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THE TOXICOLOGY AND PHYSIOLOGY OF WATERBORNE AND DIETARY SILVER EXPOSURE IN FRESHWATER FISH

By

FERNANDO GALVEZ, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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EFFECTS OF SILVER EXPOSURE ON FRESHWATER FISH

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AUTHOR: Fernando Galvez, B.Sc. (McMaster University)

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ABSTRACT

Water quality criteria regulating silver discharge to the environment have been heavily scrutinized by both the photographic industry and the regulatory community over the last decade. In the United States, there is now a general consensus that the present regulatory framework, which is based on work done in the 1970's, fails to appropriately assess the impact of silver in the environment. Nonetheless, other countries have begun developing water quality criteria for silver which are in many cases based on this same old toxicological information.

The present thesis addresses the need for new information, and specifically characterizes the toxicological, physiological and biochemical responses of freshwater fish to waterborne and dietary exposures of silver. This thesis provides concrete evidence that acute waterborne toxicity in juvenile rainbow trout is produced by the free Ag^+ ion, yielding 4 to 7-day LC50 values ranging from 3.1 to 5.5 µg/L Ag^+. In comparison, LC50 values varied by as much as 30-fold during toxicity tests in which complexing agents such as chloride and dissolved organic carbon were manipulated. Using these data, together with other recently published results from physiologically-based studies, a new acute toxicity model has been developed within a Biotic Ligand Modeling (BLM) framework where the geochemistry of the receiving water is taken into account. This new acute toxicity model is unique in that it relates toxicity to a prediction

of the binding of Ag^+ to toxic sites on the gill, rather than to a prediction of total gill silver load. This distinction is important because the total bioaccumulated silver load appears to be unrelated to the acute toxic response. Silver accumulated in the liver is shown to bind to metallothionein within the cell, possibly explaining why silver can accumulate to high levels in the liver during waterborne silver exposure with no apparent toxicity to the fish. Chronic exposure to low levels of silver results in a significant disturbance to plasma Na^+ and Cl^- at levels from 0.1 to 0.5 µg/L total silver. The ionoregulatory disturbance is mild in comparison to acute waterborne exposure to LC50 levels of silver, and requires longer to be fully manifested. Interestingly, fish show physiological acclimation to silver-induced perturbations in ion balance after only 7 to 16 days, and toxicological acclimation to 3.0 µg/L total silver exposure after 23 days. Acclimation appears to be associated with enhanced branchial Na^+ influx and Na/K-ATPase activity (the target of Ag^+), despite the continued presence of metal in the water. Considering the level of silver required to elicit toxicological acclimation, it is unlikely that acclimation will significantly impact the predictive capability of the acute toxicity model. Finally dietary exposures to silver sulfide up to 3,000 µg/g and a biologically incorporated silver up to 3.1 µg/g produced no physiological impairment in juvenile rainbow trout during long-term studies. Interestingly, the biologically incorporated form of silver was accumulated in the livers of fish over four orders of magnitude better than dietary silver sulfide. The fact that the bioavailability of dietary silver is influenced by speciation suggests that a Biotic Ligand Modeling approach may be plausible for dietary silver exposure.

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Wow! So this is the time when in a few short sentences I am suppose to acknowledge everyone who has been instrumental in the completion of my Ph.D. Not really an easy task considering the length of my stay here.

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THESIS ORGANIZATION AND FORMAT

This thesis is presented in an "open-faced" format approved by McMaster University and with the recommendation of my supervisory committee. This thesis consists of nine chapters. Chapter 1 provides a brief introduction, and a summary of the scope and implications of the major findings from the studies enclosed within. Chapters 2-9 have either been published or submitted for publication in scientific journals.

Chapter 1: Background, scope and implications of the study.

Chapter 2: The relative importance of water hardness and chloride levels in modifying the acute toxicity of silver to rainbow trout (*Oncorhynchus mykiss*).

Authors: F. Galvez and C.M. Wood

Date accepted: April 1997

Journal: *Environmental Toxicology and Chemistry* 16: 2363-2368. (1997) Copyright Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL

Comments: This study was conducted and written by F.G. under the supervision of C.M.W.

Chapter 3: Effects of chloride, calcium and dissolved organic carbon on silver toxicity: comparison between rainbow trout and fathead minnows.

Authors: N.R. Bury, F. Galvez, and C.M. Wood

Date accepted: July 1998

Journal: *Environmental Toxicology and Chemistry* 18: 56-62. (1999) Copyright Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL

Comments: This study was performed jointly by N.R.B. and F.G. Each contributed equally to the design and completion of the study. The manuscript was written by N.R.B. (based on lot), with support from F.G. and supervision from C.M.W.

Chapter 4: A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to rainbow trout in fresh waters.

Authors: J.C. McGeer, R.C. Playle, C.M. Wood, and F. Galvez

Date submitted: October, 1999

Journal: *Environmental Science and Technology*

Comments: The modelling scenarios were initially presented by F.G. and J.C.M. at a policy meeting held at Eastman Kodak (April 1999). Some of the key ideas came from C.M.W. and R.C.P. The ideas were further developed during the course of the meeting from discussion with many people. Authorship was based on lot. J.C.M. wrote the bulk of the manuscript with input from F.G., C.M.W., and R.C.P. Much of the Materials and Methods, and Results sections was written by F.G.

Chapter 5: Physiological responses of juvenile rainbow trout to chronic low level exposures of waterborne silver

Authors: F. Galvez, C. Hogstrand, and C.M. Wood

Date accepted: June 1997

Journal: *Comparative Physiology and Biochemistry,* 119C: 131-137. (1998) Copyright Elsevier Science Inc, Oxford, England.

Comments: This study was performed jointly by F.G. and C.H. at McMaster University during a visit by C.H. from the University of Kentucky. Each contributed equally in the design and completion of the study. The manuscript was written by F.G. with input from C.H. and supervision from C.M.W.

Chapter 6: The mechanisms and costs of physiological and toxicological acclimation to waterborne silver in juvenile rainbow trout (*Oncorhynchus mykiss*).

Authors: F. Galvez and C.M. Wood

Date submitted: January 2000.

Journal: Aquatic Toxicology

Comments: This study was conducted and written by F.G. under the supervision of C.M.W.

Chapter 7: The distribution of waterborne silver-110m in juvenile rainbow trout.

Authors: F. Galvez, G.D. Mayer, C.M. Wood, and C. Hogstrand.

Date submitted: January 2000.

Journal: *Comparative Physiology and Biochemistry, C.*

Comments: This study was conducted jointly by F.G. and G.D.M. in the laboratory of C.H. at the University of Kentucky. Each contributed equally in the design and completion of the study. The paper was written by F.G. with input from G.D.M. All work was done under the supervision of C.H. and C.M.W.

Chapter 8: The physiological effects of dietary silver sulfide exposure in rainbow trout.

Authors: F. Galvez and C.M. Wood

Date accepted: August 1998.

Journal: *Environmental Toxicology and Chemistry*, 18: 84-88. (1999) Copyright Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL

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Chapter 9: The physiological effects of a biologically incorporated silver diet in rainbow trout (*Oncorhynchus mykiss*).

Authors: F. Galvez, C. Hogstrand, J.C. McGeer, D. Bureau and C.M. Wood.

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CHAPTER 1

BACKGROUND, SCOPE, AND IMPLICATIONS OF THE STUDY

BACKGROUND

Several reviews have recently been published highlighting the research on silver performed over the last 7 years (Hogstrand and Wood, 1998; Purcell and Peters, 1999; 1999; Ratte, 1999; Wood *et al.*, 1999). This work has been funded predominantly by the photographic industry due to their growing need to understand the fate, transport and effects of silver in the environment. The photographic industry represents the major source of silver waste to the aquatic environment and is therefore expected to be significantly influenced by environmental regulations. Silver regulations were first established in the United States in the early 1960's to control the levels of silver in drinking water according to the Safe Drinking Water Act. Although it was well established at the time that oral exposure to silver posed few health risks to humans, limits of 50-100 µg/L were nonetheless set to counteract potential cosmetic effects ("argyria") resulting from silver accumulation in tissues. At these high concentrations, the photographic industry was not severely impacted (Purcell and Peters, 1999). This scenario changed with the inauguration of the Clean Water Act of 1965, which mandated that Ambient Water Quality Criteria (AWQC) be established for all chemicals deemed to be "priority pollutants". The U.S. Environmental Protection Agency (US EPA) formulated this category of pollutants from the chemicals controlled under the Safe Drinking Water Act.

Ambient Water Quality Criteria were promulgated in the late 1970s and early 1980s to protect aquatic organisms from the effects of anthropogenic discharge of toxic compounds. In the case of silver, a round-robin program was established by the US EPA to evaluate the toxicity of waterborne silver (Lemke, 1981). Toxicity tests were performed using silver nitrate under laboratory conditions. Results from these studies indicated that silver, as silver nitrate, is one of the most toxic metals to aquatic organisms. As a result of this report, an AWQC was promulgated for silver (US EPA, 1980). The AWQC sets a limit for allowable total silver concentration in natural water and is calculated according to the following equation:

$$[Ag] = e^{(1.72*[\ln \text{hardness}]-6.52)} \qquad \text{Eq. 1}$$

where hardness is expressed in mg/L of $CaCO_3$ equivalents. At representative hardness levels of 50, 100 and 200 mg/L, the US EPA AWQC for silver is 1.2, 4.0, and 13.4 µg/L. The AWQC was designed to protect against acute toxicity, representing a concentration which should not be exceeded for more than 1 h, every 3 years.

In Canada, a much more stringent national guideline of 0.1 µg/L total silver, designed to protect against both acute and chronic toxicity, has been developed for the protection of aquatic organisms (CCME, 1995). It essentially represents a value which should never be exceeded in natural waters. Australia (NWQMS, 1999) and Europe (RIVM, 1999) have recently proposed a similar limit. This value is based on two direct studies performed on early life stages with freshwater fish (Davies *et al.*, 1978; Nebeker *et al.*, 1983), and on indirect extrapolation from acute toxicity values by various acute-to-chronic (ACR) approaches. Most importantly, these studies were also performed using silver nitrate in pristine laboratory water. It is the photographic industry's contention that

the present set of environmental criteria for silver are overly stringent and have produced an unnecessary economic burden to the regulated community. This is partly supported by the fact that regulations were all derived from studies performed with silver nitrate. The photographic industry discharges most of its silver waste as silver thiosulfate, which is known to be much less toxic than silver nitrate (LeBlanc *et al.*, 1984; Hogstrand *et al.*, 1996).

Prior to the start of the silver toxicology research program at McMaster University in 1993, virtually nothing was known of the physiological mechanism of silver toxicity to aquatic organisms. In the first study, Hogstrand *et al.* (1996) made several important observations which would be central to a large portion of my doctoral thesis work.

- The toxicity of silver in rainbow trout is greatly dependent on the speciation of the metal in water. The authors suggested that silver nitrate is extremely toxic to fish because it dissociates readily to form free ionic silver, Ag^+. Reciprocally, complexes such as silver thiosulfate are relatively benign to freshwater fish due to the high affinity of Ag^+ for thiosulfate anion.
- Silver accumulates readily in internal tissues such as the liver, although this elevated tissue silver burden does not appear to be of toxicological importance, at least in an acute sense.
- Bioaccumulated silver is a powerful inducer of metallothionein in fish.
- The equation of the US EPA AWQC (Eq. 1) for silver appeared to be inherently flawed due to over-dependence on hardness as a modifier of acute silver toxicity. Instead, re-analysis by Hogstrand *et al.* (1996) of data used to derive the criterion

(Lemke, 1981) suggested that chloride should be incorporated into the regulation, as a much more effective protective agent than hardness. Like the thiosulfate ion, the Cl^- ion also complexes Ag^+, although its affinity is lower.

Shortly thereafter, Wood *et al.* (1996a,b) demonstrated that acute toxicity associated with silver nitrate exposure severely impaired sodium and chloride regulation in the blood plasma of rainbow trout. This reduction in plasma Na^+ and Cl^- concentrations elicited a cascade of responses leading to reduced plasma volume, and death, likely from cardiovascular collapse. Furthermore, physiological effects were only apparent when fish were exposed to silver nitrate, but not silver thiosulfate, even though the exposure concentration in the latter was over 3,000-fold higher. This again supported the importance of speciation in modifying silver toxicity.

These three studies form the main foundation of my Ph.D. thesis and will be quoted extensively throughout. Moreover, the work presented in this thesis represents only a portion of the work completed at McMaster University, and more recently by collaborators at Wilfrid Laurier University (Waterloo, ON) and the University of Kentucky (Lexington, KY). As a result, the scope of my Ph.D. thesis (given below) is intertwined with the findings of other investigators in order to form a coherent story.

SCOPE OF THESIS

This thesis employed a controlled laboratory approach to elucidate the biochemical, physiological and toxicological responses of freshwater fish to waterborne

and dietary silver. The thesis can be divided in three sections that include: i) acute waterborne toxicity, ii) chronic waterborne toxicity and iii) bioaccumulation and food route toxicity. It is composed of 8 papers from 7 separate studies.

Acute waterborne toxicity

This section contains 3 papers from 2 separate series of experiments. One of the goals of Chapter 2 (Galvez and Wood, 1997) was to ascertain the relative importance of aqueous calcium and chloride levels in modulating the acute toxicity of silver nitrate to juvenile rainbow trout. This work directly tested suggestions made by Hogstrand *et al.* (1996) that chloride rather than hardness is the protective agent against silver toxicity. The overall objective was to evaluate the validity of hardness-based Ambient Water Quality Criteria for silver currently employed by the US EPA (1980) and British Columbia (BC MOELP, 1995). Toxicity tests were performed at a total silver concentration of 100 μg/L in synthetic soft water generated by reverse osmosis. Calcium and chloride concentrations in the test water were independently varied from 50 to 5,000 μM with either $Ca(NO_3)_2$ or $CaSO_4$, and NaCl or KCl, respectively. Median lethal time (LT50 or ET50) was calculated using log probit analysis. Chloride was over ten-fold more protective against acute silver toxicity than calcium on an equimolar basis. Moreover, both Ca salts protected equally well, whereas NaCl was slightly more protective against acute silver toxicity than was KCl.

The second portion of Chapter 2 outlines work performed to test the hypothesis that silver toxicity is solely due to the free Ag^+ concentration and not a function of total silver concentrations in the water. This hypothesis was tested using 7-day toxicity tests

under static-renewal conditions. LC50 represents the concentration of a toxicant resulting in 50 % mortality in the specified time period. In the first series, Ag^+ concentrations were manipulated by maintaining the total silver concentration at 100 µg/L but varying chloride concentrations from 1000 to 3,750 µM using NaCl. In the second series of tests, toxicity tests were performed at either 50 or 225 µM Cl^- but with varying total silver concentrations. Geochemical speciation analysis with the modelling program MINEQL+ (Schecher and McAvoy, 1992) was used to calculate the Ag^+ concentrations at each of the measured LC50s. These results were the first to demonstrate that acute silver toxicity in rainbow trout is solely due to the free Ag^+ concentration of the water, with a 7-day LC50 occurring at approximately 3.1 µg/L of free Ag^+. In comparison, on the basis of total silver concentration, the 7-day LC50 varied by as much as 30-fold (i.e., 3 to 100 µg/L of total Ag) when chloride concentrations ranged from 50 to 2,500 µM (a typical range for natural freshwaters). This study proposed that any factor altering Ag^+ availability via complexation will be expected to alter acute silver toxicity to rainbow trout.

In comparison to the dramatic influence of aqueous chloride concentration on silver toxicity to rainbow trout, other laboratories have reported only a very minor role of chloride on silver toxicity in fathead minnows (Brooke *et al.*, 1996; Karen *et al.*, 1999). Prior to the study reported in Chapter 3, recent studies had suggested that dissolved organic matter (DOM) was likely an important modifier of acute silver toxicity based on its ability to complex free Ag^+ (Janes and Playle, 1995). Consequently, the first purpose of Chapter 3 (Bury *et al.*, 1999a) was to assess the influence of the silver complexing agents, Cl^- and DOM, on acute toxicity in rainbow trout versus fathead minnows.

Similarly, the validity of the hardness-based AWQC of the US EPA (1980) and BC MOELP (1995) for silver was tested using fathead minnows. The experiment was unique in that test chambers were designed so that both fish species could be tested simultaneously within one chamber. Tests were performed in synthetic soft water generated by reverse osmosis. Aqueous chloride, calcium and DOM concentrations were varied from 50 to 1,500 µM with NaCl, 50 to 2,000 µM with $CaNO_3$, and 0.3 to 5.8 mg carbon/L with humic acid, respectively. These results demonstrated that chloride is much more protective against silver toxicity in rainbow trout compared to fathead minnows. In comparison, increasing the concentration of DOM in water protected both species almost equally well against silver, whereas calcium was only slightly protective. The latter finding confirmed the inadequacy of hardness-based criteria for silver.

The second objective of Chapter 3 was to test the hypothesis that Ag^+ is the primary toxic moiety. Geochemical speciation analysis (using measured water chemistry at the LC50 concentrations) corroborated the findings from Chapter 2. In rainbow trout, the 96-hour LC50 occurred at ~5.5 µg/L Ag^+ whereas LC50 values based on total silver concentration ranged from 7.5 to 27.7 µg/L. In contrast, the free Ag^+ concentration at the LC50 progressively decreased with increasing aqueous chloride concentrations for fathead minnows. Nevertheless, Ag^+ levels were predictive of toxicity in fathead minnows when DOM was used as the complexing agent. These data suggest that weak silver complexes such as AgCl (Log $K = 3.2$) may be available to fathead minnows, whereas strong silver complexes such as AgDOM (Log $K = 9.0$) are relatively non toxic.

The results from Chapter 2 and 3 demonstrate that geochemical speciation of silver in the water column must be considered when deriving water quality guidelines.

Moreover, the chapters conclude that the present hardness-based equation for silver is not predictive of acute silver toxicity in fish. In contrast, Ag^+ (and possibly other weak silver complexes) is/are the main silver species driving acute silver toxicity in freshwater fish. In the absence of appropriate technologies for measuring Ag^+ at environmental concentrations, geochemical speciation modelling as performed in Chapter 2 and 3 may help overcome this void. There are however problems with simply adopting a Free Ion Activity Model (Campbell,1995) approach in the development of environmental guidelines. Most notably, the ameliorating effects of competitive cations (such as Ca^{2+}) on acute silver toxicity are not incorporated into its modelling framework. Because (1) Ca^{2+} does not complex with Ag^+ and (2) the concentrations of all inorganic and organic ligands for Ag remained constant, the concentration of Ag^+ must have increased as the LC50 of total Ag in those exposure waters increased. Thus, the concentration of Ag^+ was not a constant predictor of acute toxicity (*sensu* Meyer *et al.*, 1999), and competition (*sensu* Playle, 1998) between Ca^{2+} and Ag^+ for binding to the fish gills must have been an important modifier of Ag toxicity.

One way of overcoming this discrepancy is to use a gill-loading model as first proposed by Pagenkopf (1983) and Morel (1983), and later developed by Playle *et al.* (1993b) for Cu and Cd and by Janes and Playle (1995) for Ag. The model operates on the premise that, in order for a metal to elicit toxic effects, it must first interact with negatively-charged sites on or in the organism. Considering the gill is the primary target of metals, it has been suggested that gill metal burden may be a useful biomarker of acute toxicity. The gill model as developed by Playle (1998) and others uses conditional stability constants (log *K*) for the interaction of metals and competing cations at the gills

of freshwater fish. These values can then be incorporated into geochemical speciation programs such as MINEQL+ (Schecher and McAvoy, 1992) to predict short-term metal accumulation on the gills of freshwater fish. In essence, this Biotic Ligand Modelling approach is similar to the Free Ion Activity Modelling approach except that conditional equilibrium stability constants for interactions of metals with other cations on the biological surface (i.e. gill) are also included into the modelling framework.

Janes and Playle (1995) have derived a series of conditional stability constants for silver and competing cations, and used them to successfully predict silver accumulation at the gills of fish exposed to silver in laboratory and natural waters. Although, in concept, their model could be used as a regulatory tool, it assumes that a link exists between gill silver accumulation and acute toxicity (Bergman and Dorward-King, 1997). Results presented in Chapters 2 and 3 demonstrate that chloride concentrations as low as 250 µM protect rainbow trout against acute silver toxicity, whereas Janes and Playle (1995) suggest that over 1,500 µM chloride are required to prevent accumulation of silver on the gill. Furthermore, a recent study by McGeer and Wood (1998) found that increasing waterborne chloride significantly protected gill Na/K-ATPase against waterborne silver but did not influence gill silver load at 48 hours. Bury *et al.* (1999b) also noted that aqueous chloride concentrations as low as 300 µM and DOC concentrations of 1.25-2.5 mg C/L protected against Ag-induced gill Na/K-ATPase inhibition, whereas gill silver burdens at 6 hours were unaffected by these treatments. Geochemical speciation modelling demonstrated an extremely good correlation between gill Na/K-ATPase inhibition and Ag^+ concentration. In both cases, 50 % inhibition of Na/K-ATPase activity (IC50) was produced by ~1.72 µg/L (16 nM) of free Ag^+.

Moreover, the extent of Na^+/K^+-ATPase inhibition was not related to the magnitude of silver accumulation on the gills.

The goal of Chapter 4 (McGeer *et al.*, 2000) was to develop a series of modelling scenarios for use within a predictive Biotic Ligand Model for acute silver toxicity. Conditional stability constants (log *K*) used in Chapter 4 were either taken directly from the scientific literature or calculated by us from available published data (Janes and Playle,1995; Morgan *et al.*, 1997; Hogstrand and Wood,1998; McGeer and Wood, 1998; Bury *et al.*, 1999a,b). All modelling scenarios were initially tested against the measured LC50 data presented in Chapter 3. Predicted LC50 estimates were compared to measured LC50 values using correlation analysis. The acute toxicity model used the geochemical speciation program MINEQL+ (Schecher and McAvoy, 1992) with the addition of values for interactions of metals and competing cations on the gills of freshwater fish. LC50 data from Chapter 3 were best predicted when a log *K* value of 7.6 for the affinity of Ag^+ on the gill was used together with cationic competition log *K* values of 2.9 and 2.3 for Na^+ and H^+, respectively. Correlation analysis of the predicted versus measured LC50 values yielded a r value of 0.94 ($p<0.001$) and a slope of 1.13. The log *K* for the gill-Ag interaction was determined based on the concentration of Ag^+ required to inhibit Na/K-ATPase by 85 % (McGeer and Wood, 1998; Bury *et al.*, 1999b), which according to Morgan *et al.* (1997) represents the gill Na/K-ATPase inhibition seen in rainbow trout exposed to LC50 levels of silver. This modelling scenario was further validated against a larger selection of published LC50 data for rainbow trout from studies in which water chemistries were adequately reported. Once again, the acute toxicity model presented in Chapter 4 provides a good match with 31 LC50 values from 10

studies. This acute toxicity model represents an alternate set of conditional stability constants derived from physiologically-based studies, and accounts for the effect of concentration, complexation and competition reactions of waterborne ligands at sites on the gill of toxicological relevance to silver.

The acute toxicity model in Chapter 4 represents a method for developing site-specific acute AWQC by simply characterizing water chemistry. An analogous Biotic Ligand Model (Paquin *et al.*, 1999) based to a large extent on our data, has been provisionally adopted by the US EPA as a site-specific modification to current acute AWQC. The eventual intention is to entirely replace the current AWQC with this modelling approach.

Chronic waterborne toxicity

It has been known since the advent of clean analytical techniques that normal environmental concentrations of silver are extremely low, ranging from 10 to 100 ng/L (Shafer *et al.*, 1998). It is therefore necessary to understand the physiological effects of silver exposure at more environmentally relevant concentrations of silver. Chapters 5 and 6 present two papers consisting of six long-term exposures (> 23 days) on juvenile rainbow trout exposed to relatively low concentrations of waterborne silver nitrate in Hamilton dechlorinated tap water (moderately hard water).

In Chapter 5 (Galvez *et al.*, 1998), two separate 28-day exposures were performed at total silver concentrations of 0.5 and 2 μg/L under flow-through conditions. Fish were fed to satiation daily in order to assess the effects of silver on food consumption and fish growth. Other parameters of interest included plasma Na^+ and Cl^- balance, liver silver

burdens and hepatic metallothionein levels. Exposure to 0.5 μg/L silver resulted in a slight increase in food consumption, although growth rates remained unaltered. Both plasma Na^+ and Cl^- levels were significantly decreased by day 16. In comparison, exposure to 2 μg/L silver resulted in a dramatic reduction in food consumption and fish growth. In addition, the reductions in plasma ion concentrations were more pronounced than at 0.5 μg/L Ag, and occurred more rapidly, a significant effect occurring by 7 days. In both silver exposures, plasma Na^+ and Cl^- returned to control concentrations by the end of the experiment, suggesting that fish were acclimating to silver, at least in a physiological sense. Low concentrations of silver produced ionoregulatory disturbances qualitatively similar, although quantitatively smaller than those seen during acutely lethal silver exposures (Wood *et al.*, 1996a). Moreover, the recovery in ion regulation was not related to the induction of metallothionein in the liver since elevated concentrations of the protein were only seen in fish exposed to 2 μg/L Ag.

The purpose of Chapter 6 (Galvez and Wood 2000) was to assess the nature of the acclimatory response observed in Chapter 5. True acclimation, in a toxicological sense, is characterized by an increased tolerance to an elevated, otherwise lethal concentration of a toxicant, arising from previous chronic exposure to that toxicant (McDonald and Wood, 1993). Chapter 6 contains results from 2 separate experiments in which juvenile rainbow trout were exposed over 23 days to silver concentrations of 0.1, 1, 3 and 5 μg/L total Ag (as $AgNO_3$) in Hamilton dechlorinated tap water. The free Ag^+ concentrations were <0.01, 0.03, 0.15 and 0.37 μg/L, respectively. To monitor changes in lethal tolerance, either 4-day or 7-day toxicity tests were conducted throughout the experimental period. In addition, a variety of physiological parameters were investigated

on days 0, 5, 10, 15 and 23 of silver exposure, including plasma ion concentrations (Na^+, Cl^-), unidirectional Na^+ influx, and gill Na/K-ATPase activity. The physiological cost of silver acclimation was assessed using food consumption, growth, food conversion efficiency and swimming performance as indices. Only the 5 μg/L Ag treatment produced significant physiological cost of acclimation manifested in significant reductions in specific growth rate, cumulative food consumption, food conversion efficiency, and critical swimming speed. While all treatments showed signs of physiological acclimation (i.e. recovery in ion regulation), only the 3 and 5 μg/L Ag treatments produced toxicological acclimation as suggested by significant increases in LC50s by day 23. Because toxicological acclimation is elicited in freshwater trout well above the concentrations of silver found in the environment (Shafer *et al.*, 1998), the phenomena probably will not be observed in feral fish. Thus, toxicological acclimation should not greatly complicate the generation of regulatory guidelines by modelling approaches. Furthermore, sublethal physiological effects might occur at total silver concentrations as low as 0.1 μg/L, in accord with recent guidelines in Canada (CCME, 1995), Europe (RIVM, 1999) and Australia (NWQMS, 1999).

