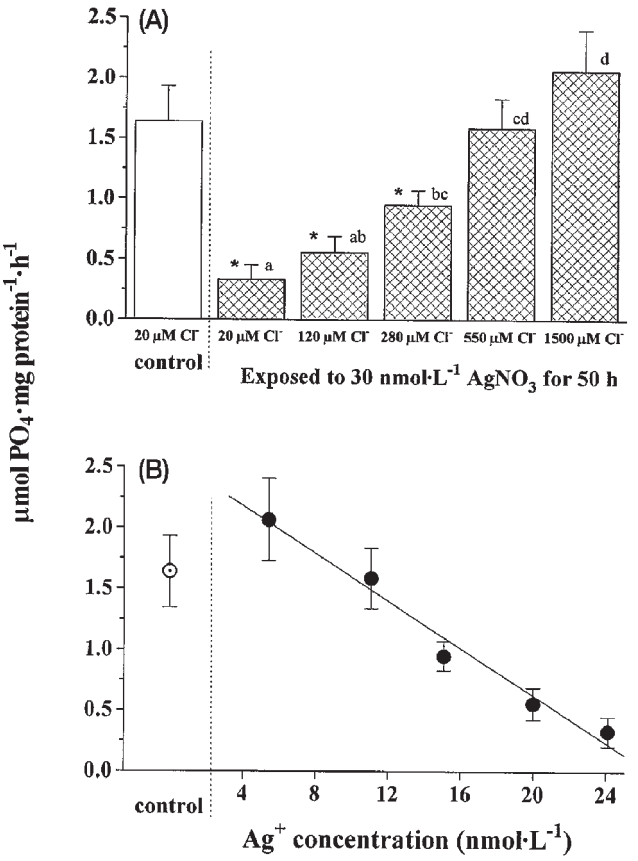
The direct relationship between Ag^+, acute toxicity (Hogstrand et al. 1996; Galvez and Wood 1997), and in this study, sublethal Ag^+ and gill Na^+/K^+ ATPase activity indicates that responses of trout to waterborne silver conform to the gill surface interaction (Pagenkopf 1983) or free ion activity model (reviewed by Campbell 1995). This model is based on the assumptions that different chemical species of a metal have differing toxicities, that the species that are toxic bind to the gill, and that they then exert a biological effect that is exhibited, ultimately, as a toxic effect. Our work indicates that ionic Ag^+ acts on the gill at a specific site, the Na^+/K^+ ATPase enzyme system, in a dose-dependent manner resulting in disruption of Na^+ balance in the fish. The mechanism of sublethal toxicity may therefore be similar to that of Cu^{2+} (Pagenkopf et al. 1974; Laurén and McDonald 1987;

Table 2. Blood and plasma variables from rainbow trout exposed to 30 nmol·L^{-1} total Ag (as $AgNO_3$) over 48 h in different water Cl^- treatments ranging from 20 to 1500 µmol·L^{-1}.

	Treatment		Time of exposure (h)				
	Total [Ag] (nmol·L^{-1})	Water [Cl^-] (µmol·L^{-1})	Pre-exposure	1	6	24	48
Blood pH	0	20	7.86±0.03	7.92±0.05	7.91±0.06	7.82±0.02	7.83±0.03
	30	20	7.88±0.02	7.89±0.02	7.86±0.03	7.90±0.01	7.88±0.04
	30	120	7.85±0.04	7.83±0.03	7.86±0.04	7.91±0.04	7.93±0.05
	30	300	7.84±0.04	7.82±0.03	7.87±0.03	7.87±0.03	7.89±0.04
	30	500	7.88±0.02	7.91±0.04	7.89±0.06	7.92±0.05	7.83±0.05
	30	1500	7.89±0.02	7.86±0.02	7.85±0.03	7.87±0.02	7.84±0.04
Plasma P_{CO_2} (torr)	0	20	2.8±0.2	2.1±0.2	2.4±0.4	3.1±0.5	2.6±0.2
	30	20	2.4±0.1	2.9±0.3	2.9±0.2	2.2±0.4	2.4±0.2
	30	120	2.5±0.1	2.5±0.1	2.2±0.2	2.2±0.1	1.8*±0.1
	30	300	2.5±0.3	3.0±0.2	2.9±0.3	2.9±0.2	2.1±0.2
	30	500	2.8±0.2	2.5±0.2	2.4±0.2	2.1±0.2	1.9±0.4
	30	1500	2.5±0.2	2.6±0.3	2.5±0.2	2.1±0.2	1.7±0.3
Plasma HCO_3^- (mmol·L^{-1})	0	20	7.5±1.2	6.5±1.0	7.2±1.2	7.3±1.2	6.4±0.2
	30	20	6.7±0.5	7.9±0.8	7.7±0.8	6.6±1.1	6.9±0.7
	30	120	6.6±0.6	6.4±0.6	5.8±0.6	6.7±0.4	5.6±0.6
	30	300	6.1±0.7	7.3±0.6	7.5±0.7	7.9±0.6	6.0±0.8
	30	500	7.6±0.6	7.7±0.9	7.0±1.0	6.5±0.8	4.6±0.9
	30	1500	7.1±0.8	6.9±0.8	6.4±0.7	5.6±0.3	4.1*±0.7
Plasma Na^+ (mmol·L^{-1})	0	20	142±2.7	142±5.0	138±4.8	136±3.8	138±4.3
	30	20	137±2.6	134±3.1	130±3.3	129±3.3	121*±3.1
	30	120	139±2.3	138±3.2	138±2.8	135±3.4	131±5.3
	30	300	138±2.7	137±3.7	137±3.4	133±2.8	134±5.2
	30	500	137±5.0	141±5.4	142±3.6	135±3.1	136±2.5
	30	1500	138±4.3	141±4.4	145±5.6	140±6.0	139±5.7
Plasma Cl^- (mmol·L^{-1})	0	20	119±3.9	119±3.5	124±4.5	120±4.8	119±4.8
	30	20	116±4.5	117±4.1	117±3.6	113±3.7	104±6.8
	30	120	121±3.0	122±4.3	121±3.7	118±4.5	113±6.6
	30	300	118±4.6	114±3.6	117±3.5	113±3.6	112±4.1
	30	500	120±3.5	121±3.4	121±3.0	120±3.2	119±4.1
	30	1500	117±3.7	120±4.9	116±5.9	115±6.0	112±6.9
Plasma T_{amm} (µmol·L^{-1})	0	20	78±30	60±24	78±28	41±31	29±18
	30	20	69±15	137*±14	152*±14	161*±22	185*±30
	30	120	41±6	62±13	116*±16	258*±33	181*±28
	30	300	68±8	106±14	154*±18	214*±28	190*±35
	30	500	51±14	108±24	174*±35	280*±49	268*±39
	30	1500	52±10	75±9	149*±5	199*±49	170*±39
T_{amm} excretion (µmol·kg^{-1}·h^{-1})	0	20	343±80	391±73	438±111	375±109	404±104
	30	20	334±27	316±26	339±28	372±43	456±59
	30	120	231±23	246±25	252±26	334±31	328±49
	30	300	258±63	319±57	345±64	394±72	364±53
	30	500	224±27	188±43	271±39	317±31	393±13
	30	1500	300±58	295±39	293±57	403±35	377±47
Hematocrit (%)	0	20	33±1.7	34±1.7	32±3.2	27±2.6	26±3.5
	30	20	31±1.4	29±1.4	30±1.3	32±1.7	30±2.2
	30	120	29±2.1	29±2.1	27±1.8	27±1.9	28±2.3
	30	300	30±2.8	30±1.9	28±1.9	26±2.2	27±2.6
	30	500	33±2.5	31±2.1	30±2.6	27±2.2	28±1.8
	30	1500	27±3.4	27±2.9	27±2.6	29±2.7	34±3.0

Note: Control fish were kept in 20 µmol·L^{-1} [Cl^-], in the absence of $AgNO_3$. Total ammonia excretion was measured over 1.5 to 2 h immediately after blood sampling. Within each treatment, samples were collected before exposure began (0 h) and at 1.5, 6, 24, and 48 h. Mean (± SEM) from at least six fish (except for controls where n = 4) are shown. * indicates a significant difference from the pre-exposure mean for that treatment.