Bioaccumulation and food route toxicity

Several studies have suggested that waterborne silver is readily accumulated in freshwater fish regardless of the speciation of the metal (Hogstrand *et al.*, 1996; Wood *et al.*, 1996b; Galvez *et al.*, 1998). Wood *et al.* (1996b) noted extremely high concentrations of silver in the liver of fish exposed to silver thiosulfate. Nonetheless, the accumulated metal did not appear to elicit acute toxic responses to fish. The benign

nature of the bioaccumulated silver may in part be due to its ability to significantly induce metallothionein production, especially in the liver (Cosson, 1994; Hogstrand *et al.*, 1996; Wood *et al.*, 1996b; Galvez *et al.*, 1998). Metallothionein is a low molecular weight protein (6,000-7,000 daltons) known to bind strongly to intracellular metals. Metallothionein is believed to play a role in the metabolism of essential metals such as Cu and Zn, and may be involved in the detoxification of non-essential metals (e.g., Ag, Cd, Hg). Unfortunately, no studies have addressed whether bioaccumulated silver in fish is actually bound to metallothionein *in vivo*. As a result, the objectives of Chapter 7 (Galvez *et al.*, 2000a) were to investigate the uptake, internal distribution, fate and elimination of silver in juvenile rainbow trout during chronic waterborne exposure. Fish were given a 2-day pulse of radioactive ^{110m}Ag (as $AgNO_3$) and subsequently placed in a tracer-free silver nitrate solution (3.8 μg/L Ag) for another 19 days in Lexington, Kentucky dechlorinated tap water (moderately hard water). The radioactive pulse was found to accumulate predominantly in the liver by the end of the experiment. Subcellular fractionation of the liver showed that 35 to 72 % of the total tissue burden of silver distributed within the cytosolic fraction. Further fractionation of the liver suggested that the cytosolic ^{110m}Ag pulse was primarily associated with proteins of molecular weight characteristic of metallothionein. In contrast, gill silver was distributed predominantly within the nuclear fraction of the cell. Moreover, gel chromatography of the gill cytosol showed that ^{110m}Ag eluted almost exclusively in the heavy molecular weight fractions, and only marginally with metallothionein.

As previously mentioned, the final form of most of the silver in the natural environment is silver sulfide, which tends to accumulate in sediments. The

concentrations of silver in sediments make it possible for accumulation of silver by benthic organisms. These organisms may be subsequently consumed by pelagic fish. The objective of Chapter 8 (Galvez and Wood, 1999) was to investigate the physiological effects of dietary silver sulfide exposures to ~0, 3.0, 30, 300 or 3,000 μg/g total Ag for 58-days. Diets were prepared from commercial feed, which was ground into a fine powder and was then mixed with various amounts of silver sulfide. Fish were fed once daily, and food consumption, growth and silver accumulation measured throughout. Daily food consumption rates were lowered by 14 to 22 % in all the silver sulfide treatments relative to the control diet. Despite the reduction in food consumption no significant effects on fish growth were noted. Overall, dietary silver sulfide exposure at concentrations up to 3,000 μg/g Ag elicited few effects except for a four-fold increase in liver silver concentrations by day 58.

Chapter 9 (Galvez *et al.*, 2000b) expands on our investigation of the effects of dietary silver exposure in rainbow trout. In this study, a silver-enriched diet containing 3.1 μg/g Ag (i.e., the low end of the range used for silver sulfide) was prepared from the carcasses of rainbow trout previously exposed for one week to 100 mg/L total Ag as waterborne silver thiosulfate. In addition, a series of control diets was prepared similarly, except that fish were exposed to either sodium thiosulfate or Hamilton dechlorinated tap water. The final concentrations of silver in these control diets were approximately 0.05 μg/g. Diets were subsequently fed daily to juvenile rainbow trout for a four-month period. Two treatments of fish were fed either the control or silver diets to satiation. The remaining three treatments were fed either the control, thiosulfate or herring diets, at a ration identical to the silver treatment (pair fed controls). Fish fed the

silver-laden diet showed no physiological impairment based on food consumption rates, growth rates, ammonia and urea excretion rates, oxygen consumption, sodium influx, plasma ions and intestinal Na/K-ATPase and amylase activities. In contrast, biologically incorporated silver was readily accumulated in the liver and intestines. Liver silver concentrations were elevated approximately 12-fold after only 36 days. Despite this accumulation, hepatic zinc and metallothionein concentrations remained constant, whereas liver copper concentrations were significantly lower on day 16 only. The Concentration Specific Accumulation Rate in the livers of fish fed the biologically incorporated diet (Chapter 9) was about 4.6 orders of magnitude greater than for the livers of fish fed the 3,000 μg/g dietary silver diet (Chapter 8). It appears that the 'speciation' of dietary silver may be an important modifier of the bioavailability of silver via the food route. Consequently, a Biotic Ligand Modelling Approach as discussed in Chapter 4 may be potentially developed for dietborne exposure to silver.

CHAPTER 2

THE RELATIVE IMPORTANCE OF WATER HARDNESS AND CHLORIDE LEVELS IN MODIFYING THE ACUTE TOXICITY OF SILVER TO RAINBOW TROUT (*Oncorhynchus mykiss*)

ABSTRACT

Static-renewal 7-d toxicity tests for silver nitrate ($AgNO_3$) were performed with juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum). The relative influences of calcium and chloride concentrations on median lethal time (LT50) were assessed. Calcium concentrations were controlled by adding either $Ca(NO_3)_2$ or $CaSO_4$, whereas chloride concentrations were adjusted with either NaCl or KCl. For both calcium salts, a 100-fold elevation in concentration increased the LT50 approximately 10-fold. However, a 100-fold elevation in KCl ameliorated silver (Ag) toxicity at least 100-fold, while NaCl protected against Ag toxicity even more substantially, demonstrating the much greater protective effect of chloride relative to calcium. In a separate series of bioassays, fish were exposed to 0.92 µM Ag (100 µg/L as $AgNO_3$) with varying amounts of NaCl titrated into each tank to alter the free [Ag^+]. The 7-day LC50 occurred at a [NaCl] of 2,500 µM. Using MINEQL$^+$ (a geochemical speciation program), the predicted free [Ag^+] at this LC50 value is 0.0285 µM. Further bioassays were performed in which [chloride] was maintained at either 50 or 225 µM, while total silver concentration was independently varied between 0.0092 to 0.0694 µM (1.0 to 7.5 µg/L). The 7-day LC50

value was calculated at 0.0294 μM Ag (3.18 μg/L) at a chloride concentration of 50 μM, very similar to the free [Ag^+] value of 0.031 μM calculated from an earlier LC50 test at a fixed [chloride] of 730 μM. According to MINEQL$^+$, the estimated [Ag^+] at this LC50 value is 0.0289 μM. Although a 7-day LC50 value could not be determined at 225 μM chloride, it was estimated at slightly above 0.0277 μM Ag^+. Elevating chloride concentrations from 50 to 225 μM did not alter the accumulation of Ag in the liver. In addition, there were no significant differences in hepatic Ag accumulation between any of the Ag-exposed fish, irrespective of the total Ag concentration used during the exposure. Overall, Ag accumulated to approximately 185 μmol/kg wet weight in all Ag-exposed groups (approximately a 10-fold increase above controls). These results, together with a reanalysis of published data, suggest that Ag toxicity can be correlated with the free silver ion [Ag^+], and that any factors altering Ag^+ availability (i.e., chloride) will be expected to modify acute silver toxicity.

Keywords: Silver nitrate, Rainbow trout, LC50, Chloride, Hardness

INTRODUCTION

Current water quality criteria established by the U.S. Environmental Protection Agency have assigned a critical role for water hardness (primarily as Ca) in the guidelines designed to protect aquatic life from acute silver toxicity (US EPA, 1980). In accordance with this view, the US EPA has quantitatively incorporated water hardness into the following equation, which is used by regulatory agencies for enforcing maximum total recoverable silver in water

$$\text{Max. Total Recov. Ag } (\mu g/L) = e^{(1.72[\ln \text{hardness}]-6.52)}$$

where hardness is expressed in units of mg/L of $CaCO_3$ equivalents. The primary impetus behind the derivation of this equation appears to have come from data tabulated by Lemke (1981), all of which were derived from interlaboratory studies of $AgNO_3$ toxicity in daphnids, fathead minnows, and rainbow trout. In recent years however, there has been an increasing amount of evidence suggesting that in some cases this equation fails to adequately assess the impact of silver in the aquatic environment. More specifically, it has been proposed that the ambient water quality criteria are overly conservative when applied to waters of low hardness, while underprotective at elevated hardness concentrations (Wood *et al.*, 1995).

Physiological studies observing the effects of $AgNO_3$ exposure in rainbow trout (Wood *et al.*, 1996a) suggest that the toxic mechanism involves a large inhibition of active Na^+ and Cl^- uptake at the gills. This is further supported by radioisotopic flux studies from Morgan *et al.* (1997), which correlate a decrease in ^{22}Na influx with a

concurrent inhibition in activity of the branchial transport enzymes Na^+/K^+ ATPase and carbonic anhydrase following $AgNO_3$ exposure. Overall, these transport processes are expected to be influenced only mildly by water hardness. Wood *et al.* (1996a) also reported that acute $AgNO_3$ exposure had no influence on ^{45}Ca uptake. These results are consistent with other studies that have shown that most metals exert toxic effects on either Na^+ (Laurén and McDonald, 1987a, b; Wood *et al.*, 1996a) or Ca^{2+} uptake mechanisms (Spry and Wood, 1985; Verbost *et al.*, 1987), but not both. Furthermore, Davies *et al.* (1978) found only a two-fold difference in the 4-day 50 % lethal concentration (LC50) for $AgNO_3$ on trout, when water hardness was varied between 26 and 350 mg/L. Based on the US EPA equation (US EPA, 1980), this variation in hardness should have produced a >80-fold difference in the LC50 values. Goettl and Davies (1978) observed only negligible effects of water hardness in other fish species exposed to $AgNO_3$. Even though these data were available when the above equation was derived, they were not used for its formulation due to their lack of correlation with other existing data.

Although it has been suggested that chloride may modify Ag toxicity to some degree (US EPA, 1980), extensive correlative studies have not been performed. However, re-analysis of Lemke's tabulated data suggests that chloride rather than calcium may be the true protective agent against silver toxicity (Hogstrand *et al.*, 1996). It is believed that chloride is able to influence toxicity by complexing with Ag^+, forming species such as $Ag(Cl)_n$ or cerargyrite (Schecher and McAvoy, 1992), both of which have been shown to be far less toxic than Ag^+, at least on an acute basis (LeBlanc *et al.*, 1984; Hogstrand *et al.*, 1996). Data presented in this manuscript and other citations

(Dufficy *et al.*, 1993) question the scientific validity or appropriateness of the US EPA hardness equation for Ag.

The objectives of this study were to evaluate the relative roles of calcium and chloride as modifiers of $AgNO_3$ toxicity. In addition, computer-based geochemical speciation modelling with MINEQL$^+$ (Schecher and McAvoy, 1992) was used in the design of several acute toxicity bioassays to test the assumption that Ag^+ is the predominantly toxic form of the metal. Finally, MINEQL$^+$ was employed for the re-analysis of Lemke's tabulated data to assess further the validity of the hardness equation (US EPA, 1980).

MATERIALS AND METHODS

Test organisms

Juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum; 5.17 ± 0.16 g, *n*=600) were obtained from a local hatchery (Rainbow Springs Hatchery, Thamesford, ON, Canada) and placed in a 300-L fiberglass tank (Living Stream, Toledo, OH, USA). Ion-depleted water (not deionized) ([calcium] ~ 15; [chloride] ~13; [potassium] ~ 23; [sodium] ~ 13 µM) was produced from Hamilton, Ontario, Canada dechlorinated tap water by reverse osmosis (Culligan MD-1000). Water was then delivered to a head tank before being gravity fed to the holding tank at a rate of 0.75 L/min. A stock solution containing 16,100 µM NaCl (BDH) and 15,000 µM $Ca(NO_3)_2 \cdot 4H_2O$ (BDH) (analytical-grade salts) was pumped into the water at a rate of 1.75 mL/min. Salts were added at the head tank and well mixed before delivery to the holding tank. Fish were acclimated to this synthetic soft water ([Na^+]= 50.0; [Cl^-]= 50.0, [Ca^{2+}]=50.0 µM, 15.5 °C ± 0.9°C) for at least 2 weeks prior to experimentation. Fish were fed dry trout pellets (Ziegler's Bros., Gardners, PA, USA) to satiation daily. The particle-reactive nature of silver required that fish feeding be discontinued 2 d prior to the start of experimentation and during the bioassays. This ensured that the measured concentrations of Ag in the water were maintained near nominal levels.

Experimental design

For each series of bioassays, five treatments plus a simultaneous control were performed in duplicate. Ten fish per replicate (20 per treatment) were tested in each bioassay.

Experimental protocol

All bioassays were performed in 60-L covered tanks. Each was filled with 50 L of synthetic soft water (as described above) and provided with gentle aeration. Bioassays were static-renewal with 80% (40 L) of this water renewed daily. Water samples were taken daily, both before and after each water replacement for analysis of total and dissolved silver and total chloride and calcium. Ten fish were randomly placed in each tank approximately 30 min prior to the start of experiments, at which time the appropriate amount of $AgNO_3$ stock was added to each tank. Henceforth, fish mortality was monitored continuously over the first 4 h, after which observations were made three times daily for a total of 7 d. Cessation of opercular movement and lack of movement during gentle prodding verified mortality. Dead fish were immediately removed and their weights and lengths recorded.

Bioassay for $AgNO_3$ (Effects of NaCl and $Ca(NO_3)_2$)

The relative roles of calcium and chloride in ameliorating $AgNO_3$ toxicity were tested at various water qualities. Eleven tanks were used in this study; nine were spiked with 100 mL of a $AgNO_3$ stock solution (0.46 mM; 78.7 mg/L) (Fisher Scientific, Phillipsburg, NJ, USA; analytical grade) to give a final total [Ag] of 0.92 μM. Chloride (as NaCl) was added to four of these tanks yielding chloride concentrations of 100, 500, 1,000, and 5,000 μM. In four other tanks, Ca (as $Ca(NO_3)_2$) was independently varied to yield the same concentrations (100, 500, 1,000, 5,000 μM). Ag (0.92 μM) was also added to a tank containing only synthetic soft water (50 μM of chloride and calcium). Two control tanks containing no $AgNO_3$ and either 5,000 μM of chloride or calcium were

tested. The bioassay was performed over 7 d as outlined in the experimental protocol section.

Bioassay for $AgNO_3$ (effects of KCl and $CaSO_4$)

To verify that the ameliorating effects on $AgNO_3$ toxicity could be solely attributed to calcium and chloride, irrespective of their counter ions, the same experiment was performed using $CaSO_4$ (Sigma Chemical, St. Louis, MO, USA) and KCl (BDH) salts. As before, calcium and chloride concentrations were independently varied from 50 to 5,000 µM. $AgNO_3$ was added to each of these nine tanks to a final concentration of 0.92 µM Ag. Two control tanks were employed with calcium and chloride concentrations independently maintained at 5,000 µM. Fish mortality was observed over a 7-d period.

$AgNO_3$ LC50 at varying [chloride]

Geochemical speciation modelling with $MINEQL^+$ (Schecher and McAvoy, 1992) was used to estimate the [Ag^+], $[AgCl]_n$ and cerargyrite formed at each of the Ag concentrations used in a LC50 test for $AgNO_3$ on juvenile rainbow trout, recently performed in our laboratory (Hogstrand *et al.*, 1996). The test had yielded a 7-day LC50 value of 0.0842 µM Ag (9.1 µg/L; as $AgNO_3$) in Hamilton city water ([Na^+]= 600; [Cl^-]= 730; [Ca^{2+}]= 1,000; [HCO_3^-]= 1,900 µM; pH 7.9- 8.2). According to $MINEQL^+$, an ionic Ag^+ concentration of 0.031 µM Ag^+ would be expected at this total [Ag] in Hamilton water. With this information in mind, two separate series of bioassays were designed. In the first, $AgNO_3$ was added to give a total [Ag] of 0.92 µM, while the [chloride] was

manipulated appropriately to give free Ag^+ concentrations, bracketting the 0.031 µM 7-d LC50 value. The concentrations of free Ag^+ tested were 0.019, 0.0255, 0.0319, 0.0451, 0.0712 µM. These concentrations were obtained by altering the chloride concentration to 3,750, 2,800, 2,230, 1,560, 1,000 µM, respectively, using NaCl. Except for variations in Na^+ and Cl^- concentration, the ionic composition of the bioassay water was identical to that of the synthetic soft water used previously.

In the second series, the [chloride] was maintained at either 50 or 225 µM, while concentrations of total Ag (i.e. $AgNO_3$) were varied to give [Ag^+] surrounding the 7-d LC50 value. The total Ag concentrations tested were 0.0092, 0.0167, 0.0296, 0.0518 and 0.0694 µM (1.0, 1.8, 3.2, 5.6 and 7.5 µg/L). These yielded nominal free Ag^+ concentrations of 0.0084, 0.0150, 0.0267, 0.0468, 0.0626 µM (at 50 µM chloride) and 0.0062, 0.0111, 0.0198, 0.0346, 0.0463 µM (at 225 µM chloride) in the two water qualities, respectively. These concentrations used a logarithmic scale to adhere to general procedures for performing bioassays, as outlined by Sprague (1991).

Analysis of Ag in fish liver

Livers from the survivors of the second LC50 series (50 and 225 µM chloride series) were removed after completion of the 7-d bioassay. Ag analysis was performed on control, 0.0167 and 0.0518 µM Ag-exposed fish at 50 µM chloride and 0.0167 and 0.0694 µM Ag-exposed fish at 225 µM chloride. Livers were individually homogenized in 1.0 mL of 50 mM Tris-HCl (pH 8.0) (Sigma Chemical), at 0°C in a glass tube fitted with a Teflon®-tipped pestle (Thomas Scientific, Philadelphia, PA, USA). A subsample of each homogenate (400 µL) was withdrawn and transferred to previously acid-washed

test tubes. Homogenates were digested for 2 h with five volumes of 70 % HNO_3 at 120°C. Samples were then allowed to cool to room temperature, after which 0.75 volumes of H_2O_2 was added. Digests were evaporated to dryness without allowing the digested residue to become ashed. Five mL of 0.5% HNO_3 was added to each tube and analyzed for Ag using atomic absorption spectroscopy.

Water and tissue Ag analyses

All water samples were immediately acidified with 0.5 % (v/v) of HNO_3 (J.T. Baker, Phillipsburg, NJ, USA; trace metal grade). Both water and tissue samples were analyzed for Ag by atomic absorption spectroscopy, using a graphite furnace (Varian GTA-95) for silver concentrations less than 0.23 µM (25 µg/L) or by flame atomic absorption (AA) (Varian AA 1275) for samples having a concentration greater than 0.23 µM Ag. The graphite furnace operation was set at the following temperature ramping profile: injected at ambient temperature, ambient temperature to 75°C over 5 s, 75°C to 90°C over 12 s, 90°C to 120°C over 30 s, with atomization occurring at 2,000°C. The graphite tube was flushed with ultra-pure N_2 gas between samples to eliminate contamination. An automated sample injector was used to dispense 10-µl samples. Both graphite furnace and flame AA analyses were performed at a wavelength of 328.1 nm and band slit width of 0.5 nm.

Nonfiltered water samples were used for analysis of total Ag. Water samples were also immediately passed through a 0.45-µm filter (Gelman Sciences, Ann Arbor, MI, USA) for measurement of dissolved [Ag]. Water chloride concentrations were measured in unfiltered samples using the mercuric thiocyanate colorimetric assay (Zall *et*

al., 1956). Prior to calcium analysis by flame AA, $LaCl_3$ was added to a final dilution of 0.2% to eliminate Na^+ interference.

Statistical methods

Log-probit analysis of mortality plots was employed to determine LT50 values for each exposure (Sprague, 1991). Calculation of 95% confidence limits was performed by the nomographic methods of Litchfield and Wilcoxon (1949). Regression lines were fitted through the probit-versus-dose curves using the method of least squares (Biosoft Corp., Ferguson, MO, USA). LC50 calculations were based on measured total silver concentrations (e.g., Table 1).

RESULTS

Exposure to 0.92 μM Ag (as $AgNO_3$) was most toxic when water chloride concentrations were low. When both NaCl and $Ca(NO_3)_2$ concentrations were maintained at 50 μM, the LT50 was 42 min (95% confidence limits: 34.1-51.7 min) (Fig. 1). The LT50 increased to 6,600 (3,882 to 11,220) min at a [NaCl] of 1,000 μM ([calcium] unchanged). An LT50 value at 5,000 μM chloride could not be determined because less than 50 % mortality was observed by the end of the 7 d. Manipulation of water [Ca] had much less overall effect. Independently varying the [Ca] (using $Ca(NO_3)_2$) from 50 to 5,000 μM (at a constant [chloride] of 50 μM) increased the LT50 from 42 (34.1-51.7) min to only 470 (395.0 to 559.3) min (Fig. 1).

Bioassays using KCl and $CaSO_4$ to independently vary [chloride] and [Ca] confirmed that increasing chloride concentrations decreased silver toxicity more so than increasing [Ca]. When both [chloride] and [Ca] were maintained at 50 μM the LT50 was 68 (49.3-93.8) min. At a [chloride] of 5,000 μM, the LT50 increased to 7,100 (6,484 to 7,774) min. Independently varying the [Ca] to 5,000 μM increased only moderately the LT50 value to 540 (461 to 632) min (Fig. 2), a change of less than 10-fold.

$AgNO_3$ LC50 values

When the total silver concentration was maintained at 0.92 μM, while the [chloride] was varied to yield free Ag^+ concentrations, the resulting 7-d LC50 value occurred at a [chloride] of approximately 2,500 μM (Fig. 3A). The estimated [Ag^+] at the 7-d LC50 value was 0.0285 μM based on speciation modelling with MINEQL+ (Fig. 3B).

Seven-day LC50 tests at 50 and 225 μM [chloride] were also performed. At 50 μM [chloride], the LC50 was calculated at 0.0294 μM, while at 225 μM [chloride] an LC50 value could not be directly calculated because there was only 40 % mortality at the highest silver concentration (0.0416 μM) (Table 1). The LC50 value for the 50 μM chloride exposure, in terms of Ag^+ concentration is 0.0289 μM, as determined using measured water quality parameters in MINEQL+, while at 225 μM [chloride] it was expected to be slightly above 0.0227 μM Ag^+ (at [Ag]= 0.0416 μM). Silver analysis of water samples showed that as [chloride] increased the proportion of dissolved silver decreased (Table 1). The dissolved silver proportion (as percent recovered) also decreased as total silver increased regardless of the chloride concentration.

Livers of fish exposed to $AgNO_3$ during the 7-d bioassays showed a 10-fold increase in [Ag] compared to controls (Table 2). Overall, accumulation was not affected by changes in [chloride] (50 versus 225 μM) with silver concentrations ranging between 174.3 to 195.6 μmol/kg. Hepatic silver concentrations of control fish were 17.5 ± 4.64 μmol/kg. In addition, there were no significant differences in silver liver burdens when $AgNO_3$ concentrations were varied between 0.0167 μM and 0.0694 μM.

Speciation modelling

Varying the [chloride] from 50 to 5,000 μM (as performed in initial bioassays) dramatically influenced Ag speciation, decreasing Ag^+ content from 90% to 1% of the total [Ag] (total [Ag]= 0.92 μM), while cerargyrite and $AgCl_n$ percentages increased reciprocally. This speciation effect was predominantly observed at a [chloride] ranging from 125 to 800 μM (Fig. 4A-C). In contrast, variations in water [Ca] over the range

tested in the bioassay exerted only negligible effects on Ag speciation. Furthermore, the effects of chloride on Ag speciation are also dependent on the total Ag in the water. For instance, at a total silver concentration (as $AgNO_3$) of 0.092 μM, cerargyrite is not formed between 125 and 800 μM chloride (Fig. 4A). Instead, a greater percentage of Ag will be found as $Ag(Cl)_n$ species. As [$AgNO_3$] is increased to 0.46 and 0.92 μM Ag, cerargyrite starts to form at approximately 100 and 200 μM chloride, respectively (Fig. 4B,C).

DISCUSSION

Elevating the [Ca] (as $Ca(NO_3)_2$) 100-fold increased survival time approximately 10-fold (Fig. 1). In comparison, only a two-fold increase in [chloride] (as NaCl) was required to produce the same protective effect to an acutely toxic exposure of 0.92 μM total Ag (as $AgNO_3$) and a similar 100-fold elevation of chloride concentrations increased the LT50 approximately 100-fold. A parallel experiment to this one was performed in which [chloride] and [Ca] were manipulated using KCl and $CaSO_4$ salts, respectively. Once again, a 100-fold elevation in [Ca] resulted in only a 10-fold increase in LT50, whereas similar increases in [chloride] had greater than a 100-fold protective effect. These results show no differences in acute Ag toxicity between either of the Ca^{2+} salts used, whereas, NaCl was shown to have a slightly greater protective effect than KCl.

Wood *et al.* (1996a) have demonstrated that acute Ag (as $AgNO_3$) resulted in a significant decrease of plasma Na^+ and Cl^- concentrations in freshwater rainbow trout. This ionoregulatory disfunction was attributed to impairment of Na^+ and Cl^- uptake mechanisms at the gill (Morgan *et al.*, 1997). It seems likely that an increased [Na^+] in the water might slightly alleviate this ionic disturbance by either competing with Ag^+ at the gill surface or by creating an improved gradient for Na^+ entry (Pagenkopf, 1983; Janes and Playle, 1995). In contrast, acute $AgNO_3$ exposure did not produce any observable effects on plasma K^+ concentrations (Wood *et al.*, 1996a), suggesting that the branchial uptake mechanisms of this electrolyte were unaffected. Consequently, elevating the waterborne [K^+] would not be expected to decrease Ag toxicity to the same extent as [Na^+] would. This is supported by results from this study that appear to indicate

that chloride is somewhat more protective when introduced as NaCl as compared to KCl. In addition, no effect on either the branchial uptake rate of Ca^{2+} or on plasma Ca concentrations was produced in response to acute $AgNO_3$ exposure (Wood *et al.*, 1996a). Consequently, [Ca] (hardness) should not greatly affect the specific sites of toxicity. Nevertheless, Ca^{2+} may help to decrease ion permeability at epithelial tight junctions due to its role as a gill membrane stabilizer. This "tightening" of paracellular junctions would result in reduced Na^+ and Cl^- efflux rates (Evans, 1987), possibly helping to counteract the acutely toxic effects of $AgNO_3$ exposure and explaining the small protective effect seen here.

Speciation modelling with MINEQL$^+$ has suggested that Cl^- may exert an ameliorating role on silver toxicity by complexing with Ag^+, thus reducing the amount of Ag^+ bioavailable to the organism (Hogstrand *et al.*, 1996). Although increasing [chloride] reduces Ag toxicity, it does not appear to affect the rate at which Ag is accumulated in hepatic tissue. In this study, Ag concentrations in the liver were consistently around 185 µmol/kg wet weight (after 7-d of exposure), regardless of the [chloride] used in the study (50 or 225 µM) (Table 2). Hogstrand *et al.* (1996) observed similar silver concentrations in livers of fish exposed to Ag concentrations surrounding the 7-d LC50 value (~185 µmol/kg or 20 µg/g), a chloride concentration of 730 µM. In addition, small variations in total silver concentrations did not appear to alter Ag accumulation levels. This suggests that Ag uptake kinetics become saturated at relatively low concentrations of Ag.

Several earlier studies have indicated that silver toxicity is best correlated with free [Ag^+], rather than total silver (Cooper and Jolly, 1970; Terhaar *et al.*, 1977; LeBlanc

et al., 1984; Hogstrand *et al.*, 1996). In the present study, the estimated Ag^+ concentrations at the calculated LC50 values ranged between 0.0277 and 0.0289 μM. This narrow range was obtained despite LC50 values (as total silver) varying as much as 31-fold (0.0294- 0.92 μM Ag) and chloride concentrations varying at least 50-fold (50-2500 μM). In comparison, the 7-day LC50 value at 730 μM chloride was 0.0842 μM Ag, translating to an [Ag^+] of 0.0310 μM (Hogstrand *et al.*, 1996).

With this background in mind, Lemke's tabulated data (Lemke, 1981) were reanalyzed using MINEQL$^+$ to determine the free Ag^+ concentrations that should correspond to each of the reported 4-day LC50 values for fathead minnows and rainbow trout, based on the given ionic composition of the test water. Although LC50 values varied substantially (from 0.0361 μM to 2.590 μM total Ag), speciation analyses with MINEQL$^+$ using water chemistry data reported by Lemke (1981) gave [Ag^+] estimates from 0.025-0.314 μM, with the majority of values below 0.100 μM (Fig. 5). The relatively close agreement in Ag^+ concentrations between these data and our 7-day LC50 results support the view that toxicity is best correlated with [Ag^+].

Free ionic Ag^+ concentrations ranging between 0.0009 to 1.85 nM have been measured in natural water samples using potentiometric techniques (Chudd, 1983). In all cases, the highest waterborne Ag^+ concentrations were observed in water bodies receiving industrial discharges. Thus, despite having silver concentrations as high as 0.35 μM in surface waters of highly polluted sites (Taylor *et al.*, 1980), complexation processes are expected to reduce Ag^+ concentrations to well below acutely toxic concentrations (Cooley *et al.*, 1988; Dufficy *et al.*, 1993). When LC50 values (as total Ag) are low, Cl^- likely ameliorates Ag toxicity by complexing with Ag^+ to form $Ag(Cl)_n$

(Fig. 4a). At high LC50 values it is probably cerargyrite formation that is the driving force behind the amelioration of Ag toxicity in response to elevated chloride concentrations (Fig. 4B,C). Consequently, the present regulatory framework based on the "hardness-equation" (US EPA, 1980) fails to assess correctly the true toxicity of Ag species in the natural environment. Clearly more research is needed on the exact geochemical requirements of future AWQC for Ag.

Acknowledgements

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Table 1. Measured total and dissolved silver concentrations in bioassay water.

Chloride concentration = 50 μM

Nominal [Ag] (μM) (x10⁻³)	*Total [Ag] (μM) (x10⁻³)* [a]	*Dissolved [Ag] (μM) (x10⁻³)* [b]	*% of Total Ag in Dissolved Fraction*
9.2	13.5	12.0	89.0
16.7	13.3	13.1	97.9
29.6	29.4	24.2	82.3
51.8	38.5	28.2	73.3
69.4	52.4	37.4	71.4

Chloride concentration = 225 μM

Nominal [Ag] (μM) (x10⁻³)	*Total [Ag] (μM) (x10⁻³)* [a]	*Dissolved [Ag] (μM) (x10⁻³)* [b]	*% of Total Ag in Dissolved Fraction*
9.2	11.4	8.5	74.8
16.7	20.6	16.1	77.7
29.6	23.1	13.4	58.0
51.8	31.6	21.1	67.0
69.4	41.6	28.1	67.5

[a]Observed [Ag] represents the arithmetic mean obtained from all water samples taken.

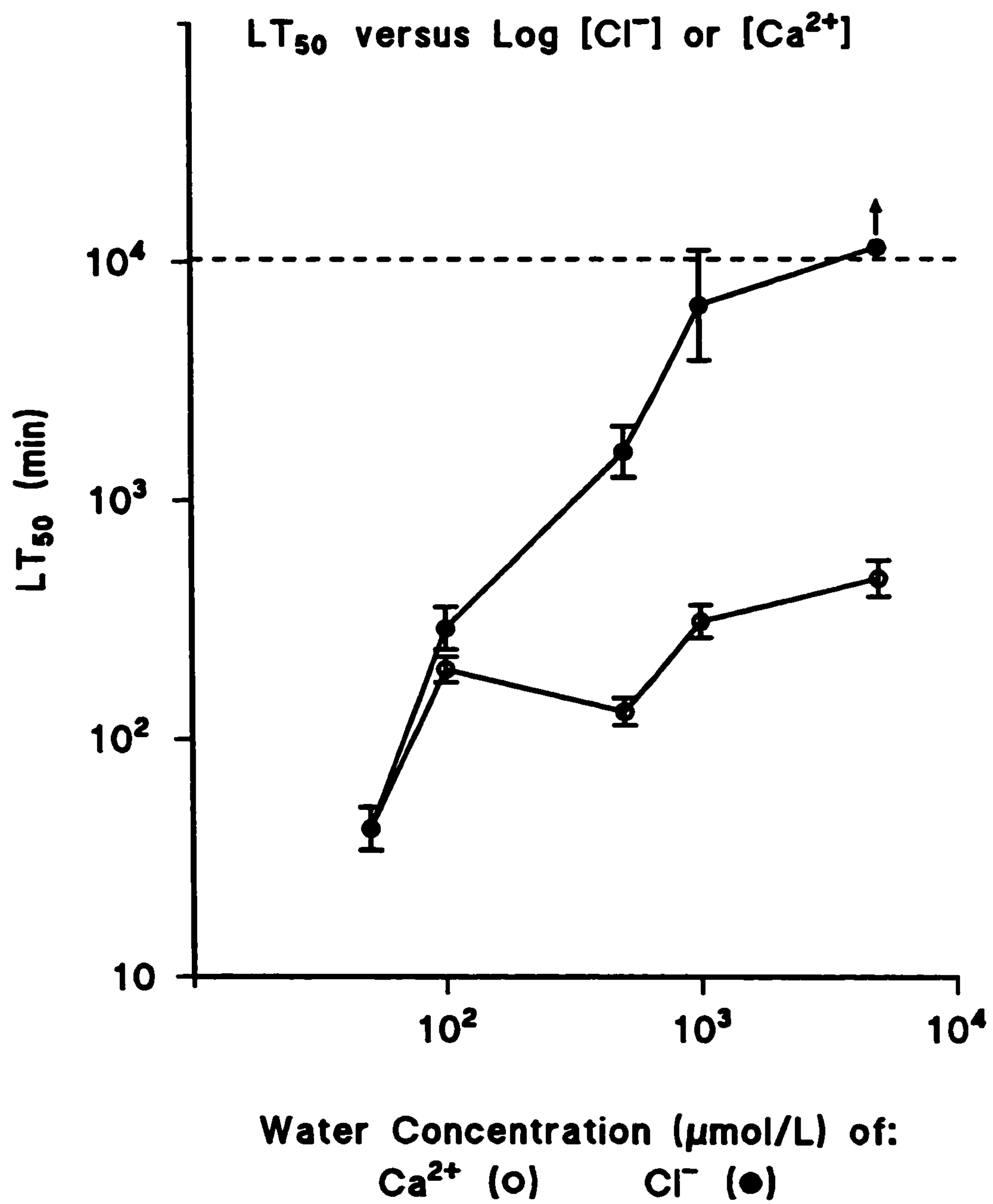
[b]Dissolved [Ag] represents all Ag passing through a 0.45-μm filter.

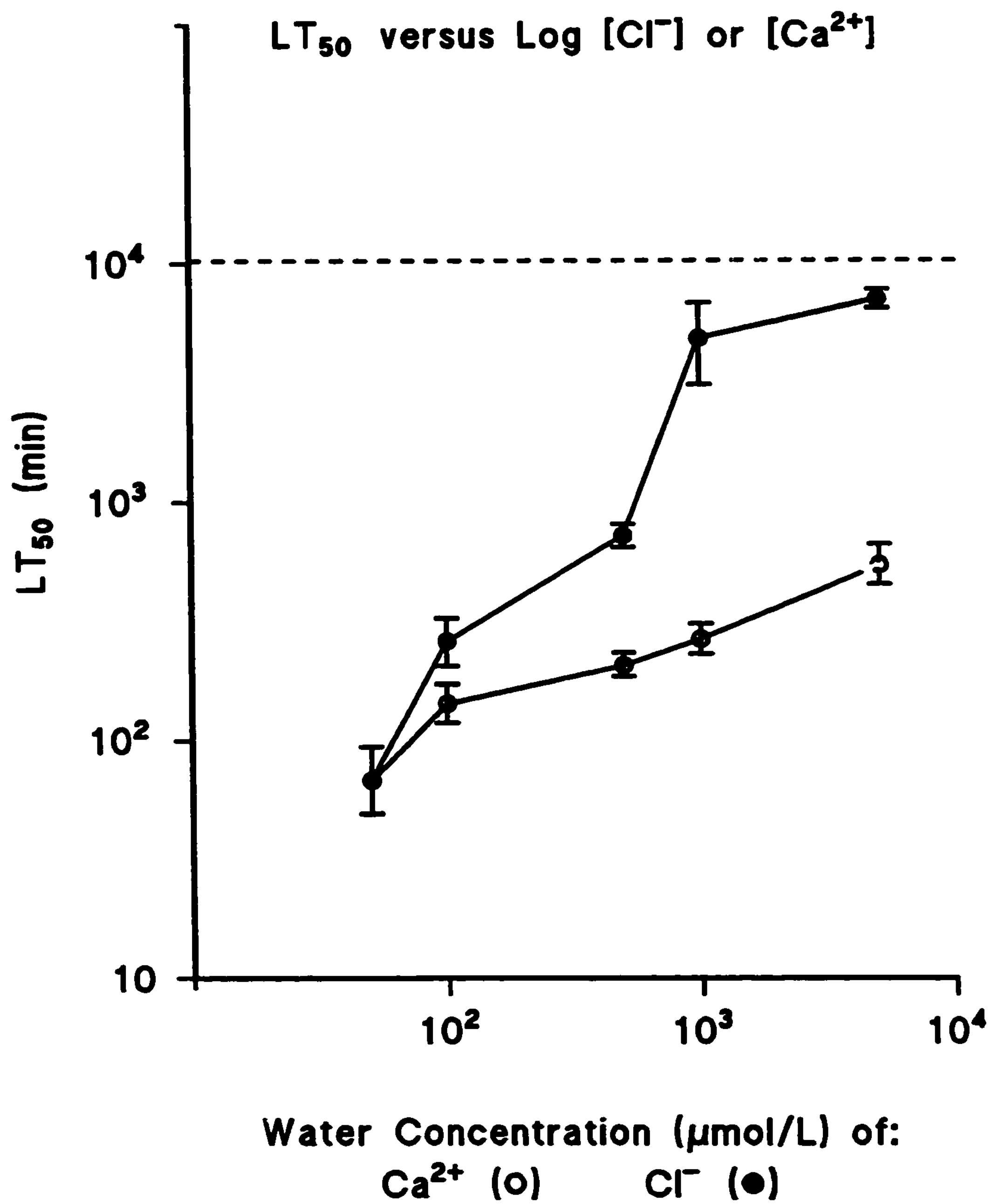
Table 2. Hepatic silver concentrations in juvenile rainbow trout

Nominal [Ag] (μM) (x 10^{-3})	Hepatic silver concentrations[a]	
	50 μM chloride	225 μM chloride
Control	17.52 ± 4.64 (n = 9)	na[b]
16.7	195.61 ± 87.70 (n =7)	174.29 ± 26.05 (n = 7)
51.8	176.23 ± 17.24 (n = 4)	na[b]
69.4	na[b]	193.66 ± 33.10 (n = 2)

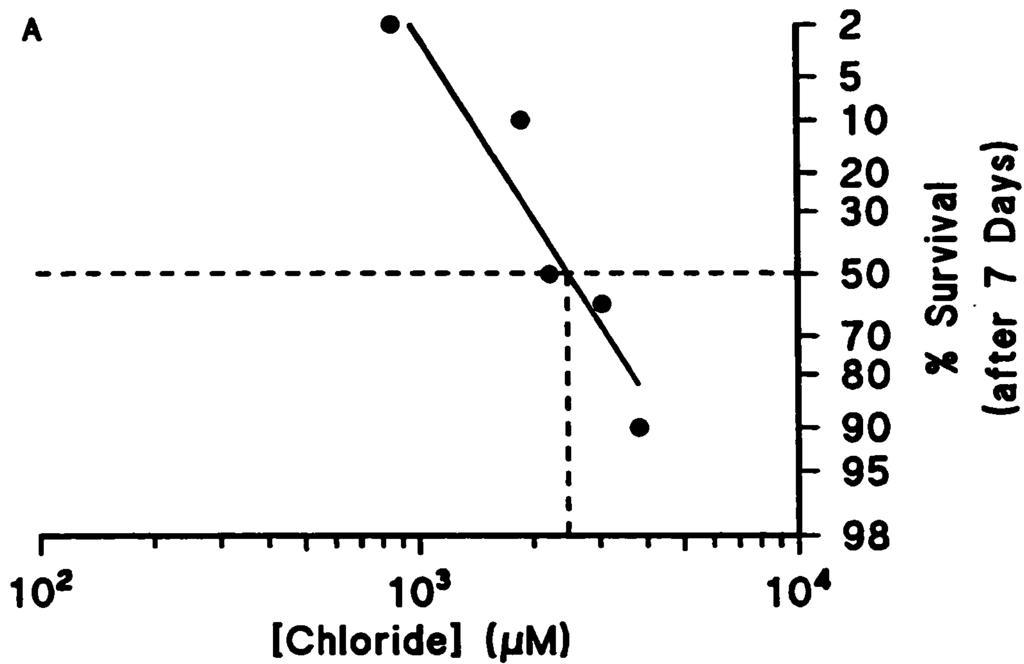
[a]Hepatic Ag concentration measured as μmol per kg wet weight

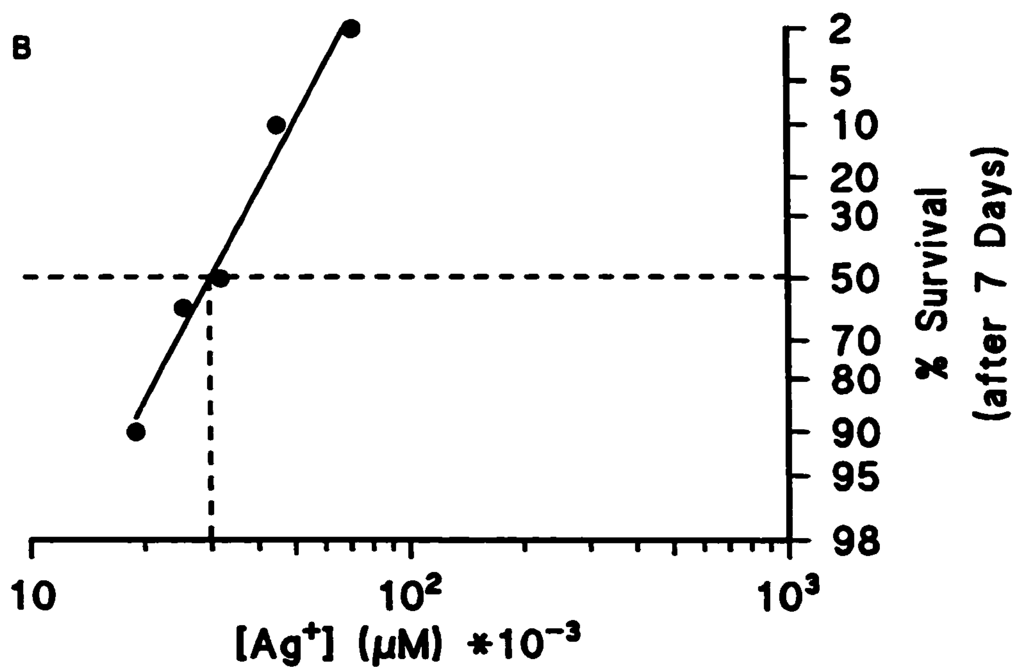
[b]Not analyzed

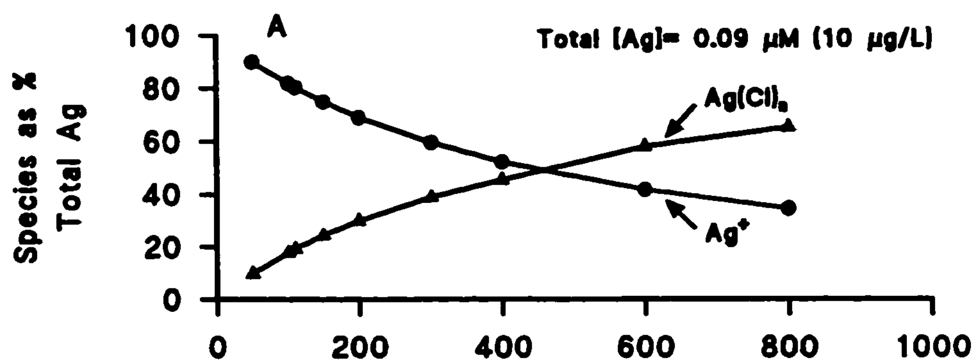

LT50 versus Log [Cl⁻] or [Ca2+]
10⁴
10³
10²
10
LT50 (min)
10²
10³
10⁴
Water Concentration (µmol/L) of:
Ca2+ (o) Cl⁻ (●)

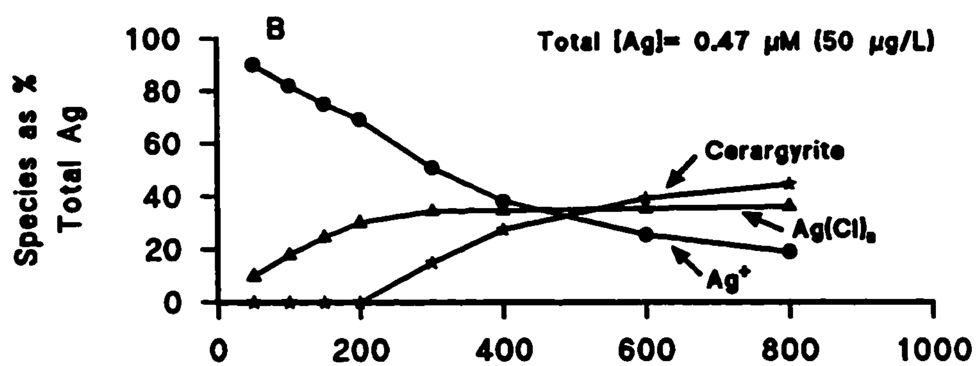


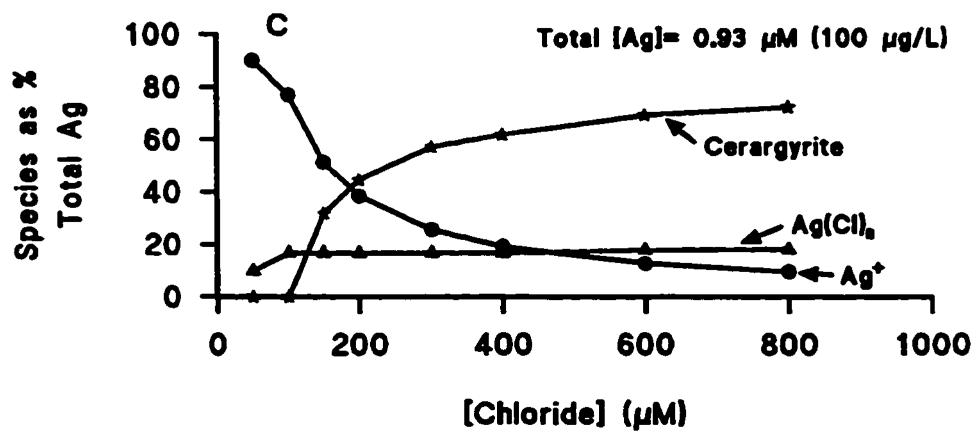
LT50 versus Log [Cl⁻] or [Ca2+]
10⁴
10³
10²
10
LT50 (min)
10²
10³
10⁴
Water Concentration (µmol/L) of:
Ca2+ (o) Cl⁻ (●)

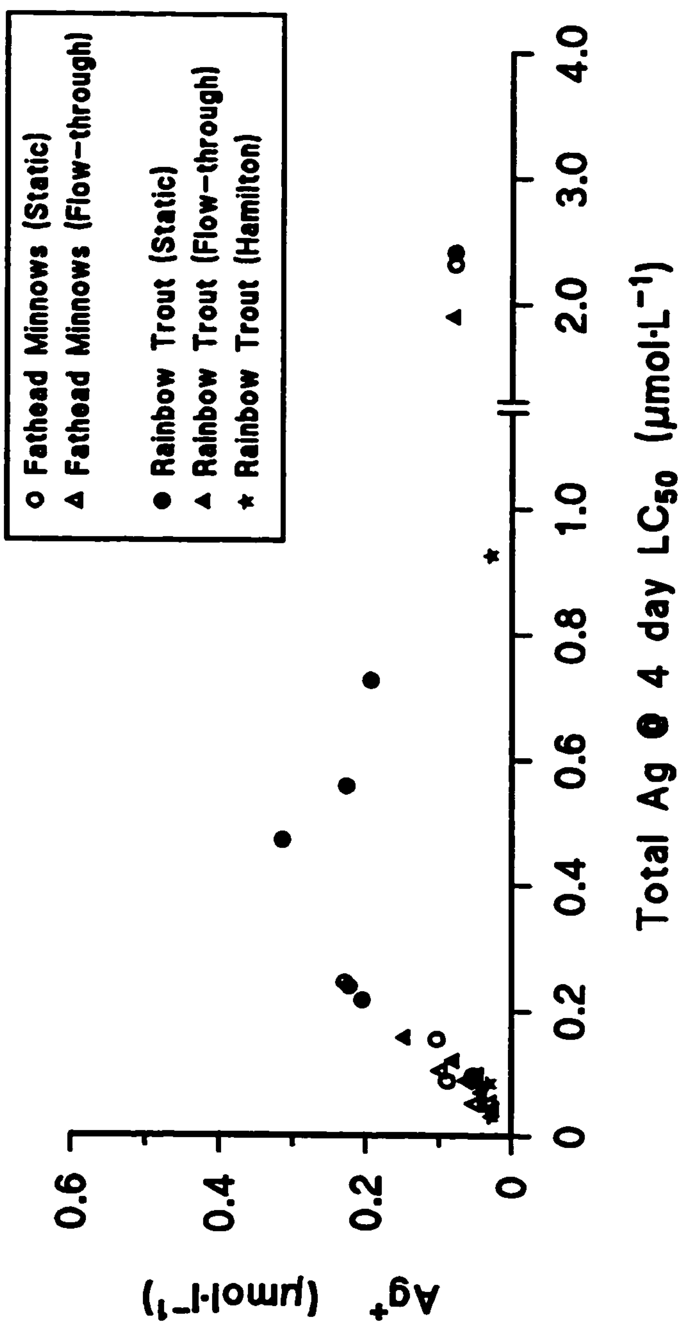

A
2
5
10
20
30
50
70
80
90
95
98
% Survival
(after 7 Days)
10²
10³
10⁴
[Chloride] (µM)


B
2
5
10
20
30
50
70
80
90
95
98
% Survival
(after 7 Days)
10
10²
10³
[Ag⁺] (µM) *10⁻³


A
Total [Ag]= 0.09 μM (10 μg/L)
Species as % Total Ag
Ag(Cl)n
Ag+
0
20
40
60
80
100
0
200
400
600
800
1000


B
Total [Ag]= 0.47 μM (50 μg/L)
Species as % Total Ag
Cerargyrite
Ag(Cl)n
Ag+
0
20
40
60
80
100
0
200
400
600
800
1000


C
Total [Ag]= 0.93 μM (100 μg/L)
Species as % Total Ag
Cerargyrite
Ag(Cl)n
Ag+
0
20
40
60
80
100
0
200
400
600
800
1000
[Chloride] (μM)



Fathead Minnows (Static)
Fathead Minnows (Flow-through)
Rainbow Trout (Static)
Rainbow Trout (Flow-through)
Rainbow Trout (Hamilton)
Ag+ (μmol·l-1)
0
0.2
0.4
0.6
0
0.2
0.4
0.6
0.8
1.0
2.0
3.0
4.0
Total Ag @ 4 day LC50 (μmol·L-1)

CHAPTER 3

EFFECTS OF CHLORIDE, CALCIUM AND DISSOLVED ORGANIC CARBON ON SILVER TOXICITY: COMPARISON BETWEEN RAINBOW TROUT AND FATHEAD MINNOWS

ABSTRACT

The effects of independently altering chloride, calcium and dissolved organic carbon (DOC) on the toxicity of silver (presented as $AgNO_3$) were compared between rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*). The 96-h median lethal concentration (LC50) toxicity tests for both species were performed under the same conditions, within the same containers. In addition, the effect of altering [Cl^-] on silver-induced perturbations to body Na^+ influx and gill silver load was studied. Toxicity tests were conducted in synthetic soft water (50 μM Na^+, 50 μM Cl^-, 50 μM Ca^{2+}, 0.3 mg DOC/ L). The [Cl^-], [Ca^{2+}], and [DOC] were adjusted by the addition of NaCl, $CaNO_3$, or humic acid, respectively. On the basis of total silver, increasing [Cl^-] over a range of 50 μM to 1,500 μM resulted in a 4.3-fold increase in the 96-h LC50 values (decrease in toxicity) for rainbow trout, but did not significantly affect the 96-h LC50 values for fathead minnows. Increasing water [Ca^{2+}] (from 50 to 2,000 μM) had only a small influence (1.5-fold increase) on the 96-h LC50 values in either species. However, increasing DOC levels (from 0.3 to 5.8 mg DOC/L) significantly increased the 96-h LC50 values (2.7- to 4.1-fold increases) in both species. If the 96-h LC50 values

are calculated on the basis of ionic silver, Ag^+, (utilizing the aquatic geochemical computer program MINEQL+), then, in the case of rainbow trout, toxicity correlates to Ag^+. However, this correlation does not exist for fathead minnows. Increasing [Cl^-] did not affect the degree of perturbation of Na^+ influx during acute exposure (first 4 h) to 8 μg Ag/ L in either species, nor did it affect the whole-body silver uptake rates, but it did reduce the gill silver load. These results demonstrate that differences exist in the way in which water chemistry ameliorates silver toxicity between rainbow trout and fathead minnows.