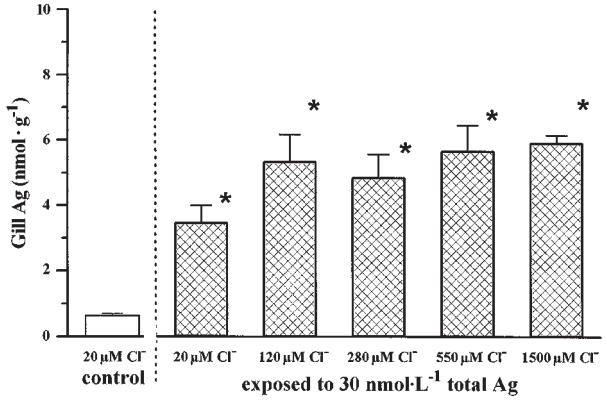
Fig. 2. Relative Na^+/K^+ ATPase activity of gill tissue from rainbow trout exposed to 30 nmol·L^{-1} total Ag (as $AgNO_3$) for 50 h expressed in relation to (A) water Cl^- treatments (cross-hatched bars) and (B) water ionic Ag^+ concentration (solid circles). Ionic Ag^+ concentration was calculated using MINEQL+ software and measured water quality values (Table 1). A group of unexposed fish similarly treated but not exposed to $AgNO_3$ is also shown (controls, open bar in A and open circle in B). Means (± SEM) from at least six fish (except for controls where $n = 4$) are shown. In panel A, * indicates a significant difference from controls ($P < 0.05$), whereas for silver-exposed fish, means tagged with the same letter are not significantly different. In panel B, a best-fit regression line is included (Na^+/K^+ ATPase activity = 2.57 – 0.097 ([Ag^+]), $r^2 = 0.98$, $n = 5$).



Allen and Hansen 1996; Li et al. 1996). This similarity between Ag^+ and Cu^{2+} occurs in spite of the fact that inorganic speciation in freshwater for copper is dominated by hydroxide and carbonate complexation (i.e., pH and alkalinity) whereas that for silver is influenced primarily by Cl^- complexation (Morel and Hering 1993).

Three physiological measures of Na^+ balance were used in these experiments, with activity of gill Na^+/K^+ ATPase being the most sensitive in illustrating the effect of Ag^+ (Fig. 2B). The protective effect of Cl^- on Na^+ balance, as measured by plasma Na^+ values and net flux of Na^+, was significant but less distinct compared with the protection of gill Na^+/K^+ ATPase activity. This may be because plasma Na^+ and net flux of Na^+ are secondary, or more general, measures of Na^+ balance. Plasma Na^+ levels will be influenced by water balance and movements of Na^+ from other internal compartments (e.g., Audet et al. 1988). Net Na^+ flux is the result of unidirectional Na^+ uptake as well as unidirectional Na^+ loss and, therefore, will be a less sensitive measure because Ag^+ exposure affects only unidirectional Na^+ uptake (Morgan et al. 1997; Webb and Wood 1998).

Fig. 3. Total Ag burden in gills of rainbow trout exposed to 30 nmol·L^{-1} total Ag (as $AgNO_3$, cross-hatched bars) over 50 h in water with Cl^- concentrations ranging from 20 to 1500 μmol·L^{-1}. Mean (+ SEM) from at least six fish are shown (except for controls where $n = 4$); * indicates a significant difference from controls. There were no significant differences in gill total Ag burden among silver-exposed fish ($P < 0.05$).



An important finding of the present study was that with increasing water Cl^- concentration, there was no relationship between water Ag^+ concentration and accumulation of silver on the gills. Furthermore, there was no relationship between the gill silver burden and one of the principal physiological effects of Ag^+, the inhibition of branchial Na^+/K^+ ATPase activity. All trout exposed to $AgNO_3$ experienced similar gill total Ag burdens after 50 h (Fig. 3). These results are similar to the data of Janes and Playle (1995), who showed that total Ag accumulation on the gills of rainbow trout after 3 h of exposure to 110 nmol·L^{-1} (12 μg·L^{-1}) $AgNO_3$ in soft water was not affected by water Cl^- concentrations up to 1500 μmol·L^{-1} (53 mg·L^{-1}). Janes and Playle (1995) interpreted this as a result of the higher conditional equilibrium binding constant for Ag^+–gill (log K value 10) compared with that for Ag^+–Cl^- (log K value of 5.3). However, Janes and Playle (1995) interpreted all gill accumulation, including that occurring at high Cl^- levels, as toxic Ag^+ binding. In contrast, the current study suggests that accumulation of Ag in the gill may occur as a result of both Ag^+–gill binding (toxic) and AgCl accumulation (relatively non-toxic). The latter apparently predominated at high water Cl^- levels. The mechanisms of accumulation of AgCl complexes in the gill are unknown but could occur as AgCl–gill binding and (or) as diffusion of neutral $AgCl^0$ into the gill epithelium. The possibility of mixed ligand–metal complexes (e.g., Cl–Ag–gill) was recognized as a potential complicating factor in the development of the free ion activity model (Newman and Jagoe 1994; Campbell 1995). Studies on the gill uptake characteristics of waterborne AgCl complexes (e.g., AgCl–gill conditional equilibrium binding constant or $AgCl^0$ diffusion coefficient) and a further exploration of the relationships between gill total Ag burdens resulting from different