Keywords: Silver toxicity, Water chemistry , Rainbow trout, Fathead minnows, Physiology

INTRODUCTION

Silver, when presented as silver nitrate in laboratory water, is one of the most toxic metals to freshwater fish, with median lethal concentration (LC50) values of between 5 and 60 μg Ag/L (Coleman and Cearley, 1974; Davies *et al.*, 1978; Buccafusco *et al.*, 1981; Lemke, 1981; Nebeker *et al.*, 1983; LeBlanc *et al.*, 1984; Diamond *et al.*, 1990; Hogstrand *et al.*, 1996; Galvez and Wood, 1997). However, inorganic complexes of silver, such as silver thiosulphate, silver sulfide, and silver chloride, have been shown to be relatively benign in acute toxicity tests. Both LeBlanc *et al.* (1984) and Hogstrand *et al.* (1996) have found that Ag, when presented in the form of silver thiosulfate or silver chloride, is less toxic by at least orders of magnitude than silver nitrate for both fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*). Both studies illustrate the importance of speciation in silver toxicity.

Regulations for silver discharge in the United States acknowledge the importance that water chemistry may play in influencing toxicity. US Environmental Protection Agency (US EPA) guidelines, designed to help authorities in the regulation of silver, have designated hardness (principally the calcium content of the water) as the sole factor affecting silver toxicity and have incorporated it into the following equation:

$$\text{maximum total recoverable Ag } (\mu g/L) = e^{(1.72\,[\ln \text{hardness}]-6.52)} \qquad (1)$$

This equation is derived from interlaboratory studies of $AgNO_3$ toxicity to daphnids, fathead minnows and rainbow trout (Lemke, 1981). However, the validity of this hardness equation has been recently questioned (Hogstrand *et al.*, 1996; Galvez and

Wood, 1997; Hogstrand and Wood, 1998). Galvez and Wood (1997) showed that, for juvenile rainbow trout, water chloride had a much greater effect in ameliorating silver toxicity than did water hardness. Analysis of the water geochemistry in the toxicity test by the aquatic geochemical computer program MINEQL+ (Schecher and McAvoy, 1992) showed that silver toxicity correlated to Ag^+. Furthermore, Davies *et al.* (1978) found that when hardness was increased from 26 mg/L (as $CaCO_3$) to 350 mg/L, a 2-fold increase occurred in the LC50 value for juvenile rainbow trout. Based on the above US EPA hardness equation, an increase in hardness of this magnitude should result in a >80 times increase in the LC50 value. Erickson *et al.* (1998) reported a significant protective effect of hardness (2.5-fold increase in LC50) in 30-day-old fathead minnows over the hardness range of 48 to 249 mg/L, but this effect again was much less than that predicted by the hardness equation (17-fold).

Further evidence that water geochemistry influences silver toxicity comes from studies investigating the physiological and biochemical perturbations induced by silver exposure in rainbow trout. In freshwater rainbow trout the toxic mechanism of $AgNO_3$ involves the inhibition of Na^+ and Cl^- uptake at the gills (Morgan *et al.*, 1997; Webb and Wood, 1998), and, more specifically, the inhibition of the sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase) enzyme which is situated on the basolateral membrane of the gill (Morgan *et al.*, 1997). Bury *et al.* (1999b) and McGeer and Wood (1998) showed that an increase in water chloride reduces the degree of silver-induced disruption to Na^+ balance. Moreover, an increase in water calcium had no effect on the silver inhibition of Na^+ influx and Na^+/K^+ATPase activity (Bury *et al.*, 1999b).

Analysis of the water geochemistry by computer programs such as MINEQL+ (Schecher and McAvoy, 1992) shows a good correlation between the water ionic silver (Ag^+) concentration and the silver-induced physiological disruptions in rainbow trout (McGeer and Wood, 1998; Bury *et al.*, 1999b).

In contrast to the profound protective effects that chloride has on silver toxicity to rainbow trout (Galvez and Wood, 1997; McGeer and Wood, 1998; Bury *et al.*, 1999b), Brooke *et al.* (1996) and Karen *et al.* (1999) reported that chloride has only a very minor protective influence on silver toxicity to 4-d-old fathead minnows. Erickson *et al.* (1998) reported a modest increase in silver toxicity to 30-d-old fathead minnows when water chloride was increased 2.5-fold. Consequently, the first purpose of this study was to ascertain the effect of chloride on silver toxicity to fathead minnows and rainbow trout by conducting toxicity tests under the same conditions. The second purpose of this study was to compare the effect of water chloride on silver-induced perturbations to Na^+ balance and silver accumulation in the gills and body of both rainbow trout and fathead minnows. In addition, the relative influences of calcium and dissolved organic carbon on silver toxicity were assessed. Calcium is included because of its importance in the US EPA hardness equation (US EPA, 1980) (see above). Dissolved organic carbon is prevalent in freshwater systems and has been shown to reduce heavy metal toxicity (Welsh *et al.*, 1993; Erickson *et al.*, 1996; Hollis *et al.*, 1997). Specifically, Erickson *et al.* (1998) found that elevating DOC from 1.5 mg/L to 10.5 mg/L increased the silver LC50 values for 30-d-old fathead minnows by more than 4-fold. Moreover, DOC has also been shown to reduce the accumulation of silver on the gills (Janes and Playle, 1995;

Bury *et al.*, 1999b), as well as prevent silver-induced physiological perturbation to Na^+ balance in rainbow trout (Bury *et al.*, 1999b).

MATERIALS AND METHODS

Toxicity tests

Juvenile rainbow trout, weight 2.2 ± 1.2 g, were obtained from Humber Spring Hatchery, Orangeville, Ontario, Canada and fathead minnows, weight 0.23 ± 0.28 g, were obtained from Aquatic Biosystems, Fort Collins, Colorodo, USA. Fish were kept in dechlorinated Hamilton (ON, Canada) tap water ([Na^+], 0.5 mM; [Cl^-], 0.7 mM; [Ca^{2+}], 1 mM; [Mg^{2+}], 0.2 mM; [K^+], 0.05 mM, pH 7.8-8.0, 17 °C) for 2 weeks before acclimation to test water. Fish were gradually acclimated to synthetic soft water, generated by reverse osmosis (RO, Andersen, Dundas, ON, Canada) of Hamilton dechlorinated tap water, over a period of 2 weeks. During this period the ratio of soft water to hard water that the fish received was increased. The water temperature was maintained at 17 °C. After 2 weeks, the fish received RO water with the addition of NaCl and $CaNO_3$ to attain a final soft water composition of 50 μM, Na^+, 50 μM Cl^- and 50 μM Ca^{2+} (K^+, Mg^{2+} levels and pH of the soft water are reported in Table 1; titratable alkalinity to pH 4 was 8.25 mg $CaCO_3$/L). Fish were maintained in this synthetic water for at least 2 weeks before testing, during which time both species were fed a commercial ration (Martin Mills Inc., Tavistock, ON, Canada) at 1.5% of their body weight daily.

All toxicity tests were performed in a static-renewal system and followed American Society for Testing and Materials guidelines (ASTM, 1993). To enable direct comparison between the toxicity of silver to rainbow trout and fathead minnows, tests were conducted in the same container in a temperature-controlled room at 17 °C, a

temperature that both species could experience in the natural environment, with a 16-h light and 8-h dark light regime. Test media, consisting of RO water and the appropriate salts (NaCl or $CaNO_3$, respectively, to obtain the desired Cl^- or Ca^{2+} concentrations) were stored in 200-L containers in the experimental room with constant aeration for 24-h before use. DOC was added as humic acid (Aldrich Chemical, Milwaukee, WI, USA)/ For acclimation of fish to the various test waters, 60 rainbow trout and 60 fathead minnows were placed in separate 72-L tanks containing 50-L of the aforementioned medium for 48-h. After 24-h, 50 % of the test water was exchanged with fresh media and the tanks were well aerated throughout the acclimation period.

Experimental tanks were designed so that 2 nylon mesh chambers (approximate size 15 x 15 x 40 cm) were suspended in 20-L of test medium. During the 96-h experiment, the test solutions were continuously aerated to maintain high dissolved oxygen levels (Table 1) and to enable thorough mixing of the eluents. For each test, at least 6 (maximum of 9) concentrations over the range of 3.2 to 40 μg $AgNO_3$/L were used to elucidate a 96-h LC50 value. Each experimental tank was assigned a silver concentration and then randomly distributed in the temperature-controlled room. Clean techniques ensured that the controls never experienced silver. Ten rainbow trout and 10 fathead minnows were placed in separate chambers within the same tank, and the total weight of the fish to water volume ratio never exceeded 2g/L.

Silver was added from a stock of 1M $AgNO_3$ (in 0.1 M HNO_3) that was stored in the dark. The water silver concentrations were monitored frequently (every 3-4 h over the first 48-h and periodically thereafter) and the concentrations were maintained by the

addition of an appropriate volume of the stock $AgNO_3$ solution. The silver concentration to which the fish were exposed was calculated as a time-weighted average of the total silver measured throughout the experimental period. Each day 80 % of the medium from each tank was replaced with fresh medium supplemented with an appropriate volume of the 1M $AgNO_3$ stock so as to maintain the desired silver levels. Two 10-mL water samples were taken before and after the medium change. The first water sample was used for analysis of water ions, total ammonia and total silver levels (see below), whereas the second water sample was filtered through 0.45 μM filters (Millipore, Bedford, MA, USA) for measurement of dissolved silver levels.

Water analysis

Aliquots from the 10-mL daily water sample were taken for Cl^- and total ammonia analysis, and the remainder of the sample was acidified with 0.5% by volume of trace metal grade concentrated HNO_3 for analysis of ions and silver. Water Na^+, Ca^{2+}, K^+ and Mg^{2+} concentrations were measured by flame atomic absorption (Varian AA 1275, Varian Ltd., Mississauga, ON, Canada). Water silver levels were measured by graphite furnace atomic absorption spectrophotometry (Varian AA 1275 fitted with a GTA-95 atomiser). Water Cl^- levels were measured by the colorimetric mercuric thiocyanate method (Zall *et al.*, 1956) and water ammonia by the salicylate-hypochlorite method (Verdouw *et al.*, 1978). Water pH (GK2401C electrode) and dissolved oxygen levels (E5046 electrode) were measured using Radiometer equipment (Radiometer

Instruments, Copenhagen, Denmark). Dissolved organic carbon levels were determined by a Rosemount Analytical DC-180 automated total organic carbon analyser (Folio Instruments, Kitchener, ON, Canada).

Flux measurements

Juvenile rainbow trout, weight 4.22 ± 1.89 g, were obtained from Humber Spring Hatchery, and fathead minnows, weight 2.71 ± 0.75 g, were obtained from Spring Valley trout farm, Petersburg, Ontario, Canada. Fish were kept in dechlorinated Hamilton tap water (for composition see above) for at least 2 weeks before conducting experiments. Fish were fed 1% of their body weight per day during this period.

Whole-body Na^+ and silver influxes were performed in 100-mL of test medium within light-shielded plastic bags. About 24-h before commencement of the experiment, 100-mL of synthetic soft water containing 0.01 μCi $^{110m}AgNO_3$/L (94.3 MBq, Amersham International, Courtaboeuf, France) was placed in the plastic bags to saturate silver binding sites on the plastic. Fluxes were performed in synthetic soft water supplemented with 0.5 mM $CaNO_3$ and 0.25 mM $NaNO_3$, and adjusted to pH 7.6 with 1 M KOH. The [Cl^-] was adjusted to 50 μM in the control series and 50, 250, or 800 μM in the experimental series by the addition of KCl. To each container 2 μCi/L $^{24}Na^+$ (nuclear reactor at McMaster University, Hamilton, ON, Canada) was added in the form of a trace amount of sodium carbonate, which had negligible effect on water total Na^+ level. In

addition, in all experimental series, 1 μCi ^{110m}Ag/L was added in the form of $AgNO_3$ (equivalent to 8 μg total Ag/L, as measured by graphite furnace atomic absorption spectrophotometry, see above). Individual rainbow trout or fathead minnows were then added to the plastic bags. The flux period lasted for 4-h, during which time the medium was continuously aerated. The first 5-mL water samples were taken 15 min after the fish had been added to the plastic bag; this delay allowing for mixing of the eluent. Further water samples were taken at 4-h. All water samples were immediately acidified with 0.5% HNO_3 and Na^+ and silver concentrations were measured as described above (see *Water analysis*). After 4-h the fish were euthanised by an overdose of MSS-222 (Syndel Pharmaceuticals, Vancouver, BC, Canada) and then washed in 150 mg/L $Na_2S_2O_3$ for 1 min, 250 μg $AgNO_3$/L for 1 min, dechlorinated Hamilton tap water for an additional 1 min and then blotted dry. This wash regime removed loosely bound ^{110m}Ag and $^{24}Na^+$ from the surface of the fish. The gills were dissected from the body and counted separately. Radioactivities of the water, body and gills were measured on a gamma counter (Packard Instruments, Downers Grove, IL, USA).

The radioactivity in the samples emitted by $^{24}Na^+$ was calculated from the initial total counts per minute (cpm) (composed of both $^{24}Na^+$ and ^{110m}Ag cpm) minus the cpm after 10 half- lives (150-h) of $^{24}Na^+$ (representing the proportion attributable to ^{110m}Ag alone). This value was then corrected for the decay of the $^{24}Na^+$ isotope during counting.

Gill loads (of Ag) and whole-body influx rates of sodium and silver were calculated from the appearance of radioactivity in the tissues as follows:

$$\text{whole-body influx} = q / (SA \cdot t \cdot wt) \qquad (2)$$

where q represents the cpm for gill or whole body, t is the time (h), wt the wet weight of the sample, and SA is the specific activity of the water calculated from:

$$SA = [(cpm_i / [ion]_i) + (cpm_f / [ion]_f)] / 2 \qquad (3)$$

where cpm_i represents the initial cpm per mL in the water and cpm_f represents the final CPM per ml in the water, and $[ion]_i$ and $[ion]_f$ represent the initial and final sodium or silver concentrations of the water, respectively. The division by time was omitted for calculation of gill silver load.

Calculations and statistics

The LC50 values were calculated either by probit analysis (SPSS 6 computer package, Chicago, IL, USA) or by the methods of Litchfield and Wilcoxon (1949) and are reported with the 95 % confidence limit. Values are considered different when the CLs did not overlap. Water geochemical analysis was performed using the MINEQL+ computer program (Schecher and McAvoy, 1992), with the addition of the conditional equilibrium constants for Ag-DOC and H-DOC taken from Janes and Playle (1995). A one-way analysis of variance followed by a least significant difference (LSD) test (STATISTICA, StatSoft, Tulsa, OK, USA) was used to test differences (at $\alpha = 0.05$) among treatments in Na^+ influx rates and gill silver loads .

RESULTS

Measured water [Cl^-] and [Ca^{2+}] were in the ranges of 95 to 114 % and 99.8 to 115.8 %, respectively, of the predicted values. Measured DOC concentrations were slightly higher than predicted; a predicted value of 1mg DOC/L corresponded to a measured value of 1.63 mg DOC/L, and 5 mg DOC/ L corresponded to 5.82 mg DOC/L (Table 1). Water ammonia values averaged 12.74 ± 10.1 μM total ammonia (n=294) and measured dissolved silver values (the silver concentration after filtration through a 0.45 μm filter) were between 81 and 94 % of the measured total silver values for the Cl^- and Ca^{2+} and 71 to 75 % of the measured total silver concentration in the DOC experiment.

Influence of chloride

For rainbow trout, an increase in water [Cl^-] resulted in an increase (decrease in toxicity) in the 96-h total silver LC50 values. A 30 times increase in Cl^- increased the LC50 values by 4.3 times (Fig. 1 and Table 2). In contrast, for fathead minnows, the 96-h total silver LC50 values remained virtually constant (1.3-fold increase, not significant) over a water [Cl^-] range of 50 to 1,500 μM (Fig.1 and Table 2). Thus, at higher [Cl^-] levels (800 μM, 1,500 μM) total silver was significantly more toxic to fathead minnows than to rainbow trout. For rainbow trout, the 96-h LC50 value based on ionic silver (Ag^+ as calculated from MINEQL+), remained constant, at about 5 μg/L. However, for fathead minnows, the 96-h ionic silver LC50 values decreased significantly as [Cl^-]

increased (Fig. 1 and Table 2).

Influence of calcium

In both species, increasing [Ca^{2+}] over the range of 50 to 2,000 μM resulted in only a very small change in the 96-h LC50 (1.5-fold increase, not significant), for both total silver and ionic silver (Fig. 2 and Table 2).

Influence of DOC

For both species, an increase in the water DOC levels from 0.3 mg/L to 5.8 mg/L resulted in a substantial increase in the 96-h total silver LC50 values (Fig. 3 and Table 2). At 5.8 mg DOC/L there was a 4.1 times increase at the 96-h total silver LC50 value for rainbow trout (7.5 to 27.7 μg/L) and a 2.7 times increase occurred for fathead minnows (6.7 to 18.0 μg/L). At this highest DOC level (5.8 mg/L) the 96-h LC50, expressed as total silver, was significantly greater (less toxic) in rainbow trout than in fathead minnows. For rainbow trout, different DOC concentrations did not significantly affect (based on geochemical model calculations, Schecher and McAvoy, 1992) the 96-h ionic silver LC50 values, which remained close to 5 μg/L. However, for fathead minnows, the highest DOC level (5.8 mg/L) resulted in a decrease in the 96-h ionic silver LC50 values (Fig. 3 and Table 2).

Flux measurements

The total silver concentration for the flux experiments was set at 8 μg $AgNO_3$/L. Altering the chloride concentration to 50, 250 or 800 μM corresponded to changes in the [Ag^+] of 6.2, 4.5 and 2.4 μg $AgNO_3$/L, respectively (calculated from MINEQL+, Schecher and McAvoy, 1992). The whole-body Na^+ influx rate was about 50% higher in control rainbow trout compared to control fathead minnows. Acute exposure to silver resulted in a significant (30 - 40 %) reduction in the whole-body Na^+ influx rate in both species (Table 3); the inhibition was independent of the water [Cl^-]. Rainbow trout accumulated more silver in their gills and exhibited higher body silver uptake rates during the 4-h exposure period than did fathead minnows (approximately 2-fold differences; Table 4). Increasing [Cl^-] did not affect the body silver uptake rate in either species, but did tend to lower the gill silver load in both. This effect was statistically significant only at 800 uM [Cl^-] for rainbow trout (65% reduction); the 50% reduction in gill silver burden for fathead minnows at this [Cl^-] was not significant (Table 4).

DISCUSSION

Conducting toxicity tests on rainbow trout and fathead minnows under the same conditions, and within the same container, allows direct comparison of the effects of freshwater chemistry on silver toxicity between the species. Aquatic environments can vary dramatically in their ionic composition, but variation in the concentration of a single ion, as was the case in this study, is rare in the natural environment. Results from this study show that toxicity of silver to rainbow trout decreases, with respect to total silver levels, when water chloride or DOC levels are altered, but altering calcium levels does not significantly affect the 96-h LC50 total silver values. Toxicity of silver to rainbow trout correlates to the water [Ag^+], supporting the notion that it is the ionic forms of heavy metals that are toxic (Pagenkopf, 1983). Increasing DOC concentration reduces the toxicity of silver to fathead minnows, but chloride and calcium have only a minor effect on the 96-h total silver LC50 values. No clear relationship exists between toxicity and Ag^+ for fathead minnows. This dichotomy cannot be explained by any obvious difference between the species in the silver-induced disturbance to the whole-body Na^+ influx or accumulation of silver in the gills or body over a 4-h acute exposure to 8 μg Ag/L (as $AgNO_3$); trends in all were very similar in the two species (Tables 3 and 4). However, we noticed that fathead minnow mortalities generally occurred sooner than those of rainbow trout at the same silver concentration in these tests, which may indicate a difference in the mode of toxic action or pathway for silver entry.

Chloride

Chloride, when present in excess so that it forms $AgCl_n$ complexes, has been shown to significantly reduce silver toxicity to fathead minnows (LeBlanc *et al.*, 1984) and rainbow trout (Hogstrand *et al.*, 1996). In the present study, the chloride levels were chosen so that no cerargyrite formed and that only the aqueous Ag^+, and the aqueous silver chloride species, AgCl and $AgCl_2^-$, levels varied. Chloride strongly protected rainbow trout against silver toxicity when the latter was expressed as total silver. Similarly, Galvez and Wood (1997) found that chloride increased the median lethal time values for rainbow trout at a fixed silver concentration of 100 µg Ag/L. Hogstrand and Wood (1998) summarize additional data indicating that chloride protects against silver toxicity to rainbow trout. In all studies silver toxicity correlated to Ag^+. The strong relationship between Ag^+ and toxicity was also observed in physiological studies on silver-induced disturbances. Both Bury *et al.* (1999b) and McGeer and Wood (1998) showed that perturbations to Na^+ balance in rainbow trout induced by silver exposure correlate to water [Ag^+].

The 96-h total silver LC50 values for fathead minnows did not vary over a chloride range of 50 - 1,500 µM. The result is similar to those of Brooke *et al.* (1996) and Karen *et al.* (1999), which showed a very minor protective effect of chloride on silver toxicity to 4-d-old fathead minnows. In contrast, Erickson *et al.* (1998) found that the addition of sodium chloride actually increased silver toxicity to 30-d-old fathead minnows. Nevertheless, the overall conclusion is that chloride does not protect fathead minnows against silver toxicity as it does in rainbow trout. Moreover, the 96-h Ag^+

LC50 values declined with increasing chloride concentrations. This suggests that other Ag complexes, such as $AgCl^0$ and $AgCl_2^-$, contribute to toxicity in fathead minnows. Indeed, Erickson *et al.*. (1998), based on their toxicity data in fathead minnows, proposed that $AgCl^0$ may contribute to toxicity because it may be more readily transported by this species across membranes to the site of action. Copper toxicity to fathead minnows has been found to be modified by altering water chemistry (Laurén and McDonald, 1986; Welsh *et al.*, 1993; Erickson *et al.*, 1996; Hollis *et al.*, 1997). The 96-h total copper LC50 values increase as water alkalinity and pH levels are changed. However, when these values are expressed as ionic copper, the 96-h LC50 values decrease, suggesting that species of copper other than Cu^{2+} are toxic to fathead minnows (Brooke *et al.*, 1996).

Assuming that the site for silver toxicity to freshwater fish is the gill (Morgan *et al.*, 1997; Hogstrand and Wood, 1998; Webb and Wood, 1998), then the difference in the toxic response of rainbow trout and fathead minnows when water chloride concentrations are altered suggests that the gills of these two species respond differently to AgCl complexes. Physiological studies show that silver inhibits Na^+ influx in rainbow trout (Table 2; Morgan *et al.*, 1997; McGeer and Wood, 1998; Webb and Wood, 1998; Bury *et al.*, 1999b), as well as in fathead minnows (Table 3). Chloride did not protect against perturbations to Na^+ balance induced by acute exposure to 8 ug/L of silver ($AgNO_3$) in either species. In the case of rainbow trout, this contrasts to the results from two previous studies (McGeer and Wood, 1998; Bury *et al.*, 1999b), and is probably a consequence of the higher silver concentration (8 μg $AgNO_3$/L compared to 3.2 μg $AgNO_3$/L) and the shorter exposure times (4-h compared to 6- and 48-h) used in the

present study. The reduction in gill silver levels in rainbow trout, the site of silver toxicity in this species (Morgan *et al.*, 1997; McGeer and Wood, 1998; Webb and Wood, 1998; Bury *et al.*, 1999b), may explain why chloride protects against silver toxicity, but the same phenomenon occurred in fathead minnows without the accompanying protection. Silver toxicity to freshwater rainbow trout correlates to Ag^+ (Galvez and Wood, 1997; Morgan *et al.*, 1997; Hogstrand and Wood, 1998; McGeer and Wood, 1998; Bury *et al.*, 1999b), but this scenario does not appear to be apparent for fathead minnows (Brooke *et al.*, 1996; Erickson *et al.*, 1998; Karen *et al.*, 1999), because other silver complexes may be toxic. The physiological reasons for the disparity between the two species is not clear and requires further investigation.

Calcium

Increasing water calcium concentrations from 50 to 2,000 μM had only a slight, non-significant, effect on 96-h total silver LC50 values for both rainbow trout and fathead minnows (Fig. 2). This small protective effect may be a consequence of the stabilizing effect that Ca^{2+} has on the permeability of the fish gill epithelium (Evans, 1987). This will reduce the loss of ions via the paracellular pathway that occurs on exposure to silver (Wood *et al.*, 1996a). These results corroborate those of Galvez and Wood (1997) and also add to the debate raised by a number of authors (Hogstrand *et al.*, 1996; Galvez and Wood, 1997; Hogstrand and Wood, 1998; McGeer and Wood, 1998; Bury *et al.*, 1999b) concerning the validity of the US EPA hardness equation (US EPA,

1980), designed to help authorities in the regulation of silver discharge. The reported data are in contrast with Erickson *et al.* (1998) who reported a modest protective effect of hardness on silver toxicity. However, it is evident that for such an equation to be reliable, other parameters, such as chloride, DOC, sulphide and thiosulphate, which influence silver toxicity to a greater extent, must be incorporated.

Dissolved Organic Carbon

On the basis of total silver, DOC strongly protects both rainbow trout and fathead minnows against silver toxicity. This finding is in close agreement with the data of Erickson *et al.* (1998) on 30-d-old fathead minnows and Karen *et al.* (1999) for 4-d-old fathead minnows. The protective effect of DOC has also been demonstrated for copper toxicity (Welsh *et al.*, 1993; Erickson *et al.*, 1996), as well as for a mixture of copper and cadmium (Hollis *et al.*, 1997). Protection results from DOC forming silver complexes, which reduces the available metal concentration in the water. Indeed, DOC has been shown to reduce silver gill accumulation in rainbow trout (Janes and Playle, 1995; Bury *et al.*, 1999b). However, the reduction in the 96-h ionic silver LC50 values for fathead minnows at a DOC concentration of 5.8 mg/L suggests that a proportion of these Ag-DOC complexes may contribute to toxicity. A very similar pattern was observed by Erickson *et al.* (1996) for the effects of DOC on copper toxicity to fathead minnows. They suggested that the DOC-copper complexes are not absorbed by the fish, but that the

interaction of these complexes at the gill surface results in a fraction of the metal becoming available.

In conclusion, on the basis of total silver, DOC protects both rainbow trout and fathead minnows against silver toxicity, water chloride protects only rainbow trout, and water calcium has only a negligible effect. For rainbow trout, silver toxicity correlates to Ag^+, but this is not the case for fathead minnows, at least at different water chloride concentrations. The difference between the LC50 values of two species over the water chloride range and at the highest DOC level tested suggests that rainbow trout and fathead minnows react differently to inorganic-Ag and organic-Ag complexes. These differences warrant further investigation.

Acknowledgement

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Table 1. Measured water ion concentrations, pH, dissolved oxygen (DO_2), and dissolved organic carbon levels (DOC). Values are mean ± SD[a].

Predicted Cl:Ca:DOC	Cl^- (µM)	Ca^{2+} (µM)	Na^+ (µM)	K^+ (µM)	Mg^{2+} (µM)	DO_2[b]	pH	DOC[c] (mg L^{-1})
Chloride								
50:50:0.3	67.8 ± 15.8 N=40	47.6 ± 9.3 N=42	83.9 ± 8.5 N=47	10.9 ± 2.5 N= 48	4.7 ± 5.6 N=48	91.2 ± 1.1 N=6	6.2 ± 0.03 N=6	NM
250:50:0.3	240.7 ± 34.2 N=40	51.7 ± 3 N=42	257.2 ± 11.6 N=47	11.2 ± 2.4 N=47	3.8 ± 3.2 N=48	90.3 ± 3.8 N=6	6.3 ± 0.04 N=6	NM
800:50:0.3	782.2 ± 68.2 N=34	65.2 ± 22.7 N=48	868 ± 120.1 N=47	12.7 ± 3.2 N=48	14.1 ± 3.3 N=46	94.9 ± 1.1 N=6	6.6 ± 0.04 N=6	NM
1500:50:0.3	1426 ± 139 N=33	91.2 ± 71.6 N=64	1590 ± 155 N=63	14 ± 2.9) N=64	3.6 ± 3.8 N=64	92.7 ± 4.7 N=6	6.7 ± 0.2 N=6	NM

Calcium								
50:500:0.3	46.3 ± 10 N=35	499 ± 27.8 N=48	75.2 ± 10.6 N=48	11.7 ± 2.5 N=48	12.2 ± 7.9 N=48	94.9 ± 2.5 N=6	6.6 ± 0.07 N=6	NM
50:2000:0.3	58.9 ± 26.5 N=28	2316 ± 252 N=45	85.7 ± 21.1 N=45	12 ± 2 N=44	12 ± 4 N=44	79.2 ± 14.3 N=6	6.5 ± 0.03 N=6	NM
DOC								
50:50:1	74.3 ± 16.6 N=42	32 ± 13.1 N=54	110.6 ±13.7 N=46	15.2 ± 2.6 N=54	3 ± 2.7 N=54	NM	6.7 ± 0.07 N=6	1.63 ± 0.14 N=4
50:50:5	95.2 ± 19.4 N=42	34.5 ± 22.4 N=64	150.6 ± 21.6 N=62	14.6 ± 1.6 N=64	3.5 ± 3.7 N=64	NM	6.7 ± 0.08 N=6	5.82 ± 0.12 N=4

[a]N= number of samples measured; NM = not measured.

[b]Dissolved oxygen concentrations presented as a % of air saturated water.

[c]DOC levels for reverse osmotic dechlorinated Hamilton tapwater (controls) are 0.3 mg L^{-1} (Bury *et al.*, 1999b).

Table 1. Continued.

Table 2. Summary of 96-h median lethal concentrations ± 95% confidence limits.

	Fathead minnows		Rainbow trout	
Water Chemistry	Total silver	Ionic silver[b]	Total silver	Ionic silver[b]
Cl (μM): Ca (μM): DOC[a] (mg L^{-1})	(μg Ag L^{-1})	(μg Ag^{+} L^{-1})	(μg Ag L^{-1})	(μg Ag^{+} L^{-1})
Chloride Treatment				
50:50:0.3	6.7 ± 2.5	4.9 ± 2.3	7.5 ± 2.8	5.6 ± 2.7
250:50:0.3	7.5 ± 2.4	4.1 ± 1.5	9.2 ± 3.0	5.2 ± 1.8
800:50:0.3	7.7 ± 2.0	2.3 ± 0.6	18.5 ± 4.5	6.1 ± 1.5
1500:50:0.3	8.8 ± 2.3	1.7 ± 0.4	25.6 ± 3.7	5.3 ± 0.6
Calcium Treatment				
50:500:0.3	7.8 ± 2.4	6.1 ± 2.1	9.9 ± 4.8	8.0 ± 3.3
50:2000:0.3	9.9 ± 2.6	8.4 ± 1.9	10.5 ± 2.3	8.9 ± 1.6
DOC Treatment				
50:50:1.6	13.3 ± 5.5	6.2 ± 1.2	18.4 ± 2.7	10.6 ± 2.1
50:50:5.8	18.0 ± 3.2	0.4 ± 0.3	27.7 ± 2.8	5.1 ± 2.9

[a]DOC = dissolved organic carbon.

[b]Ionic silver was calculated by the geochemical speciation program MINEQL+ (Schecher and McAvoy, 1992)

Table 3. Whole body Na^+ influx rates ($\mu mol\ kg^{-1}\ h^{-1}$) for fathead minnows and rainbow trout in control conditions (in μM: 50, Cl^-; 500, Ca^{2+}; 250, Na^+) and with the addition of $8 \mu g\ Ag\ L^{-1}$ (as $AgNO_3$) at different water chloride concentrations.

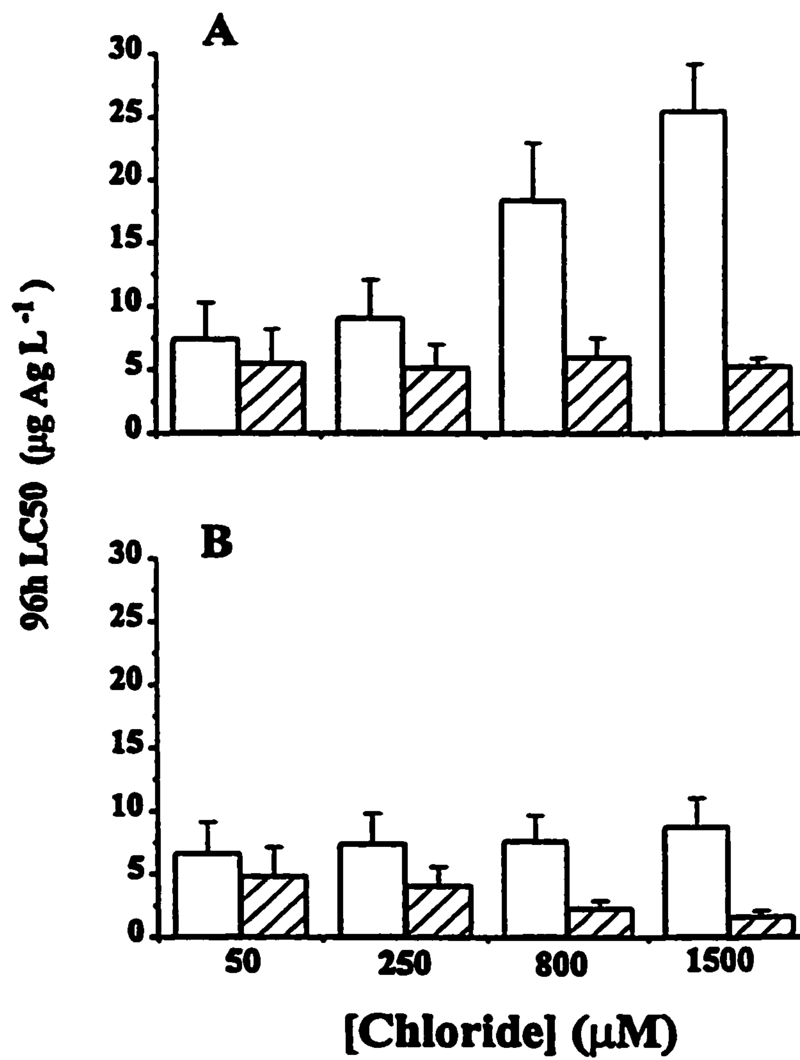
	Fathead minnows ($\mu mol\ kg^{-1}\ h^{-1}$)	Rainbow trout ($\mu mol\ kg^{-1}\ h^{-1}$)
Control	246.5 ± 19.7	360.7 ± 29.5
Chloride concentration		
50 µM	195.9 ± 21.7	269.0 ± 14.7 *
250 µM	149.7 ± 14.5*	236.9 ± 32.6 *
800 µM	169.7 ± 24.8*	221.9 ± 21.9 *

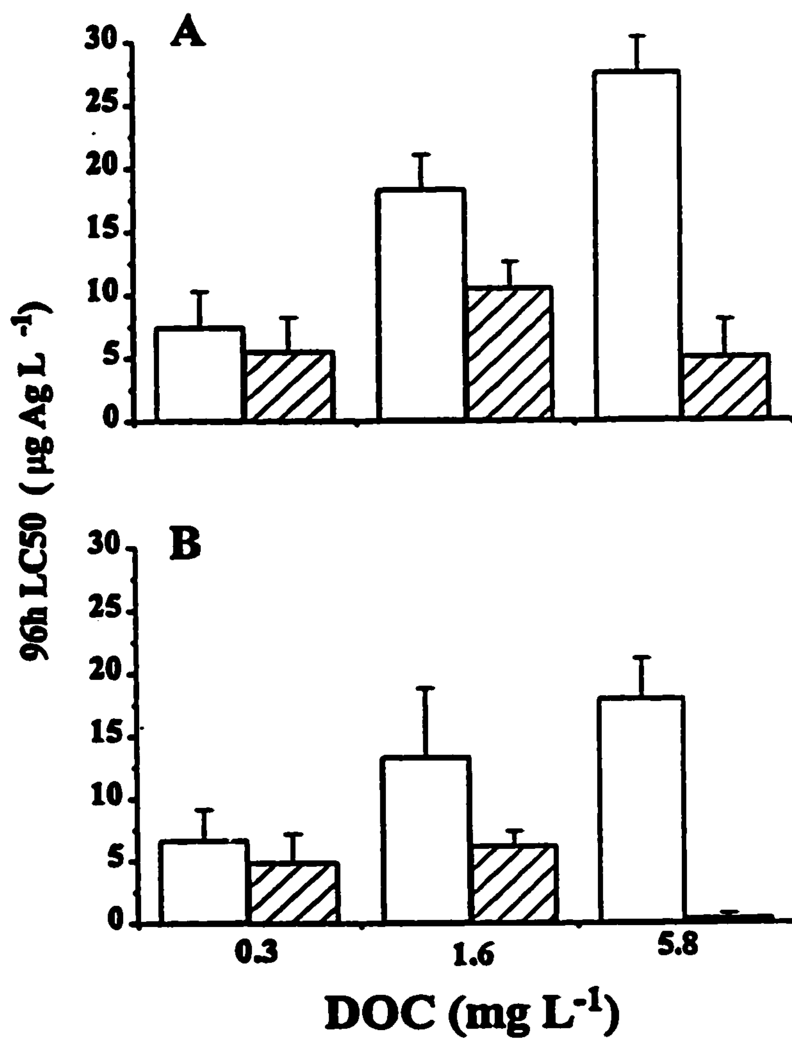
Asterisks indicate significant difference from control values for the appropriate species ($P<0.05$, one-way ANOVA followed by LSD test). Each value represents the mean ± SEM for 7 or 8 fish.

Table 4. Gill silver load (μg kg^{-1} gill) and body silver uptake (nmol kg^{-1} h^{-1}) for fathead minnows and rainbow trout exposed to 8μg Ag L^{-1} (as $AgNO_3$) labelled with ^{110m}Ag at different water chloride concentrations.

	Fathead minnows		Rainbow trout	
	Gills (μg kg^{-1})	Body (nmol kg^{-1} h^{-})	Gills (μg kg^{-1})	Body (nmol kg^{-1} h^{-})
Cl^- concentration				
50 μM	329 ± 71	13.1 ± 1.5	521 ± 69	25.3 ± 2.1
250 μM	235 ± 69	11.3 ± 3.0	562 ± 136	35.9 ± 9.0
800 μM	164 ± 37	12.2 ± 2.1	192 ± 33[a]	22.8 ± 4.7

[a] represents difference from the other values in the same column ($P<0.05$, one-way ANOVA followed by LSD test). All other values are not significantly different from each other. Each value represents the mean ± SEM for 7 or 8 fish.


A
B
30
25
20
15
10
5
0
96h LC50 (μg Ag L -1)
50
250
800
1500
[Chloride] (μM)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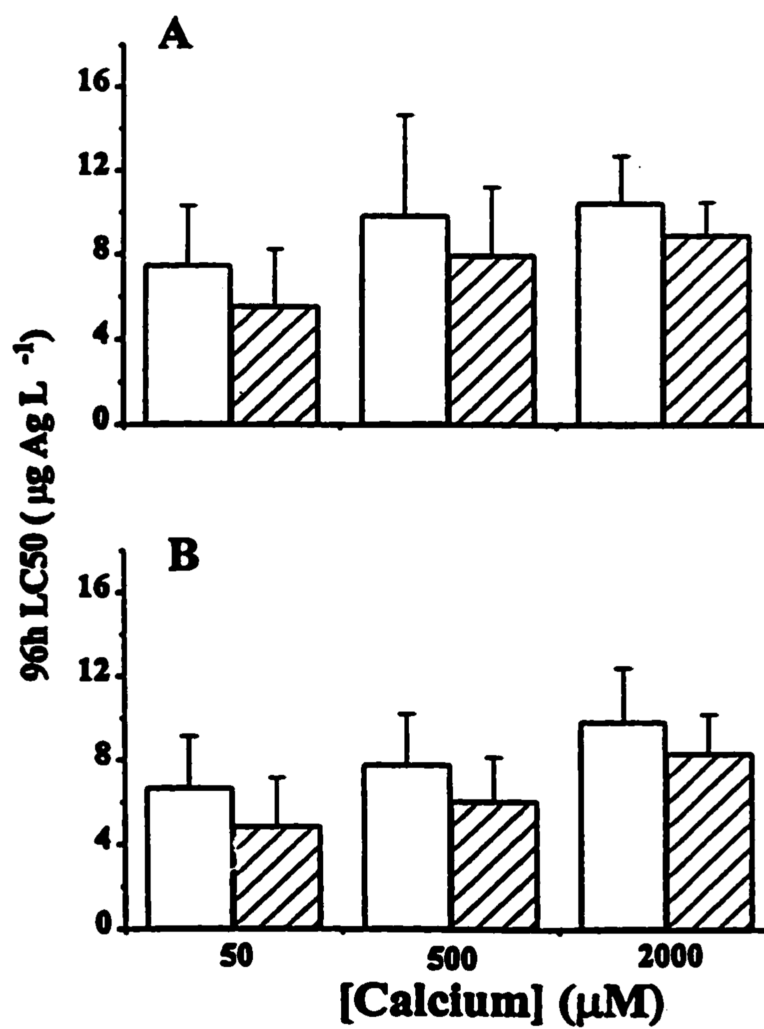
DOC (mg L-1)


A
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96h LC50 (μg Ag L -1)
0
4
8
12
16
50
500
2000
[Calcium] (μM)

CHAPTER 4

A PHYSIOLOGICALLY BASED BIOTIC LIGAND MODEL FOR PREDICTING THE ACUTE TOXICITY OF WATERBORNE SILVER TO RAINBOW TROUT IN FRESH WATERS

ABSTRACT

Previously published silver-gill binding model using conditional equilibrium binding constants (*K*) was fitted to toxicity data for rainbow trout (*Oncorhynchus mykiss*) and subsequently modified to produce a mechanistically based model for predicting acute toxicity of silver. The model used an "off the shelf" aquatic geochemistry software program (MINEQL+) and physiologically based *K* values. The final version of the model does not predict total gill silver loading but rather the binding of Ag^+ to key toxic sites on the gill and incorporates the effects of cation competition at those sites. The acute toxicity model for Ag^+ provided the best fit to toxicity data when the log *K* value for Ag-site complexes was 7.6, the log *K* values for Na-site and Ca-site complexes were 2.9 and 2.3, respectively, and the log *K* for Ag-dissolved organic matter was 9.0. The model we present is easy to use and provides a good match with previously published acute $AgNO_3$ toxicity data for rainbow trout from 31 data sets in 10 studies. The modified model is now ready for a full verification with a greater range of laboratory and natural waters.

INTRODUCTION

Modelling the interactions of metals with biological surfaces, particularly the fish gill, has recently been advocated as a method for predicting the acute toxicity of metals in freshwater systems (Bergman and Dorward-King, 1997; Renner, 1997; Playle, 1998; Meyer *et al.*, 1999). These biotic ligand models are based on the gill surface interaction model for trace metal toxicity concepts proposed by Pagenkopf (1983). The applicability of these approaches is based on the premise that waterborne metals, particularly in the free cation form, bind to specific sites on the gills, impair physiological processes related to ionic uptake, and result in acute toxicity (McDonald and Wood, 1993). This gill modelling or tissue residue based approach has the potential to provide a relatively simple, inexpensive, mechanistically based and scientifically defensible way to predict the acute toxicity of metals on a water chemistry specific basis.

The data available for the acute toxicity and gill binding of silver for rainbow trout, while not extensive, are remarkably complete and complementary (Hogstrand and Wood, 1998; Wood *et al.*, 1999). The mechanism of acute Ag^+ toxicity is inhibition of Na^+/K^+ ATPase activity in the gills and associated ionoregulatory failure (Hogstrand et al., 1996; Morgan et al., 1997; McGeer and Wood, 1998; Webb and Wood, 1998). The effects of metal complexation on both acute toxicity and gill Na^+/K^+ ATPase activity have been illustrated in a number of studies (Hogstrand *et al.*, 1996; Galvez and Wood, 1997; Morgan *et al.*, 1997; McGeer and Wood, 1998; Bury *et al.*, 1999a,b).

The work of Janes and Playle (1995) provides a suitable modelling framework using experimentally-derived conditional equilibrium binding constants (K) to model silver binding to gills of rainbow trout. When the appropriate log K values for gill-silver

loading are integrated into chemical equilibrium computer programs (e.g., MINEQL+, Schecher and McAvoy, 1992), the resulting model can be used to predict silver accumulation on trout gills and, by extension, acute silver toxicity. One of the strengths of this approach is that both the influence of competition with other cations (e.g., Ca^{2+}, H^+, and Na^+) and complexation with anions (e.g. dissolved organic matter (DOM), thiosulfate, and Cl^-) by Ag^+ are incorporated into the model (Figure 1). This approach has been used for Ag (Paquin *et al.*, 1999) as well as Cu, Cd, Co and Ni (Playle *et al.*, 1993a,b; Hollis *et al.*, 1997; Richards and Playle, 1998; Hollis *et al.*, 1999; MacRae *et al.*, 1999; Meyer *et al.*, 1999), and the overall approach has been summarized in a recent review (Playle, 1998).

The log $K_{Ag\text{-}Gill}$ of 10.0 derived from the work of Janes and Playle (1995), which modeled short-term silver accumulation on the gills (over 2 - 3 h), was never experimentally correlated with silver toxicity. Indeed, although the connection between metal accumulation on fish gills and toxicity has logical and some empirical support for Cu, Cd and Ni (Playle *et al.*, 1993b; Mayer *et al.*, 1999; MacRae *et al.*, 1999), the relationships between silver accumulation and toxicity, and especially *predicted* silver accumulation and toxicity, need to be tested thoroughly. This issue is particularly important for silver, as some studies (McGeer and Wood, 1998; Bury *et al.*, 1999b) have indicated that total loading of silver on the gills was not correlated with physiological toxicity.

A recent review (Wood *et al.*, 1999) suggested that predictive models for silver toxicity should be based on the actual mechanism of Ag^+ toxicity, inhibition of gill Na^+/K^+ ATPase activity, rather than on the Ag-gill load. The conditional equilibrium

stability constant for Ag^+ causing a 50% inhibition of Na^+/K^+ ATPase activity *in vivo* (log $K_{Ag\text{-}ATPase}$) was 7.8 (Wood *et al.*, 1999), as calculated from the results of previous studies (McGeer and Wood, 1998; Bury *et al.*, 1999b). This log K value is about 150 times lower than that of the original model (log $K_{Ag\text{-}Gill}$ =10.0; Janes and Playle, 1995). While the former value better represents the mechanism of silver toxicity, the latter represents short-term accumulation of silver by the gills, and neither has been explicitly validated in a predictive toxicity model. Clearly, these types of metal-gill binding models must be tested against experimentally-derived toxicity data from a range of water chemistries and, if necessary, adjusted in a scientifically-defensible manner before they can be considered as tools for use in regulating metals in the aquatic environment. One other version of a model for acute silver toxicity has recently been developed (Paquin *et al.*, 1999). While this model exploited the complexation and competition framework of the biotic ligand approach, the development of log K values was based on fitting the model to silver toxicity data. As such, this was a toxicity based model rather than a mechanistically based model.

The main purpose of our work was to develop a mechanistically based acute toxicity model for silver that could be tested against experimentally-derived acute toxicity data sets. The acute toxicity model used an "off the shelf" aquatic geochemistry software program (MINEQL+, Schecher and McAvoy, 1992) with the addition of values for interactions between gills, Ag^+, other cations, and DOM. Our overall objective was to test whether a mechanistic approach based on biotic ligand equilibrium modelling, incorporating both complexation and competition reactions, could be used to predict acute toxicity of silver. In addition, we evaluated whether log K values, based on gill

loading (Janes and Playle, 1995), inhibition of gill Na^+/K^+ ATPase activity (Wood *et al.*, 1999), or derived using other published data would be most suitable to an acute silver toxicity model. The model was developed by comparing predicted LC50 values with measured LC50 values from published studies under a series of different scenarios, each with a unique combination of log K values.

MODELLING SCENARIOS AND INPUTS

All modelling for acute silver toxicity was done using the geochemical equilibrium modelling software program MINEQL+, ver 4.0 (Schecher and McAvoy, 1992). Modelling simulations were run using components from the existing internal MINEQL+ database that included H_2O, H^+, Ag^+, Ca^{2+}, Cl^-, CO_3^{2-}, Mg^{2+}, Na^+, and $S_2O_3^{2-}$. Two new variables, "DOM" and "ToxAg", were incorporated into the MINEQL+ database, both with an assigned charge of -1. The variable ToxAg represents a population of theoretical sites on the gill where silver can bind and produce acute toxicity. Therefore, a major compenent of ToxAg is the silver binding sites on branchial Na^+/K^+ ATPase, but other silver binding sites which might result in acute toxicity are also included. Six additional complexation / competition reactions using these new variables were inserted into the program. These additions describe the following reactions: the complexation of Ag^+ with the ToxAg (Ag-ToxAg, which results in acute silver toxicity); the competitive influence of water Na^+, H^+ and Ca^{2+} on Ag-ToxAg interactions (Na-ToxAg, H-ToxAg, and Ca-ToxAg, respectively); and the role of waterborne DOM in complexing Ag^+ and H^+ (Ag-DOM and H-DOM). Previously reported log K values available for describing Ag-ToxAg are listed in Table 1, while those calculated in the

course of our analysis of previously reported data are given in Table 2. The concentration of the ToxAg variable that was entered into the model was 1.3 $nmol \cdot L^{-1}$. This value is the molar density of sites available for Ag toxic binding based on the mean site density of 12.5 nmol per g of gill tissue developed for juvenile rainbow trout (Janes and Playle, 1995). It is assumed that the fish have a gill tissue weight of 0.1 g and are held in 1 L of water (Janes and Playle, 1995). An alternative approach would be to use the number of Ag^+ binding sites on gill Na^+/K^+ ATPase molecules, which is thought to be in the 1 to 4 nmol per g of gill tissue range (Wood *et al.*, 1999). In practice, the concentration of ToxAg used in the model was not of primary consideration because acute toxicity predictions were based on the proportional loading (i.e., % saturation) as opposed to actual accumulation of Ag on the ToxAg variable (see below). However, it was important that the concentration of ToxAg remained less than 30 $nmol \cdot L^{-1}$ because at densities above this the variable itself had the potential to influence the mass balance of total Ag at equilibrium, an unrealistic scenario for a fish in an open aquatic system. The concentration of Ag binding sites per mg of carbon on DOM entered into the model was 35 $nmol \cdot mg\ C^{-1}$ (Janes and Playle, 1995).

With one exception, the provision of additional log K values for the Ag-ToxAg variable in the model (Table 2) was done using the waterborne Ag^+ concentrations at the 50% response level, usually the 96 h LC50. This Ag^+ concentration was subsequently converted to a log value, then used as a conditional equilibrium constant. This is similar to the calculation previously shown for Ag^+-$Gill_{ATPase}$ value (Wood *et al.*, 1999) where inhibition of gill Na^+/K^+ ATPase activity was used as the response directly related to Ag^+ toxicity (Table 1). The one exception to this calculation was the Ag^+-ATPase value in

Table 2, where we used the free Ag^+ concentration at 85% inhibition of the gill Na^+/K^+ ATPase activity *in vivo* (McGeer and Wood, 1998). The 85% inhibition level (IC85) was chosen based on results showing that exposure of rainbow trout to silver (as $AgNO_3$) at a concentration equivalent to the 96 h LC50 value in relatively hard Lake Ontario water caused an 85% inhibition, rather than 50% inhibition, of branchial Na^+/K^+ ATPase activity (Morgan *et al.*, 1997).

The acute toxicity of silver to rainbow trout was predicted in the model using the assumption that the toxic threshold (i.e., 96 h LC_{50}) occurred at 50% saturation of the theoretical gill sites with Ag^+ (i.e., Ag-ToxAg = 0.65 $nmol \cdot L^{-1}$), regardless of the nature of those sites. As such, the model predicts toxicity based on the proportion of sites available for producing toxicity that are complexed by Ag^+ rather than on the predicted total accumulation of silver in the gill tissue. The primary reasons for this simplification were that the time course of accumulation of total silver by the gill can be highly variable (e.g., Fig 4 of Wood *et al.*, 1999) and that over time, total silver accumulation does not appear to be related to the actual mechanism of Ag^+ toxicity (McGeer and Wood, 1998; Bury *et al.*, 1999b). Therefore, the model we developed is an acute silver toxicity prediction model and not a gill silver loading model, and is based on the loading dynamics of a biotic ligand, the theoretical Ag^+ binding sites on the gill that produce acute toxicity.

The process of modelling the various scenarios available started with the full model of Janes and Playle (1995) and then, in progression, a variety of Ag-ToxAg log K values were tested. The model was developed was done by comparing predicted acute toxicity values with the measured 96 h LC50 data of Bury *et al.* (1999a), a toxicity data

set in which water chemistry was systematically varied and well characterized. In combination with the testing of Ag-ToxAg log *K* values, the Na-ToxAg and Ca-ToxAg log *K* values were altered in different modelling scenarios (see Modelling Results and Discussion section and Table 4) within the constraints of published data (Bury *et al.*, 1999a). In each case, calculated and measured LC50s were compared by regression analysis. The log *K* values for Ag-DOM and H-DOM (Table 1) taken from Janes and Playle (1995) were not varied during modelling scenarios. Our modelling was bound by the requirement that all log *K* values which could be tested had to be based on measured responses to silver that had been reported in the published primary literature.