exposure chemistries and toxicity are required if a model to predict acute silver toxicity is to be successful.

The disruption of Na^+ balance in rainbow trout exposed to Ag^+ was accompanied by an increase in plasma cortisol and T_{amm} content but few other significant changes in the measured physiological variables (Table 2). The increase of plasma cortisol was only significant at 2 and (or) 6 h and tended to return towards control levels, particularly for fish in water with elevated Cl^- concentrations. The initial loss of Na^+ may have elicited a stress response because increases in plasma cortisol occurred at about the same time (Figs. 1 and 2). The correlation between cortisol mobilization and ion loss has been documented previously in silver-exposed fish (Webb and Wood 1998). The increase in plasma T_{amm} levels probably reflects cortisol-induced breakdown of proteins (van der Boon et al. 1997) and is characteristic of silver-induced stress. Our study showed a significant increase in plasma T_{amm} levels with only slight increases in T_{amm} excretion rates (not significant), suggesting the possibility that a relative inhibition of T_{amm} excretion may have occurred during $AgNO_3$ exposure (Table 2). As plasma T_{amm} increased, the the blood to water partial pressure gradient for NH_3 increased but this was not accompanied by a significant increase in NH_3 diffusion (i.e., T_{amm} excretion) from the fish. These results are contrary to the recent study of Webb and Wood (1998), which concluded that $AgNO_3$ exposure in relatively hard water did not affect T_{amm} excretion. The reason for this apparent inhibition of T_{amm} excretion in our experiments is unclear but may be related to differences in water chemistry compared with that of Webb and Wood (1998) or the fact that T_{amm} excretion rates were highly variable. The lack of other dramatic physiological effects is indicative of the very specific nature of Ag^+ toxicity and the relatively low level (in terms of acute toxicity) of total silver used during our exposures.

In conclusion, this study illustrates a mechanism through which water Cl^- concentration protects against the effects of waterborne $AgNO_3$ in rainbow trout by its effect on speciation, reducing the concentration of Ag^+ in the water column. A key physiological response, disruption of Na^+ balance as a result of poisoning of gill Na^+/K^+ ATPase activity, is directly related to the Ag^+ concentration of the water. Complexes of $AgCl_n^{1-n}$ do not appear to elicit a toxic physiological response but do result in accumulation of silver in the gill tissue. The results provide more evidence that speciation, particularly in relation to Cl^-, should be considered in assessing the acute toxicity of silver. Further, they suggest that inhibition of gill Na^+/K^+ ATPase activity, rather than total gill Ag burden, is the appropriate end-point on which to build future predictive models of acute silver toxicity (Bergman and Dorward-King 1997). While not precluding other Ag–gill binding data that may become available in the future, our experiments demonstrate that gill Na^+/K^+ ATPase activity is a very good predictor of gill–Ag^+ interactions and therefore provides a mechanism by which physiologically damaging interactions (gill–Ag^+) can be separated from those that are relatively innocuous (e.g., gill–AgCl). Incorporation of these interactions into acute toxicity prediction models, such as the gill receptor loading model, may prove to be successful for accurately predicting site-specific water toxicity.

Acknowledgements

This work was supported by grants to CMW from Kodak Canada and the Natural Sciences and Engineering Research Council of Canada (NSERC) IOR program. JCM was supported by an NSERC postdoctoral fellowship. I. Morgan and N. Bury are thanked for advice. The input of J. Gorsuch, T. Bober, K. Robillard, T. Purcell, and M. Ritter was also appreciated.

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