To develop and test the different log K values in the model, information on the acute toxicity of silver to juvenile rainbow trout was collected from a variety of published studies (see Table 3). However, the model was initially developed using only one acute toxicity data set. The data of Bury *et al.* (1999a) was chosen for this purpose because acute silver toxicity (96 h LC_{50}) was quantified over a wide range of measured water chemistries, as indicated in Table 3. Once the model was developed by fitting to the Bury *et al.* (1999) data, it was validated against the full available set of toxicity data listed in Table 3.

The criteria for selection of the toxicity studies were: (i) measurements of 96 h LC_{50} (preferably) silver concentrations for rainbow trout, (ii) relatively comprehensive measurements of test water chemistry, and (iii) acute testing protocols that conformed to standard methods. However, in spite of a reasonable number of acute silver toxicity studies on trout, complete water chemistry information was sometimes lacking. In particular, DOM was rarely reported. As a result, an effort was made to include more

studies in our validation data set by estimating water chemistry based on other information. For example, the study of Hogstrand *et al.* (1996) does not report DOM concentration in Hamilton hard water, but we concluded that the value reported in other studies from the same laboratory (McGeer and Wood, 1998, Bury *et al.*, 1999a,b) would be a reasonable approximation. While the study of Karen *et al.* (1999) only provides nominal measures of water chemistry, a full listing of measured water chemistry for the same experiment was found elsewhere (Karen *et al.*, 1997). The hardness of Karen *et al.* (1999) was used to estimate the amount of Ca^{2+}, assuming Ca^{2+} and Mg^{2+} were added in equal amounts. Similarly, the hardness reported by Nebeker *et al.* (1983) were converted to Ca^{2+} concentrations; and the Fort Collins dechlorinated water supply, which is described as being soft, was assumed to have a similar Na^{+} concentration to the reported Cl^{-} levels. In the study of Davies *et al.* (1978), water chemistry is not reported but a complete quantification (except DOM) of identical water can be found in an earlier study (Davies *et al.*, 1976). The study of Diamond *et al.* (1990) also lacks detail on water chemistry (reported as being soft water) but we were able to find average water ionic concentration for the area just upstream of the test site on the New River (Simmons and Heath, 1978), although DOM, again, was not reported. Because of the lack of accurate DOM concentrations in the data sets summarized by Lemke (1981), some generalized assumptions were made. We assigned a DOM content of 0.3 mg C $\cdot L^{-1}$ for all well water sources as well as other waters reported as being "soft", and values of 3 and 1 mg C $\cdot L^{-1}$ for the Lake Superior (Lab 1) and reservoir (Lab 4) waters, respectively (Lemke, 1981).

MODELLING RESULTS AND DISCUSSION

The calculated acute toxicity of silver in five modelling scenarios, each run with the eight different water chemistries given by Bury *et al.* (1999a; see Table 3), are shown in Table 4 and discussed below. These data allow a comparison of measured and calculated acute silver toxicity as water Cl^-, Ca^{2+} and DOM were varied. As a measure of the performance of the acute toxicity models under each specific combination (scenario) of log *K* values, the slope and correlation coefficient (r) of the regression of calculated against measured toxicity were used.

Modelling scenario 1 tested the conditional equilibrium stability constants from Janes and Playle (1995), generated using gill total silver burden as an endpoint. Although this model produced a good correlation between the predicted and measured LC50 values ($r = 0.91$), the low slope (0.37) suggested that the model was overly sensitive (i.e., calculated LC50 was consistently lower than measured LC50). This suggested that to obtain a better prediction of acute silver toxicity, a reduction in the affinity of the ToxAg sites for Ag^+ was needed (i.e., a lower log *K* value for Ag-ToxAg). Therefore, for modelling scenarios subsequent to scenario 1, the physiologically based log *K* values for ToxAg were used (see Table 1 & 2), starting with the one derived from the inhibition of branchial Na^+/K^+ ATPase (Wood *et al.*, 1999; value = 7.8, see Table 1).

Modelling scenario 2 yielded both a low r value and slope, and in two water chemistries it was not possible to achieve a 50% predicted loading of ToxAg sites with Ag^+ (see Table 4). The primary reason for the poor agreement between measured and predicted acute toxicities was the fact that lowering the log *K* for ToxAg to 7.8 without altering the log *K* values for Na-ToxAg (4.7) and Ca-ToxAg (3.3) gave Na^+ and Ca^{2+}

much more of an influence on reducing the predicted Ag toxicity than actually occurred. Therefore, in further modelling scenarios it was necessary to lower the log *K* values for Na-ToxAg and Ca-ToxAg to reflect their true effect on measured silver toxicity. This reduction in the sensitivity of the model for the effects of Na^+ and Ca^{2+} was achieved by adjusting the log *K* values for Na-ToxAg and Ca-ToxAg downwards using the data of Bury *et al.* (1999b) to provide a physiological basis. According to Bury *et al.* (1999b), Na^+ and Ca^{2+} in the range of 1,500 µM offered no protection against Ag^+ induced inhibition of gill Na^+/K^+-ATPase activity suggesting, that the log *K* values for Na-ToxAg and Ca-ToxAg must be ≤ 2.9. In addition, Galvez and Wood (1997) showed that the protective effect of Ca^{2+} on Ag^+ induced acute toxicity is about 10 fold less than that of waterborne Cl^-. Accordingly, the log *K* value for Ca-ToxAg was set at 2.3, one log unit below the log *K* value for Ag-Cl complexation. The model of Janes and Playle (1995) demonstrated that Na^+ was more effective than Ca^{2+} in keeping Ag^+ off the gills of rainbow trout (see scenario 1 log *K* values, Table 4) and therefore the log *K* value for Na-AgTox was tested at 2.9. The suitability of these log *K* values was tested in modelling scenario 3.

The adjustments for the competitive effects of Na^+ and Ca^{2+} in scenario 3 greatly improved the match between predicted and measured acute toxicity (Table 4). Although the model now reasonably predicted ($r = 0.96$) the relative effects of altered water chemistry on acute silver toxicity, it remained overly sensitive (slope of 0.92) as calculated LC50 values were always less than measured, particularly for the effects of Cl^-. In subsequent modelling scenarios the Ag-ToxAg log *K* value was decreased, first to

7.6 and then to 7.3 for modelling scenarios 4 and 5, respectively. The first of these two values represents the IC85 for the effect of Ag^+ on gill Na^+/K^+ ATPase activity (Morgan *et al.*, 1997; see Table 2) as well as the Ag^+-LC50 reported by Hogstrand *et al.* (1996). The Ag-ToxAg log *K* value of 7.3 was expected to provide the best fit as it was based on Ag^+ concentrations at the LC50 of the data set used for testing (Bury *et al.*, 1999a).

The reductions in the strength of the Ag-ToxAg interactions in scenario 4 improved the match between predicted and measured acute silver toxicity by having a highly significant correlation (r=0.94) plus a higher slope (1.13) and less undercalculation of the LC50 values (Table 4). A comparison of scenarios 3 and 4 against the Bury *et al.* (1999a) data is given in Figure 2. A further decrease in log $K_{Ag\text{-}ToxAg}$ to 7.3 (Scenario 5) did not improve the model, and now it over-predicted the protective effect of waterborne Cl^- (Table 4). In fact, for the highest Cl^- concentration, an acute toxicity value could not be calculated as cerargyrite formation was induced before 50% loading of the biotic ligand occurred (Table 4).

The results of scenarios 1 to 5 indicated that, among the available log $K_{Ag\text{-}ToxAg}$ values (Table 1,2), 7.6 would provide the best match with the measured acute toxicity data set (Bury *et al.*, 1999a). Therefore, scenario 4 was adopted as the final acute toxicity model (Table 5 and Figure 3).

The final version of the acute toxicity model was developed from the toxicity experiments of Bury *et al.* (1999a), which were run with $AgNO_3$ at eight different water chemistries. To test the predictive capabilities of the final version of the acute toxicity model (Scenario 4, Table 5), water chemistries from 31 separate toxicity measurements from ten studies (Table 3) were applied to the model. The results of this model

validation are shown in Figure 4 where the predictive capability of the acute toxicity model over a wide range of water chemistries is shown. With all data included, the calculated LC50 value falls within the reported error of the measured values for 55% of the data points. When the nine data points from the Karen *et al.* (1999) study are excluded (see below), 68% of calculated values fall within the reported error of measured LC50 values (Fig 4). Of the seven predicted values are within the error range of measured values, in all but two cases the calculated values are lower than the measured values, illustrating the slightly conservative nature of our model. The regression of predicted on measured LC50, for the full data set, gave a linear relationship of:

calculated LC50 = 0.95 × [measured LC50] + 0.84 (r=0.85, n=31, dotted line in Fig 4). When the data of Karen *et al.* (1999) were separated (see discussion below), the linear relationship for the remaining 22 studies was:

calculated LC50 = 1.01 × [measured LC50] –1.4 (r=0.90; solid line in Fig. 4).

The regression of predicted on measured just for the data of Karen *et al.* (1999) yielded the relationship:

calculated LC50 = 1.06 × [measured LC50] + 3.5 (r=0.90, n=9, P<0.001),

showing that the effect of water chemistry in this study was consistent with that of the model (slope = 1.06) but that the trout in this study were more sensitive (intercept = 3.5) to silver.

The data of Karen *et al.* (1999) as well as Hogstrand *et al.* (1996) had measured 96 h LC50 values which were particularly low (e.g., high Ag toxicity) in relation to calculated values and to other studies. The reason for the high Ag toxicity in Karen *et al.*

(1999) may be that very young (~20 d) rainbow trout were used, there was only minimal holding (48 h) after transport to the testing laboratory, and there was no acclimation to the ion-depleted test waters before silver exposure. These factors, plus the elevated pH (>9.0) and the apparent zero Na^+ content of the test waters may have stressed the fish enough to make them overly sensitive to $AgNO_3$. It is also possible that very young trout are more sensitive to the acute toxic effects of silver as the fit of the regression of modelled on measured LC50 for the Karen *et al.* (1999) was excellent but offset from the other data (see above and Fig 4). The reasons for the poor predictive capabilities of the model for the study of Hogstrand *et al.* (1996) are unknown, but the estimated DOM values used may not be accurate. It is interesting to note that a recent study by Galvez and Wood (2000a) in the same laboratory with similar water chemistry and source of trout produced 96 h LC50 values which better matched predicted values (Fig. 4, Table 3).

The acute toxicity model for rainbow trout developed here is able to accurately predict acute toxicity of silver in a variety of water chemistries and stocks of juvenile rainbow trout. Our model incorporates the best available information on the effects of water chemistry on silver toxicity in rainbow trout, as well as a log *K* for Ag-ToxAg which is derived from physiological data on toxic Ag-gill interactions and the mechanism of toxicity (Morgan *et al.*, 1997; McGeer and Wood, 1998).

Within the tested range of water chemistries, output from the final model given in Table 5 matched toxicity data over a four-fold range of measured 96 h LC50s (Fig 4). Dissolved organic mater (DOM) has the greatest effect on silver toxicity, followed by Cl^-, in accord with their log *K* values (Table 5). DOM and Cl^- both act by complexing Ag^+ in the water column (Janes and Playle, 1995). Ag-DOM complexes do not appear to

accumulate on the gill, while $AgCl_{aq}$ complexes appear to accumulate but do not cause acute toxicity (Hogstrand *et al.*, 1996; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury *et al.*, 1999b). The next important modifiers of Ag toxicity are Na^+ and Ca^{2+}, with log *K* values of 2.9 and 2.3, respectively.

As validation of the acute toxicity model (Table 5), we tested its predictive capabilities against measured LC50 data. With the exclusion of Karen *et al.* (1999), the majority of the calculated LC50 are within the reported 95% CI (or ± SEM) of measured values (Fig 4), although it must be recognized that water chemistry, in some cases, has been estimated. The calculated data that do not fit within the reported error of measured values are generally less than the measured LC50 values, showing the slightly conservative nature of the model.

The log *K* values in the final version of the model which quantify the competition and complexation reactions for Ag^+ binding to toxic sites on the gill (Table 5) are very different than those of the original model of Janes and Playle (1995), the starting point of our modelling exercise. This is particularly true for the complexation of Ag^+ with the biotic ligand (Ag-ToxAg in our model). However the movement from a log *K* value of 10.0 to a value of 7.6 (2.4 log units = 250 fold change) has a theoretical, methodological, and physiological basis.

The original derivation of the Ag-gill log *K* value in the model of Janes and Playle (1995) was from rapid Ag (as total Ag) loading experiments onto the gill. Thiosulfate and its known stability constant ($\log K_{Ag\text{-}S_2O_3} = 8.8$) was used as a complexing agent and a log *K* for Ag-gill ($\log K_{Ag\text{-}Gill}$) was derived after experimentally determining the amount of $S_2O_3^{2-}$ required to keep 100% of the available silver off of the gills, at a

total waterborne $AgNO_3$ concentration of about 0.07 μM (Janes and Playle, 1995). In a simple calculation, with 0.07 μM $AgNO_3$ in solution, it took fourteen times more thiosulfate than Ag to keep approximately 50% of the Ag off trout gills (1.0 μM thiosulphate; Fig. 2 of Janes and Playle, 1995). The log of 14 is approximately 1.1, which when added to the Ag-thiosulphate log *K* of 8.8 is 9.9, very close to the final log $K_{Ag\text{-}gillAg}$ value of 10.0. All of these subsequent experimentally derived competition (Ca^{2+}, Na^+, and H^+) and complexation (DOM and Cl^-) conditional equilibrium stability constants were based on the initial, thiosulphate derived, Ag-gill number in the model (Janes and Playle, 1995).

Relative to silver toxicity and the modelling in our work, the important features of the Janes and Playle (1995) gill loading model was that it described all gill-silver interactions and the separate and independent effects of cationic competition. Because the log *K* values are based on the absence (100% effect level) of loading of silver onto the gill, it was possible to determine the effects of each cation, independent of the effects of other complexation and competition reactions. As well, the Janes and Playle (1995) model assumed that all gill-Ag interactions produced toxicity while, in fact, both toxic and not toxic binding can occur (Wood *et al.*, 1999). The other Ag-ToxAg log *K* values that were available for use in our new model (Table 2) either represent the 50% effect level (or in one case 85%) for the physiological mechanism of Ag^+ toxicity or the level of Ag^+ at the measured acute toxicity values. These physiological and toxicological log *K* values not only represent different responses and different response levels but conceptually are based on effect rather than the absence of effect as in Janes and Playle (1995). Therefore the difference between the Janes and Playle Ag-Gill log *K* value and

the other Ag-ToxAg values presented in Tables 1 and 2 are due to the fact that they each represent different facets of the interaction of silver with the biotic ligand, the fish gill. Furthermore, the log $K_{Ag\text{-}gillAg}$ value was derived from measurements of fast (3h) binding of Ag to the gills whereas the log $K_{Ag\text{-}ToxAg}$ value interactions of Ag^+ at the gills which culminate in 50% inhibition of Na^+/K^+ ATPase activity or in 50% toxicity over 96 h. In a simple calculation, which ignores Na^+ competition, with a 0.17 μM solution of $AgNO_3$, it took 4,600 times more Cl^- than silver to keep alive 50% of the trout for 96 h (782 μM Cl^-, "SW+750 Cl", Table 3, this paper). The log of 4,600 is approximately 3.7, which when added to 3.3 (the log K of AgCl, Table 5) is 7.0, only four-fold from the final log $K_{Ag\text{-}ToxAg}$ value of 7.6.

Moving the log $K_{Ag\text{-}ToxAg}$ from 10.0 to a value of 7.8, as presented in scenario 2 (Table 4), dramatically improved the predictive accuracy of the model in very dilute waters (e.g., 12.5 μg L^{-1} compared to a measured value of 7.5 μg $\cdot L^{-1}$; Soft Water treatment, Table 4). This scenario also highlighted the potential problem of models based on a mixture of log K values. Decreasing the log K value of Ag-ToxAg while leaving other log K values unchanged resulted in a model that was overly sensitive to cationic competition. While it would be simple enough to scale down all other log K values which influence the loading of the ToxAg variable (Na-ToxAg, Ca-ToxAg and H-ToxAg) by an equivalent 2.2 log units, we opted to use previously published data to derive new values for two of these variables. This approach provided an approximation of the final log K values that provided the best fit to measured data. Thus the reduction in the Na-ToxAg and Ca-ToxAg log K values was based on experimentally derived data on the interaction between Ag^+ and either Na^+ or Ca^{+2} on gill Na^+/K^+-ATPase activity.

The H-ToxAg log *K* as well as those for Ag-DOM and H-DOM were not changed from the original Janes and Playle (1995) model for two reasons. First, there was no experimental evidence to justify an alteration and second, alterations would not have improved the model (e.g., the calculated LC50 for 1.5 and 5 mg $\cdot L^{-1}$ DOM agree very well with the measured values (Table 4).

The primary contribution that our present work makes is to adjust the original gill receptor loading model (Janes and Playle, 1995), which was based on total silver loading, to an acute toxicity model that reflects only toxic loading of Ag^+. As such, in our final model (Table 5) we did not find it necessary to redefine or alter any conditional stability constants other than those for binding of Ag^+ and its competitors at the biotic ligand, as discussed in the previous paragraph. For example it was not necessary to alter the Janes and Playle (1995) numbers related to DOM complexation nor was a redefinition of complexation reactions between Ag and waterborne Cl required. This reflects the fact that complexation of silver by waterborne DOM and Cl^- affects only the toxic fraction of dissolved silver, which is ionic Ag^+. The results of our modelling scenarios also support the conclusion that cationic competition, specifically by Na^+ and Ca^{+2}, is involved in protecting against Ag^+ toxicity; indeed it was necessary to redefine these log *K* values as well as the Ag-ToxAg value. In fact, using the log $K_{Ag\text{-}ToxAg}$ value of 7.3 (scenario 5, Table 4) did not produce a good predictive model because of cationic competition. While we expected that an apparent log *K* for Ag-ToxAg derived from the data set which we were testing would work best (i.e. circular modelling) it obviously was not (Table 4). The reason for this is that the log *K* value (7.4) was based on waterborne Ag^+ concentration at the 96 h LC50, a value which does not incorporate the effect of cationic

competition on the biotic ligand.

Thus, the original Janes and Playle (1995) model proved to be very robust, and required only some relatively simple adjustments for its transformation to a silver acute toxicity model. One of the primary strengths of our modelling is that all the adjustments in log *K* values have a physiological basis. The model we present is mechanistically based and accounts for the effect of concentration, complexation and competition. A vigorous verification of this model is now required with additional data. Future adjustments to the model may be necessary and could include additional complexation or competition reactions between waterborne ligands and Ag^{+}. As well, applicability of this model could be expanded beyond rainbow trout as information on mechanisms of silver toxicity and binding (log *K* and site density) in other aquatic species becomes available.

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Table 1. Initial data available for input into the gill-Ag toxicity model, as collected from literature sources.

Model variable	Measured Response	Conditional Equilibrium Constant	Source
Ag – ToxAg	Ag - Gill	log $K_{Ag\text{-}Gill}$ = 10.0 *	Janes and Playle, 1995
Ag – ToxAg	Ag^{+} - $Gill_{ATPase}$	log $K_{Ag\text{-}ATPase}$ = 7.8$^{\phi}$	Wood *et al.*, 1999
Na – ToxAg	Na^{+} - GillAg	log $K_{Na\text{-}GillAg}$ = 4.7 *	Janes and Playle, 1995
H – ToxAg	H^{+} - GillAg	log $K_{H\text{-}GillAg}$ = 5.9 *	Janes and Playle, 1995
Ca - ToxAg	Ca^{++} - GillAg	log $K_{Ca\text{-}GillAg}$ = 3.3 *	Janes and Playle, 1995
Ag - DOM	Ag - DOM	log $K_{Ag\text{-}DOM}$ = 9.0 *	Janes and Playle, 1995
H - DOM	H - DOM	log $K_{H\text{-}DOM}$ = 4.0 *	Janes and Playle, 1995

* derived from gill Ag loading experiments.

ϕ derived from Ag^{+} concentration at IC50 *in vivo.*

Binding site density for ToxAg was 1.3 nmol $\cdot L^{-1}$ while for Ag-DOM interactions it was 35 nmol $\cdot mg\ C^{-1}$.

Table 2. Additional data available for input into the gill-Ag toxicity model as calculated from previously published data showing the toxic effects of silver on rainbow trout.

Model variable	Measured Response	Conditional Equilibrium Constant	Source
Ag-ToxAg	Ag^+ - $Gill_{ATPase}$	7.6#	Morgan *et al.*, 1997; McGeer and Wood, 1998)
Ag-ToxAg	Ag^+ - LC50	7.8 ϕ	Karen *et al.*, 1999
Ag-ToxAg	Ag^+ - LC50	7.3 ϕ	Bury *et al.*, 1999b
Ag-ToxAg	Ag^+ - LC50	7.6 ϕ	Hogstrand *et al.*, 1996

derived from Ag^+ concentration at IC85.

ϕ derived from Ag^+ concentration at LC50.

Table 3. Measured toxicity data and associated water chemistry used to develop and validate the acute toxicity model. Water chemistry values for Cl^-, Na^+ and Ca^{2+} are in μmol·L^{-1} while DOM is mg C·L^{-1}. Measured toxicity in terms of total silver added as $AgNO_3$ is shown ± SEM or (95% CI).

Treatment	Reported Water Chemistry and Temperature						96h LC_{50} μg Ag·L^{-1}	Reference
	Ca^{++}	Cl^-	Na^+	DOM	pH	Temp.		
Soft Water (SW)	48	68	84	0.3	6.2	17	7.5 ± 2.8	Bury *et al.* (1999a)
SW + 250 Cl^-	52	241	257	0.3	6.3	17	9.2 ± 3.0	Bury *et al.* (1999a)
SW + 750 Cl^-	65	782	868	0.3	6.6	17	18.5 ± 4.5	Bury *et al.* (1999a)
SW + 1500 Cl^-	91	1426	1590	0.3	6.7	17	25.6 ± 3.7	Bury *et al.* (1999a)
SW + 500 Ca^{2+}	499	46	75	0.3	6.6	17	9.9 ± 4.8	Bury *et al.* (1999a)
SW + 2500 Ca^{2+}	2316	59	86	0.3	6.5	17	10.5 ± 2.3	Bury *et al.* (1999a)
SW + 1.5 DOM	32	74	111	1.63	6.7	17	18.4 ± 2.7	Bury *et al.* (1999a)
SW + 5 DOM	35	95	151	5.82	6.7	17	27.7 ± 2.8	Bury *et al.* (1999a)
Riso Soft water	10	10	50	1.3	NR	14	10.2 (9.6 – 11.3)	Grosell *et al.* (1999)
Lk. Ont. hard water	1000	700	600	1.3*	8.0	15	11.8 (10.9-13.8)	Hogstrand *et al.* (1996)
Lk. Ont. hard water	1000	700	600	1.3*	8.0	15	15.1 ± 5.6	Galvez & Wood (2000c)
Soft water	50	50	50	0.3	6.5	NR	4.7 ** (NR)	Galvez & Wood (1997)
Control water (CW)	106	0	0	0	9.3	13	1.5 (1.3-1.6)	Karen *et al.* (1997, 1999)
CW + 2.5 humic acid	164	0	0	2.6	9.3	11	5.6 (4.8-6.4)	Karen *et al.* (1997, 1999)
CW + 5 humic acid	140	0	0	4.6	9.1	13	9.5 (8.8-10.2)	Karen *et al.* (1997, 1999)
CW + 3 Cl^-	121	107	0	0	9.1	13	3.4 (3.0-3.8)	Karen *et al.* (1997, 1999)
CW + 20 Cl^-	110	569	0	0	9.2	10	2.4 (2.0-2.9)	Karen *et al.* (1997, 1999)
CW + 40 Cl^-	124	1276	0	0	9.0	11	3.8 (3.3-4.3)	Karen *et al.* (1997, 1999)

CW+ 3 Cl^- + 2.5 humic	162	93	0	2.9	9.0	12	17.1 (14.9-19.6)	Karen *et al.* (1997, 1999)
CW + 40 Cl^- + 5 humic	176	1344	0	6.3	9.2	13	28.4 (25.1-32.2)	Karen *et al.* (1997, 1999)
CW + Ca^{2+}	228	0	0	0	8.8	13	3.6 (2.9-4.4)	Karen *et al.* (1997, 1999)
Soft Water	269	97	409	0.3*	6.9	11	6.5 (5.3-8.1)	Davies *et al.* (1976, 1978)
Hard Water	1893	666	640	0.3*	7.9	14	13.0 (3.9-22.1)	Davies *et al.* (1976, 1978)
New River, Virginia	35	25	66	0.3*	7.8	12	4.8** (3.6-6.3)	Diamond *et al.* (1990)
Lab 1 continuous flow	337	34	48	3*	7.7	14	16.4 (12.8-19.2)	Lemke (1981)
Lab 4 continuous flow	289	313	274	1*	7.6	14	8.4 (5.9-11.9)	Lemke (1981)
Lab 5 continuous flow	367	366	265	0.3*	7.5*	14	9.7 (8.4-11.3)	Lemke (1981)
Lab 6 continuous flow	773	28	387	0.3*	7.8	14	9.7 (9.0-10.3)	Lemke (1981)
Rainbow trout 1	90	202	200**	0.3*	7	12	8.6 (8.0-9.2)	Nebeker *et al.* (1983)
Steelhead trout	112	202	200**	0.3*	7	12	9.2 (NR)	Nebeker *et al.* (1983)
Rainbow trout2	131	202	200**	0.3*	7	12	9.7 (8.4-11.3)	Nebeker *et al.* (1983)

NR indicates information was not reported.

** values are 144-168 h LC50 values

* water chemistry values estimated using available information.

Table 3 cont'd

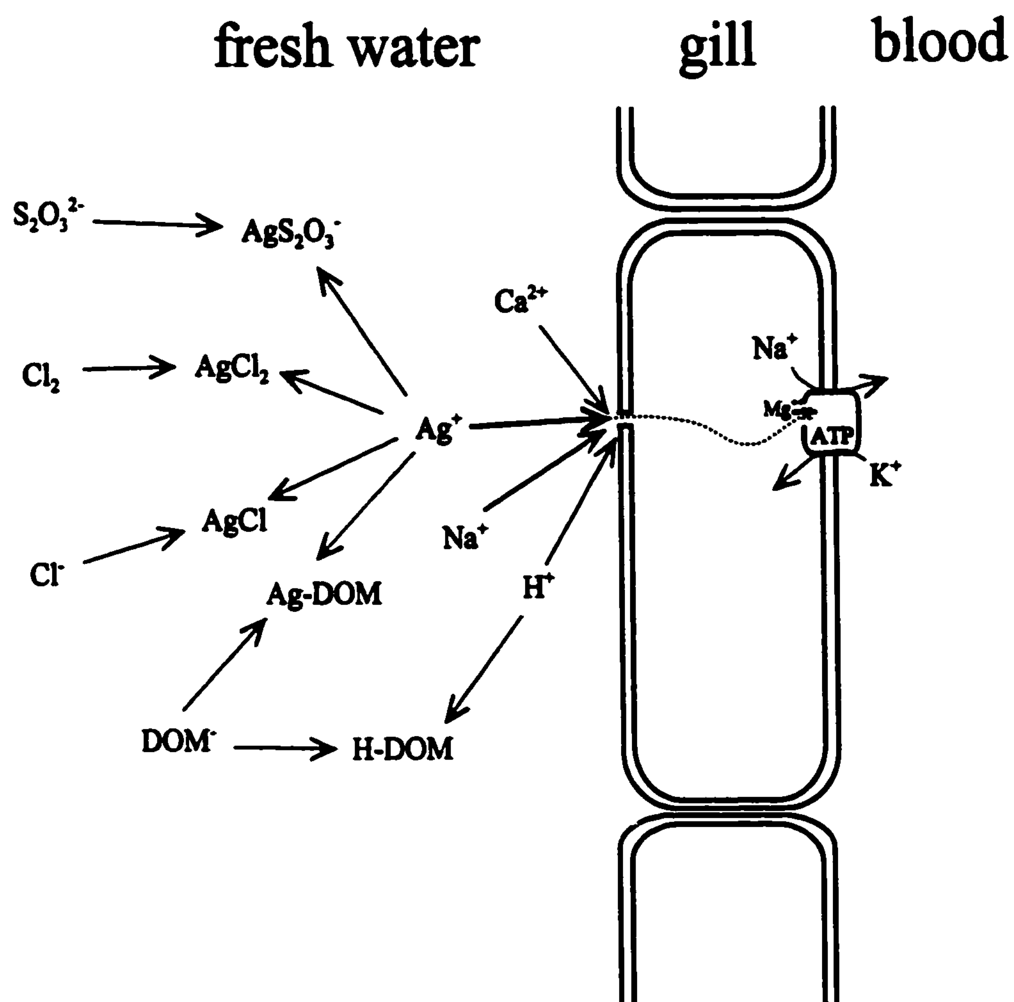
Table 4. Measured acute toxicity (96 h LC50) for rainbow trout under eight different water chemistry treatments from Bury *et al.* (1999a; see Table 3), and corresponding calculated acute toxicity using different modelling scenarios obtained by varying log *K* values. The regression of calculated against measured LC50 values was used to quantify the fit of each model with the r (* indicates P<0.05, **=P<0.01 and ***=P<0.001) and slope of each scenario shown at the bottom of the column of calculated values.

	Predicted acute toxicity values for Ag ($\mu g \cdot L^{-1}$) in modelling scenarios					Measured $AgNO_3$ 96 h LC50 ($\mu g\ Ag \cdot L^{-1}$)
Scenario	1	2	3	4	5	
Log $K_{Ag\text{-}ToxAg}$	10.0	7.8	7.8	7.6	7.3	
Log $K_{Na\text{-}ToxAg}$	4.7	4.7	2.9	2.9	2.9	
Log $K_{Ca\text{-}ToxAg}$	3.3	3.3	2.3	2.3	2.3	
Log $K_{H\text{-}ToxAg}$	5.9	5.9	5.9	5.9	5.9	
Log $K_{Ag\text{-}DOM}$	9.0	9.0	9.0	9.0	9.0	
Treatment						
Soft Water	0.6	12.5	4.3	6.2	11.1	7.5 ± 2.8
soft water + 250 μmol·L⁻¹ Cl⁻	1.1	39.2	5.4	7.9	14.8	9.2 ± 3.0
soft water + 750 μmol·L⁻¹ Cl⁻	3.0	NC	10.1	15.4	29.7	18.5 ± 4.5
soft water + 1500 μmol·L⁻¹ Cl⁻	5.0	NC	19.2	29.8	NC	25.6 ± 3.7
soft water + 500 μmol·L⁻¹ Ca⁺²	0.6	12.6	3.8	5.4	9.7	9.9 ± 4.8
soft water + 2500 μmol·L⁻¹ Ca⁺²	0.8	19.4	4.7	6.9	12.6	10.5 ± 2.3
soft water + 1.5 mg·L⁻¹ DOM	2.6	19.4	9.1	10.5	14.1	18.4 ± 2.7
soft water + 5 mg·L⁻¹ DOM	9.7	29.1	23.7	25.7	30.2	27.7 ± 2.8
r	**0.91****	**0.28**	**0.96*****	**0.94*****	**0.83***	
Slope	**0.37**	**0.37**	**0.92**	**1.13**	**0.99**	

Note: NC indicates that the it was not possible to calculate a value due to cerargyrite formation before 50% saturation of ToxAg sites.

Table 5. Selected stability constants (*K*) in the final model. Values include those added to the model as well as a selection of values from the database within the MINEQL$^+$ computer program (version 4.0).

Complex	Log *K* value	Source
Ag-ToxAg	7.6	Current model calculations
Na – ToxAg	2.9	Current model calculations
H – ToxAg	5.9	Janes and Playle (1995)
Ca – ToxAg	2.3	Current model calculations
Ag - DOM	9.0	Janes and Playle (1995)
H - DOM	4.0	Janes and Playle (1995)
$AgS_2O_3^-$	8.80	MINEQL+
AgCl	3.27	MINEQL+
$AgCl_2^-$	5.27	MINEQL+
$AgCl_3^{2-}$	5.29	MINEQL+
$AgCl_4^{3-}$	5.51	MINEQL+
$AgNO_3$	-0.29	MINEQL+
$NaS_2O_3^-$	0.60	MINEQL+
$NaCO_3^-$	1.27	MINEQL+
$NaHCO_3$	10.08	MINEQL+
CaS_2O_3	1.90	MINEQL+
$CaCO_3$	3.15	MINEQL+
$CaHCO_3^+$	11.33	MINEQL+
$HS_2O_3^-$	1.70	MINEQL+
HCO_3^-	10.33	MINEQL+
H_2CO_3	16.68	MINEQL+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blood
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$AgS_2O_3^-$
Ca^{2+}
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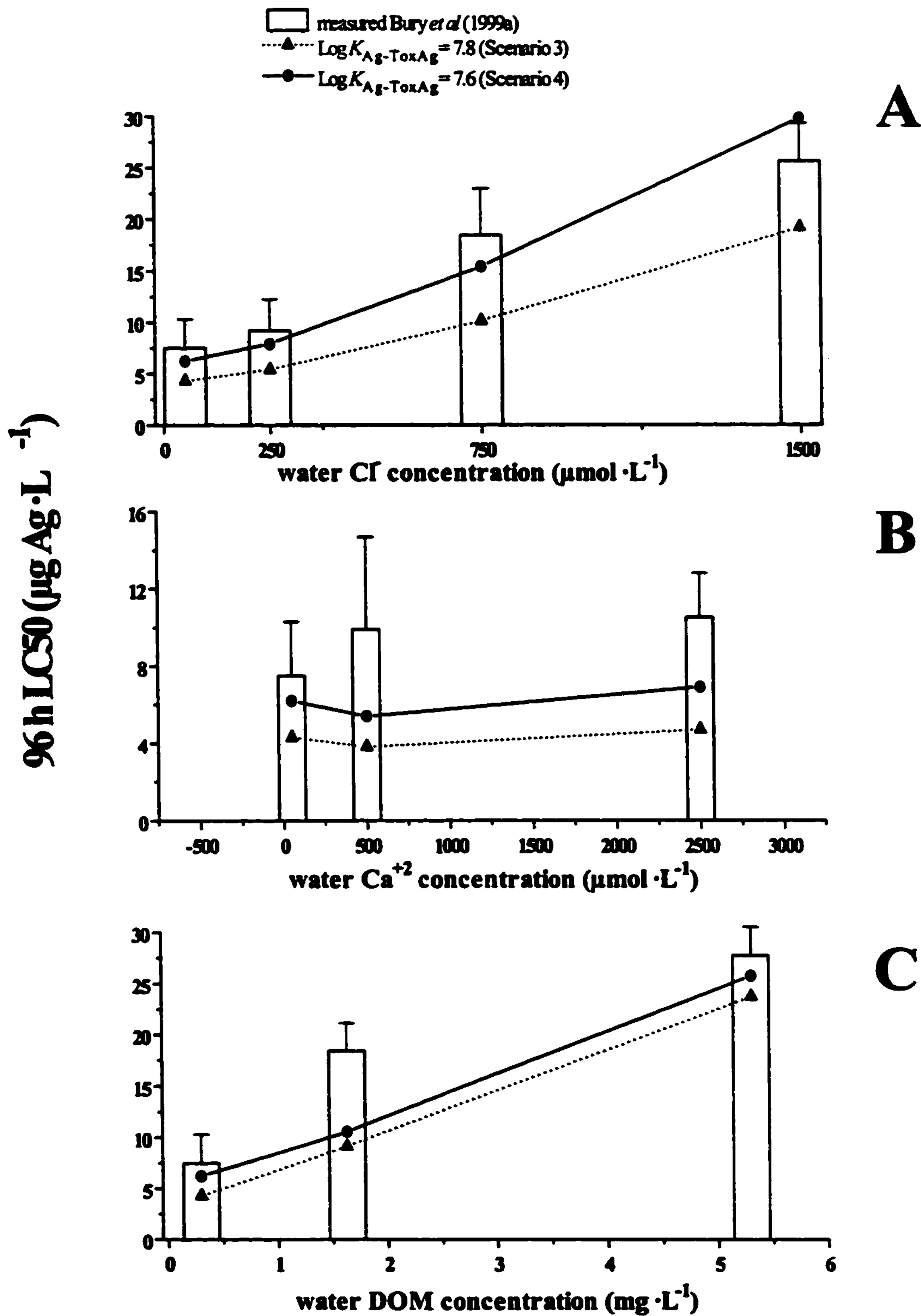
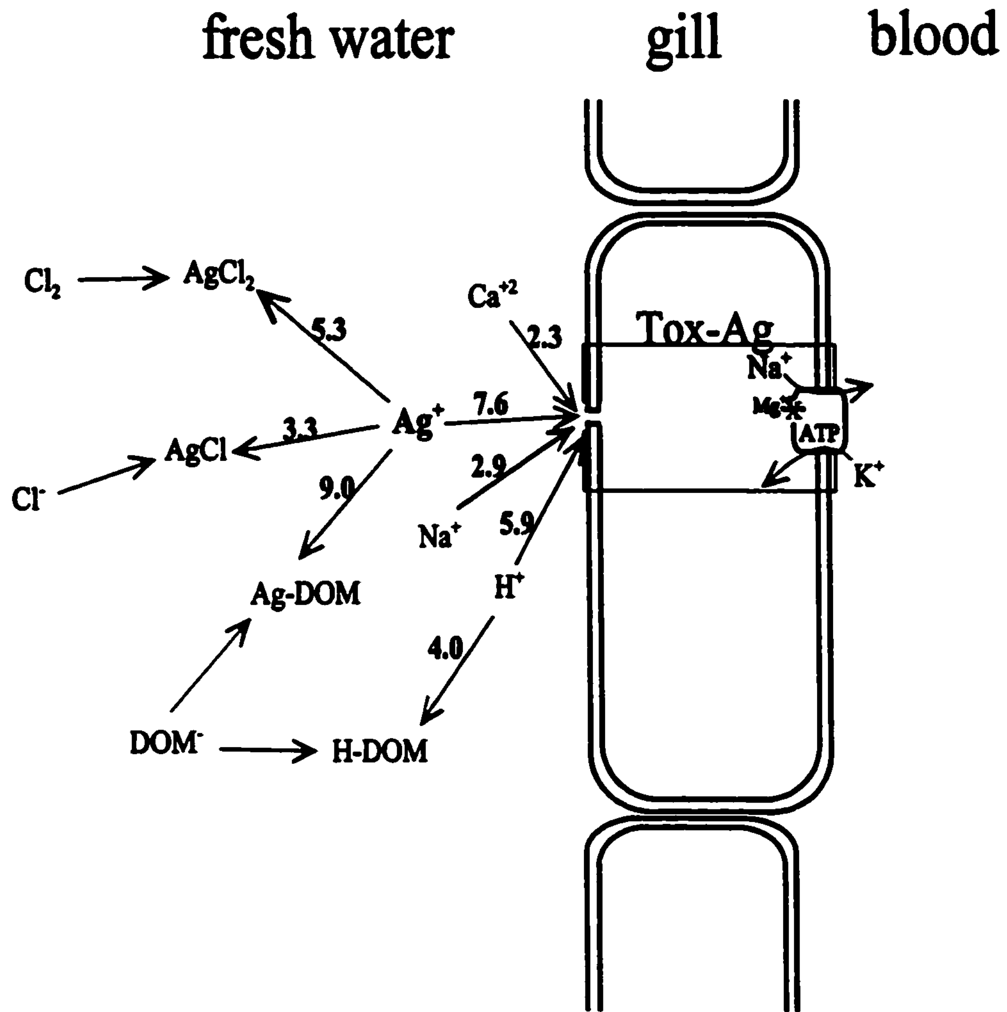
Ag^+
Mg
ATP
K^+
AgCl
Na^+
Cl^-
Ag-DOM
H^+
DOM^-
H-DOM


measured Bury et al (1999a)
Log $K_{Ag-ToxAg}$ = 7.8 (Scenario 3)
Log $K_{Ag-ToxAg}$ = 7.6 (Scenario 4)
A
B
C
96 h LC50 (μg Ag·L^{-1})
water Cl$^-$ concentration (μmol·L^{-1})
water Ca^{+2} concentration (μmol·L^{-1})
water DOM concentration (mg·L^{-1})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gill
blood
Cl2
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Mg
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5.9
H+
Ag-DOM
4.0
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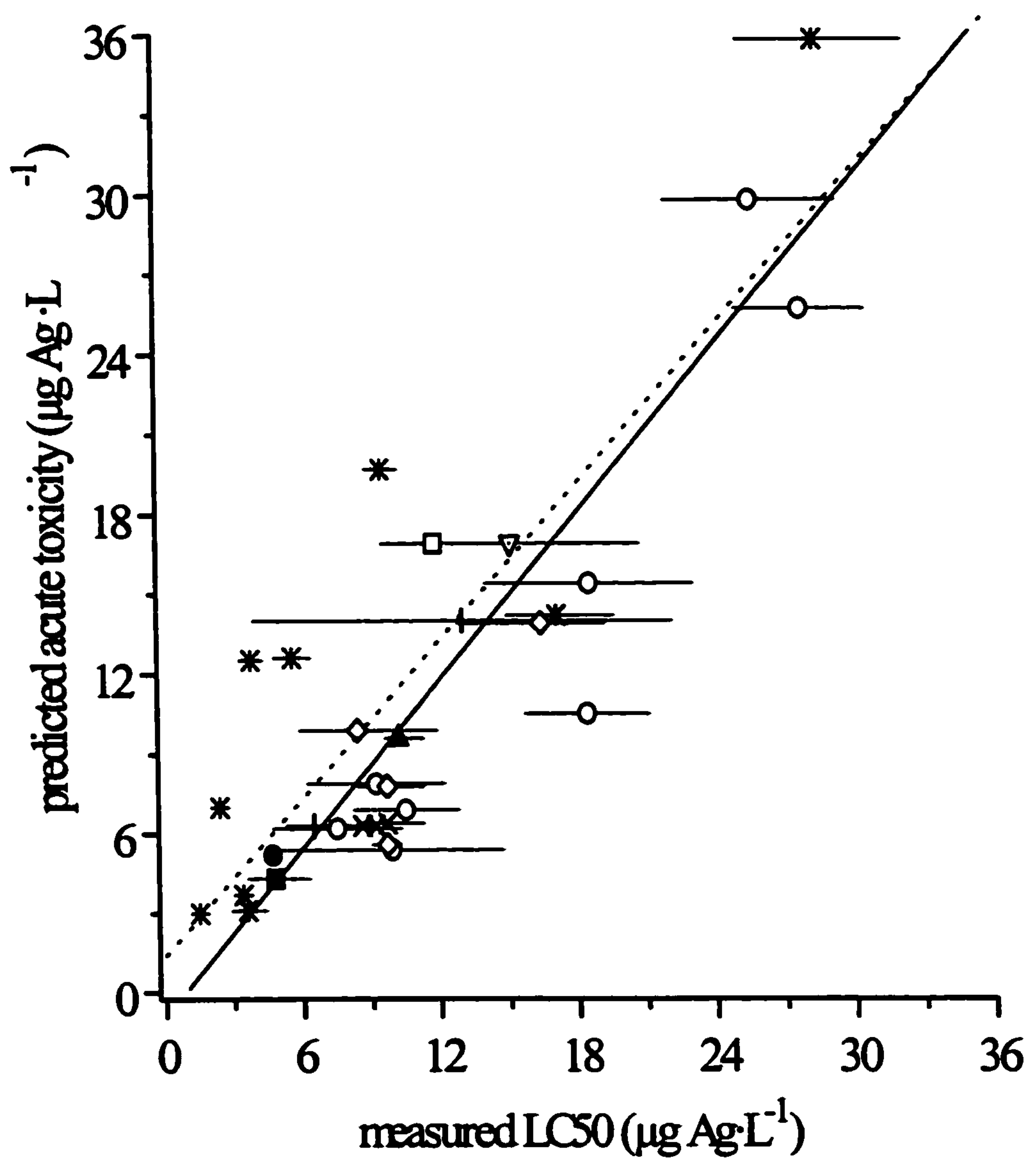
H-DOM


Bury et al. (1999b)
Grosell et al. (1999)
Lemke (1981)
Karen et al. (1999)
Davies et al. (1983)
Nebeker et al. (1983)
Hogstrand et al. (1996)
Galvez & Wood unpublished
Diamond et al. (1990)
Galvez and Wood (1997)
predicted acute toxicity (μg Ag·L⁻¹)
measured LC50 (μg Ag·L⁻¹)
0
6
12
18
24
30
36

CHAPTER 5

PHYSIOLOGICAL RESPONSES OF JUVENILE RAINBOW TROUT TO CHRONIC LOW LEVEL EXPOSURES OF WATERBORNE SILVER

ABSTRACT

The physiological effects of chronic exposure to $AgNO_3$ in moderately hard freshwater were investigated in juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum). Two separate 28-day exposures were performed at silver concentrations of 0.5 and 2.0 µg/L in flowing Hamilton dechlorinated tap water. Exposure to 0.5 µg/L Ag resulted in a slight increase (~14.9%) in food consumption, whereas growth rates remained unaltered. Both plasma Na^+ and Cl^- levels were significantly decreased by 11.8% and 9.3%, respectively at day 16 of the exposure. Hepatic Ag concentrations were elevated approximately 4-fold in 0.5 µg/L Ag-exposed fish. However, no significant increases in liver metallothionein (MT) concentrations were noted. No mortalities were observed during this 28-day exposure. In comparison, chronic exposure to 2.0 µg/L Ag resulted in a 28.8% decrease in food consumption and a 43.0% reduction in growth rate. Plasma [Na^+] was decreased by 18.3%, whereas plasma [Cl^-] was reduced by 12.2% at day 7. At both concentrations of silver, plasma ion concentrations appeared to recover thereafter. Silver accumulated steadily in the liver up until day 15 when concentrations were 39.7 µg/g wet weight (285-fold increase) above control levels. In addition, MT

levels were increased by 81.2% at day 7. Silver exposure at 2.0 µg/L resulted in approximately 15% mortality over the 28-day period.

INTRODUCTION

It has been recently suggested that the primary mechanism of acute Ag toxicity in fish is related to a severe ionoregulatory disturbance at the gills (Morgan *et al.*, 1997; Wood *et al.*, 1996a). Adult rainbow trout (*Oncorhynchus mykiss*) exposed to 10 µg/L Ag (as $AgNO_3$) showed immediate reductions of over 50% in branchial Na^+ and Cl^- influx, while complete inhibition (~99%) was produced after only 8 h. The net result was a progressive loss of Na^+ and Cl^- from blood plasma. The subsequent decrease in plasma osmolality was partially compensated for with shifts of plasma and interstitial fluids to the surrounding tissues (Wood *et al.*, 1996a). Death was postulated to occur due to cardiovascular collapse resulting from systemic plasma volume loss as has been reported in cases of acidity (Milligan and Wood, 1982; McDonald, 1983; McDonald and Wood, 1993).

Despite the dramatic physiological effects seen in adult rainbow trout at 10 µg/L Ag (as $AgNO_3$), exposure to silver concentrations as high as 30,000 µg/L (in the same water quality) failed to produce any significant effects to fish when presented as silver thiosulphate ($Ag(S_2O_3)_n$) (Wood *et al.*, 1996b) demonstrating the importance of silver speciation on toxicity (LeBlanc *et al.*, 1984). Photoprocessing effluent, the primary source of silver released into the environment, is almost entirely composed of silver thiosulphate. The two major complexes found in this include mono- and di-thiosulphate, with log K values of 8.8 and 13.7 respectively (Morel and Hering, 1993; Schecher and McAvoy, 1992). During photographic processing (fixation), most of the free Ag^+ becomes strongly complexed with thiosulphate; consequently, even though total Ag concentrations at these contaminated point sources may reach levels as high as 35 µg/L

Ag (Lytle, 1984), Ag^{+} concentrations will only range between 0.0001 and 0.2 µg/L Ag^{+} (Chudd, 1983).

In this experiment fish were exposed to either 0.5 or 2.0 µg/L Ag (as $AgNO_3$) over a 28-day period. Silver speciation modeling using a geochemical equilibrium program (Schecher and McAvoy, 1992) was used to predict nominal Ag^{+} concentrations for each of these treatments. A total [Ag] of 0.5 µg/L (in Hamilton dechlorinated tap water) was calculated to yield approximately 0.2 µg/L of free Ag^{+}, whereas a total [Ag] of 2.0 µg/L was expected to give 0.6 µg/L Ag^{+}. The 2.0 µg/L Ag exposure was performed despite yielding a high Ag^{+} so as to compare results obtained here with previous studies (Morgan *et al.*, 1997).

In this study, potential ionoregulatory disturbances were assessed by analysis of plasma Na^{+} and Cl^{-}. In addition, the performance of fish, as measured by growth and feeding, was evaluated. Finally, hepatic Ag and metallothionein content were measured. Metallothionein is a protein believed to be involved in the regulation and detoxification of trace metals. Induction of this protein may additionally serve as a useful indicator of bioavailable Ag. The aims of the present study were to expose fish to Ag^{+} concentrations near the upper range of environmental relevance (0.2 µg/L Ag^{+}) and to assess the physiological effects of such an exposure in rainbow trout.

MATERIALS AND METHODS

Animals

Juvenile rainbow trout (*O. mykiss*, 3.01 ± 0.27) were purchased from Rainbow Springs Hatchery (Thamesford, Ontario). The entire stock was initially held in a 400-L tank supplied with dechlorinated, Hamilton city tap water ([Na^+]= 0.6 mM; [Cl^-]= 0.7 mM; [Ca^{2+}]= 1.0 mM; [Mg^{2+}]= 0.2 mM; [K^+]= 0.05 mM; titratable alkalinity to pH 4.0= 1.9 mM, total hardness of approximately 140 ppm as $CaCO_3$; [HCO_3^-]= 1.9 mM; pH= 8.0) at a flow rate of 2000 mL/min. Water was vigorously aerated to maintain the dissolved oxygen concentration near saturation. Fish were kept at ambient conditions, with water temperatures ranging from 15.5°C to 17.5°C. Fish were hand-fed dry trout pellets (Ziegler Brothers Inc., Gardners, PA) to satiation daily.

Experimental protocol

The study involved two separate 28-day flow-through exposures at nominal total silver concentrations of 0.5 and 2.0 μg/L as $AgNO_3$ (Fisher Scientific, Toronto, ON). Measured Ag concentrations were 0.7 ± 0.2 and 1.7 ± 0.4 μg/L respectively. In conjunction with each Ag exposure a simultaneous control (no Ag added) was also tested. For the 0.5 μg/L Ag exposure, 450 fish were randomly selected from the original stock and divided into two equal groups of 225. For the 2.0 μg/L exposure, 300 fish were randomly selected from the original stock and divided into two equal groups of 150. In all cases, fish were placed in 400-L tanks, each supplied with 1000 mL/min of dechlorinated tap water (see above). Fish were allowed to acclimate to these conditions for at least one week before the start of the experiment.

$AgNO_3$ stock solutions were made up in light-shielded plastic carboys with Ag concentrations 1000-fold greater than the desired amount in the exposure water. Concentrated HNO_3 acid was added to the stock solution to a pH of 4.0 to help minimize adsorption of silver to the container. Silver from the stock solutions was added to their respective tanks at a rate of 1.0 mL/min using peristaltic pumps. Adequate mixing of Ag stock (1.0 mL/min) and water (1000 mL/min) at their points of addition into the tanks was achieved by vigorous aeration. Exposure tanks were initially spiked with an appropriate amount of $AgNO_3$ stock to immediately achieve nominal silver concentrations of either 0.5 or 2.0 µg/L.

Fish from the 0.5 µg/L Ag exposure and its simultaneous control were sampled on days 0, 1, 7, 16, 23, and 28, whereas fish from the 2.0 µg/L Ag exposure (and control) were sampled on days 0, 7, 15, 20 and 28. The day 0 sampling period was performed prior to spiking the tank with Ag. Ten fish per treatment were analysed for a variety of physiological parameters at each sampling period. Individually sampled fish were randomly selected and immediately sacrificed with a cephalic blow. Blood samples were withdrawn via caudal puncture using a 100 µL Hamilton syringe pre-rinsed with 50 i.u./mL ammonium heparin (Sigma, St.Louis, MO), and placed in small plastic centrifuge tubes (Eppendorf). Whole blood was spun for two minutes to obtain plasma. Samples were subsequently frozen in liquid N_2 and stored at -70 °C until analyzed for total plasma Na^+ and Cl^- concentrations. Plasma Na^+ was measured using atomic absorption spectroscopy, whereas plasma Cl^- concentrations were determined using the mercuric thiocyanate colorimetric assay (Zall *et al.*, 1956). Livers were excised, blotted dry, weighed in plastic centrifuge tubes, frozen in liquid N_2, then stored at -70 °C for analysis

of total Ag and metallothionein.

Feeding and growth

Fish were fed to satiation once daily at 17:00. A special feeding protocol was implemented to accurately assess satiety in fish (Wilson *et al.*, 1994a). In short, food was carefully sprinkled into each tank allowing it to float on the water surface. Small quantities (0.5 g-1.0g) from a pre-weighed bag were administered at one minute intervals. Attention was given to ensure that all food added was completely consumed before more food was given. Cessation of appetite was arbitrarily set as the point at which floating food was still present after two consecutive minutes. Appetite was expressed as the average cumulative amount of food consumed (in grams) during the exposure per fish. Fish weights obtained at each sampling period (n=30) were used as an index of fish growth over the course of the experiment. Growth was expressed as average weight per fish.

Analysis of silver in water

Water samples were taken daily from each tank and immediately acidified with 0.5% (v/v) of trace metal grade 70% HNO_3 (J.T. Baker; Toronto, ON) for analysis of total Ag. Samples were analysed using atomic absorption spectroscopy (Varian AA 1275, Mississauga, ON) equipped with a graphite furnace atomizer (Varian GTA-95). The graphite furnace was programmed with the following temperature ramping profile: ambient temperature to 75°C over 5 s, 75°C to 90°C over 12 s, 90°C to 120°C over 30 s, with atomization occurring at 2000°C. Between samples, the graphite tube was flushed

with pre-purified N_2 gas to eliminate contamination and memory effects. An automated sample injector was used to dispense 10 µL samples, giving a detection limit of 0.25 µg/L Ag.

Analysis of Ag and metallothionein (MT) in livers

Livers (12-160 mg) were individually homogenized in 1.00 mL of 50 mM Tris-HCl, pH 8.0 (Sigma, St. Louis, MI) , at 0°C using an ice-cold glass-teflon homogenizer (Thomas Scientific). Five hundred µL aliquots of liver homogenate were stored in individual, acid-washed test tubes for total Ag analysis. Homogenates were digested in 2.5 mL of concentrated HNO_3 for 2 hours at 120°C. Samples were allowed to cool to room temperature and 375 µL of 30% H_2O_2 (Fisher) were added to each test tube. Tubes were then slowly heated to 120°C and each of the homogenates was evaporated to dryness. Five mL of 0.5% HNO_3 were added to each tube, and subsequently analysed by graphite furnace AAS.

The remaining ice-cold homogenate was immediately centrifuged at 16,000 g for 20 min at 4 °C. The supernatant (approximately 200 µL) from each sample was collected in centrifuge tubes, frozen in liquid N_2, and stored at -70°C until analysis. The MT assay used a double antibody radioimmunoassay as described by Hogstrand and Haux (1990). This included rabbit antiserum raised against perch (*Perca fluviatilis*) as the first antibody, ^{125}I-labelled rainbow trout MT as tracer, and goat anti-rabbit IgG as the second antibody.

Statistical analysis

Mean values of both Ag-exposed groups were statistically compared to their simultaneous controls using Student's two-tailed t-test ($p<0.05$)* or ($p<0.01$)**.

RESULTS

Feeding, growth and mortality

Cumulative food eaten per fish was monitored during each of the 28-day silver exposures and simultaneous controls. Fish exposed to 0.5 μg/L Ag had a significant increase ($p<0.05$) in their average cumulative food consumption (per fish) compared to controls. Average values for cumulative food consumption were 4.27 and 3.72 g per fish respectively, representing a 14.9% elevation (Fig. 1A). Growth rates were not significantly different between controls (150.0 mg/fish/day) and fish exposed to 0.5 μg/L Ag (167.5 mg/fish/day) (Fig. 1B). In comparison, exposure to 2.0 μg/L Ag significantly decreased ($p<0.01$) average cumulative food consumption by 28.8% (Fig. 2A). Average growth rates calculated over the 28 day exposure were reduced by 43.0% (Fig. 2B). No fish mortalities were reported in either of the controls or in the 0.5 μg/L Ag-exposed group throughout the 28-day period. In comparison, exposure to 2.0 μg/L Ag resulted in a mortality rate of 15% over 28 days. Approximately 85% of these mortalities were evenly distributed between days 4 and 20 of the treatment.

Plasma ion levels

Plasma Na^+ concentrations were reduced ($p<0.05$) by 11.8% at day 16 and 15.8% at day 28 of the 0.5 μg/L Ag exposure (Fig. 3). Similarly, a decrease in plasma Cl^- levels at day 16 (lowered by 9.3%; $p<0.05$) was also seen in these Ag-exposed fish. Following day 16, plasma Cl^- concentrations were not significantly different from those of control fish. Reductions in plasma Na^+ and Cl^- were most severe during treatment to 2.0 μg/L Ag. Plasma Na^+ concentrations were decreased by 18.3% at day 7, however levels

recovered to control values by the next sampling period (day 15) (Fig. 4A). The average plasma Cl^- concentration was significantly reduced by 12.2% on day 7 and by 10.7% at day 15; complete recovery of plasma Cl^- levels was seen by day 20 (Fig. 4B).

Silver accumulation and metallothionein (MT) levels

Fish exposed to 0.5 µg/L Ag exhibited moderate accumulation of Ag in their livers only after day 23. Levels of hepatic Ag in this group was approximately four times greater than in control fish (Fig. 5). Despite the accumulation of Ag in these Ag-exposed fish, no significant induction of hepatic MT was observed. In comparison, exposure to 2.0 µg/L Ag resulted in an 130-fold elevation of liver Ag content after only 7 days (Fig 6). Silver accumulation was greatest at day 15 when levels were increased approximately 285-fold in the Ag-exposed group compared to control fish. Hepatic MT concentrations in the 2.0 µg/L Ag- exposed fish were increased by 81.2% at day 7, but elevations were not statistically significant thereafter.

DISCUSSION

Effects of Ag on plasma sodium and chloride levels

Exposure of juvenile rainbow trout to low levels of Ag elicited an ionoregulatory disturbance qualitatively similar although quantitatively smaller than that seen at acutely lethal Ag concentrations (Wood *et al.*, 1996a). At 0.5 μg/L Ag, 10% reductions in plasma Na^+ and Cl^- concentration were produced by day 16 (Fig. 3), whereas plasma Na^+ losses as high as 18% were seen following 7 days of 2.0 μg/L Ag exposure (Fig. 4). Previous studies have concluded that plasma Na^+ losses over 30% were lethal to rainbow trout (McDonald *et al.*, 1980). Similar results were seen following 6 days of exposure to 10 μg/L Ag (as $AgNO_3$) (Wood *et al.*, 1996a). Fish mortalities commenced when plasma ion concentrations were decreased by 30%. In this study, mortalities were only reported at 2.0 μg/L Ag. The majority of these deaths occurred between days 7 and 20 when plasma Na^+ and Cl^- losses were most pronounced. Furthermore, it appears that amelioration of Ag toxicity is correlated well with the recovery of Na^+ and Cl^- concentrations back to control levels (as seen by day 28 in the 2.0 μg/L Ag exposure). It is unclear whether this recovery in electrolyte homeostasis represents a true acclimatory response which would involve fish acquiring an improved resistance to subsequent lethal challenges of waterborne silver (McDonald and Wood, 1993). For example, rainbow trout which have experienced an ionoregulatory disturbance in response to low pH exposure were shown to recover back to their original ionic status. Despite this recovery, these fish were no better able to survive future low pH exposures when compared to pre-exposed fish.

Growth and food consumption

In this study, growth rates were reduced in the 2.0 µg/L Ag-exposed group (Fig. 2B), but not in fish exposed to 0.5 µg/L Ag (Fig. 1B). This is consistent with other studies suggesting that growth is impaired during waterborne silver exposure (Coleman and Cearley, 1974; Breteler *et al.*, 1982). Early life stage tests performed on steelhead trout (over 21 days) showed decreased survivorship at 1.1 µg/L Ag, and significant alterations in growth rates at concentrations as low as 0.1 µg/L Ag (Nebeker et al., 1983). Additionally, a persistent decrease in the growth rates of oysters has been observed following exposure to 2.0 µg/L Ag (Sanders *et al.*, 1990). Additionally, food consumption was significantly elevated in the 0.5 µg/L Ag exposure (Fig. 1A). It is possible that fish consume more food to increase their dietary uptake of osmolytes in order to compensate for loss of ions due to branchial impairment. For instance, trout exposed to low pH (4.4-5.2) and given a ration of 2% of their body weight per day, were able to alleviate the resulting ionoregulatory disturbance. However, if trout were starved during low pH treatment, plasma Cl^- and whole body Na^+ levels remained low (Sadler and Lynam, 1987).

Ag accumulation and MT induction

Hepatic Ag accumulation was at least 40 times greater in the 2.0 µg/L exposure, compared to the 0.5 µg/L Ag-exposed group. Ag levels in the liver, following 8 days of exposure to 2.0 µg/L Ag were similar to those seen in other studies (Hogstrand *et al.*, 1996; Galvez and Wood, 1997). Previous studies have shown that liver tissue is able to accumulate extremely high concentrations of Ag (>300 µg/g) during waterborne silver

thiosulphate exposure (Wood *et al.*, 1996b). Despite this large increase in hepatic Ag no apparent deleterious effects to these exposed fish were observed. Recently however, hepatic Ag accumulated from a Ag-contaminated diet was shown to result in a significant decrease in hepatic copper after 16 days of exposure (Galvez *et al.*, 1996). It seems feasible that as Ag accumulates in the liver, it begins to displace copper away from low-affinity ligands and metallothionein.

The long-term Ag exposures performed in this study suggest that branchial Na^+ and Cl^- uptake is a sensitive marker of Ag exposure. The fact that 15% of the 2.0 µg/L exposed group died despite plasma Na^+ reductions of only 18% (compared to 30% reductions at higher Ag concentrations (Wood *et al.*, 1996a)) may suggest that some fish in the population are more sensitive than others to chronic Ag exposure. The reduced ionoregulatory disturbance and lack of mortality observed in the 0.5 µg/L Ag treatment may indicate that a no-observed-effect-limit is being reached. Future work should attempt to better characterize this dose-response relationship.

Acknowledgements

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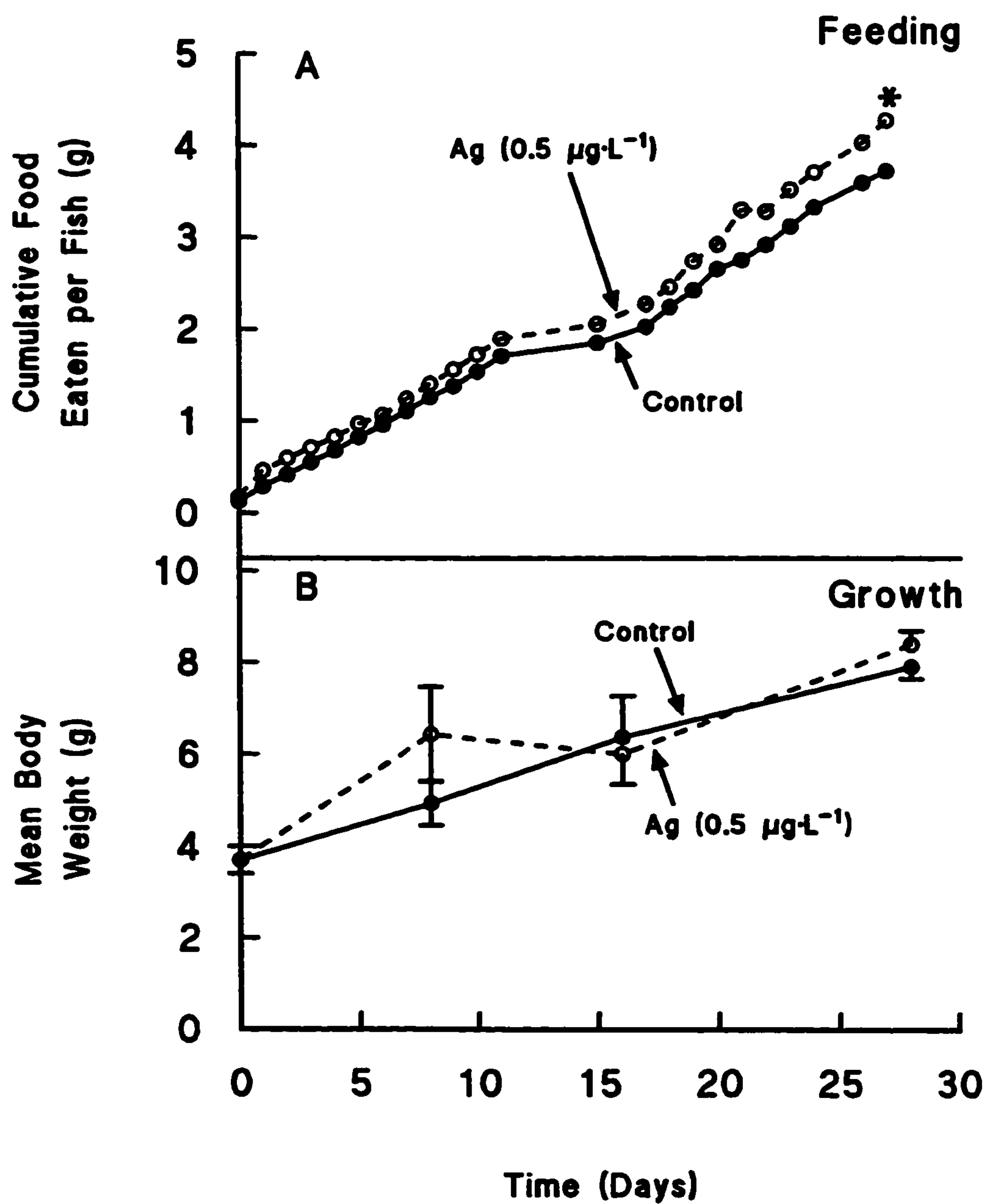
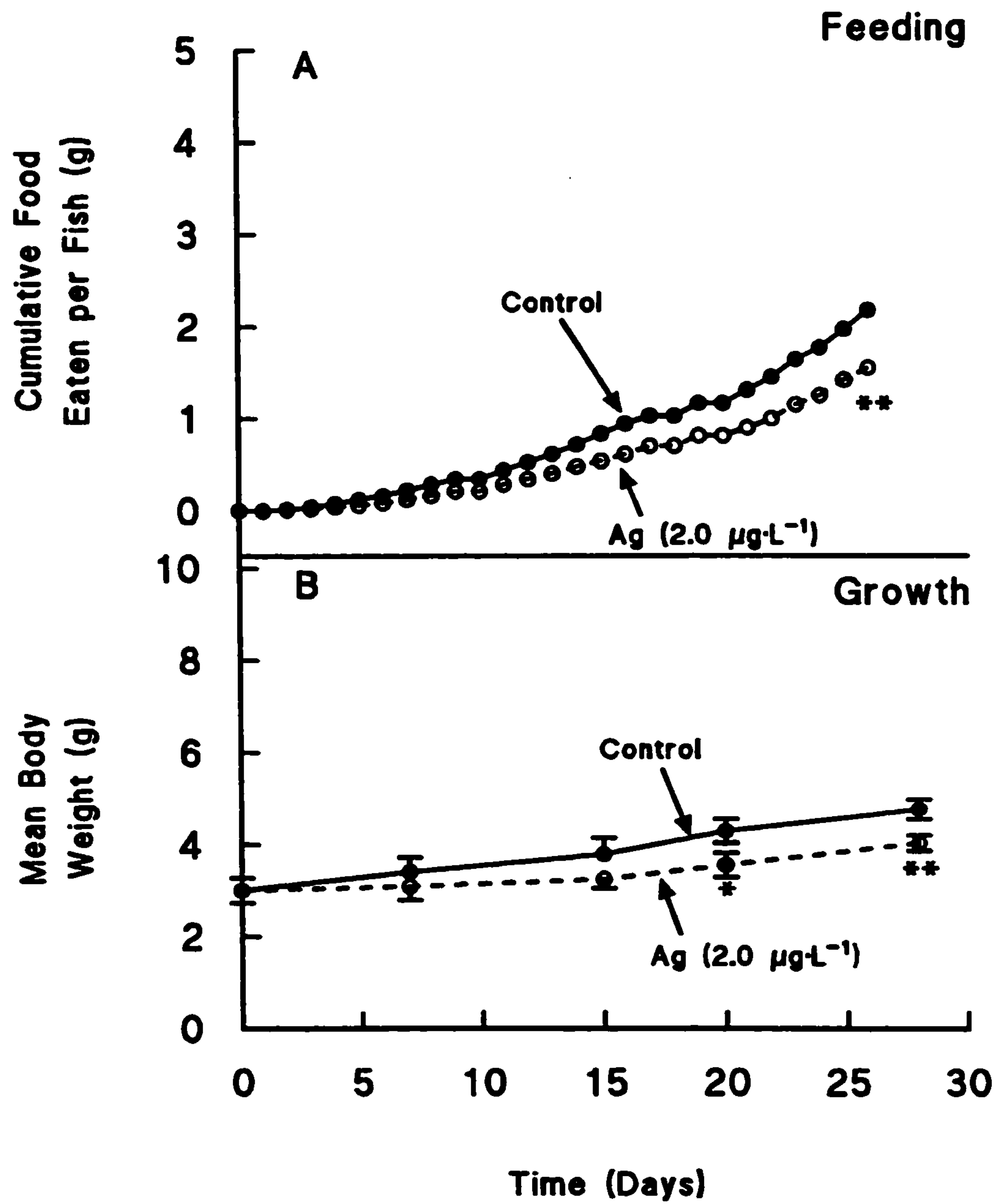
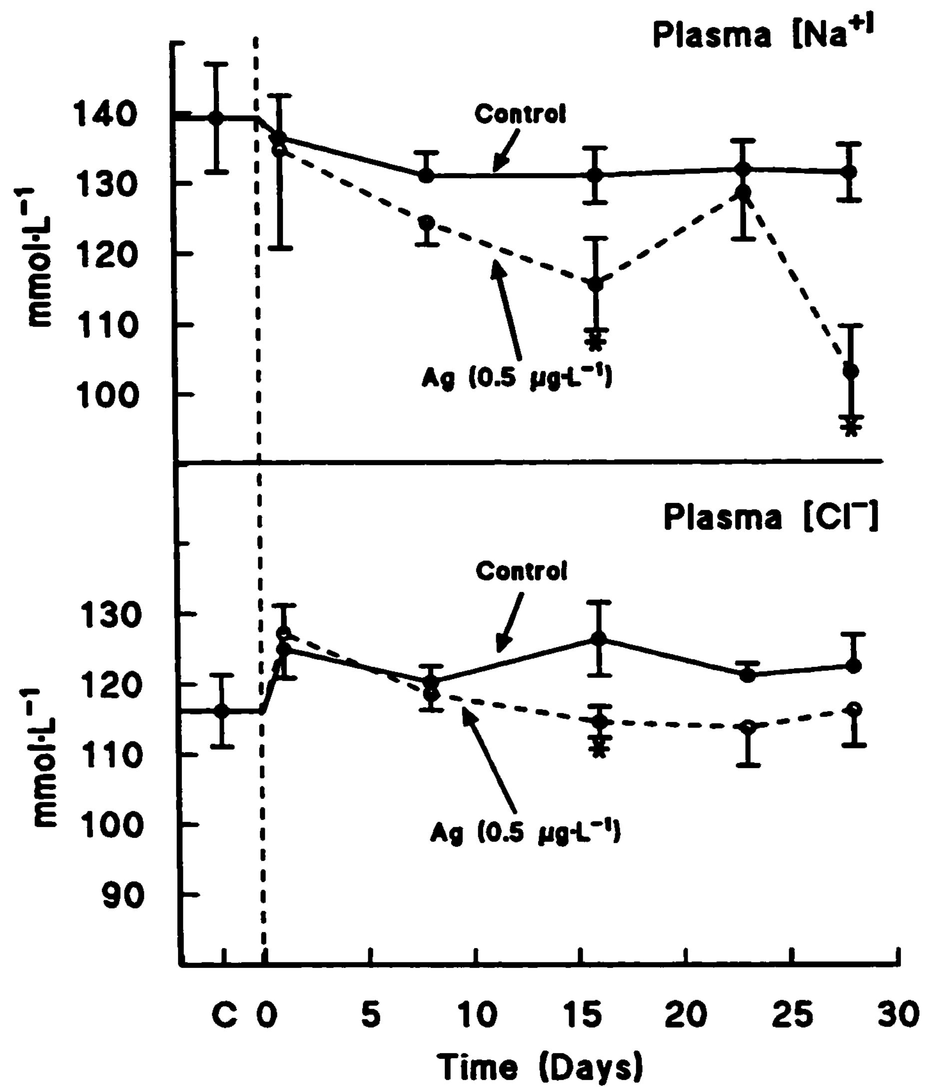
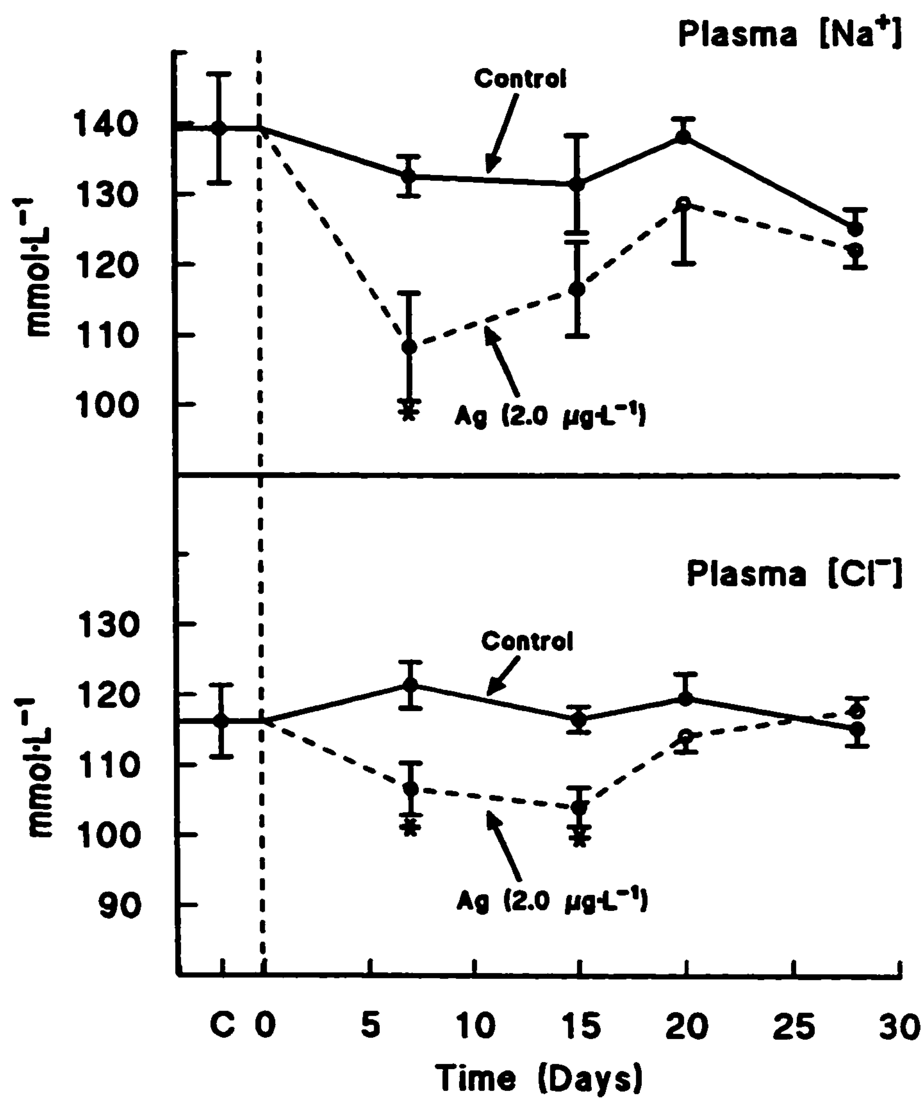


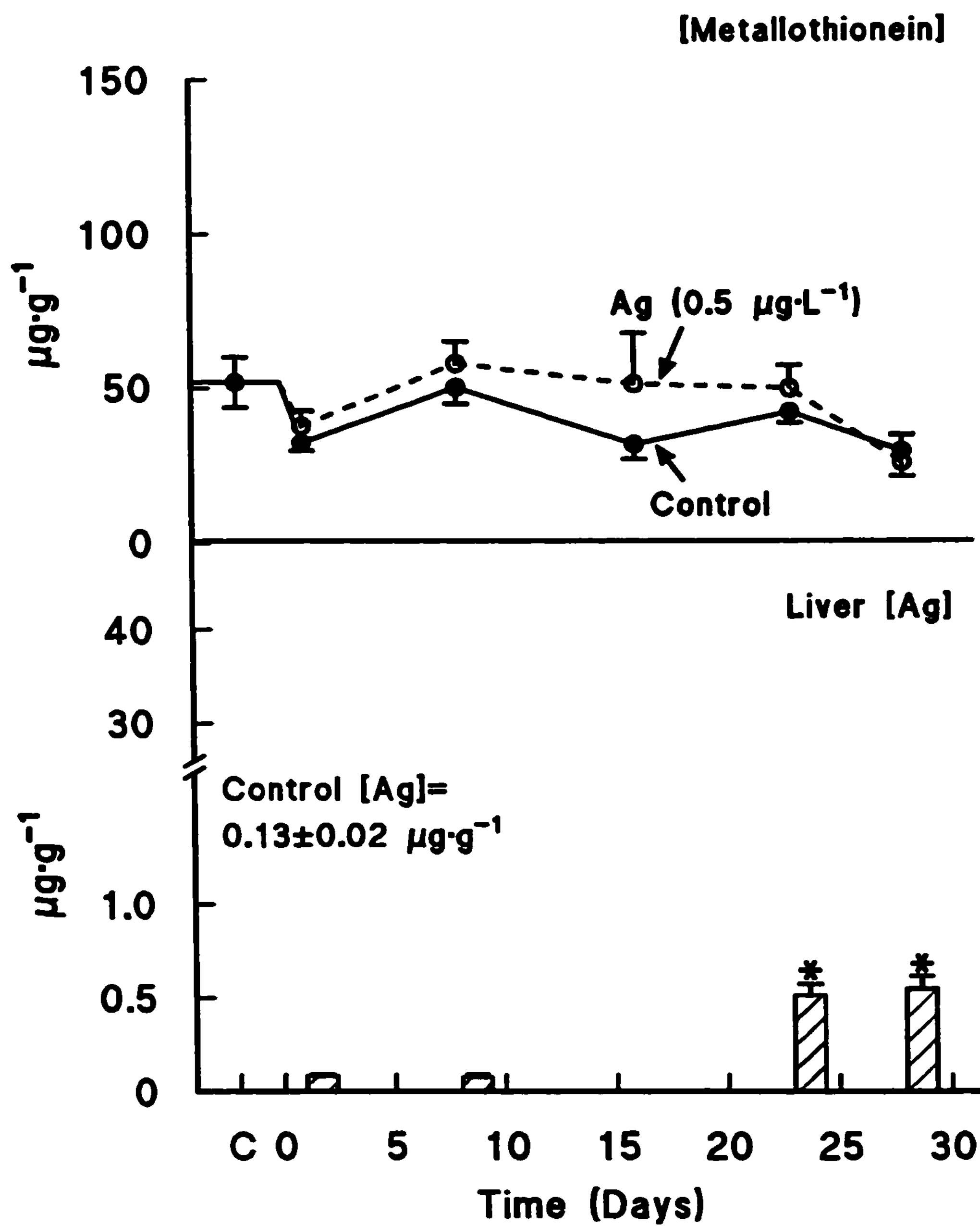
Feeding
A
Ag (0.5 µg·L⁻¹)
Control
Cumulative Food Eaten per Fish (g)
*
B
Growth
Control
Ag (0.5 µg·L⁻¹)
Mean Body Weight (g)
Time (Days)

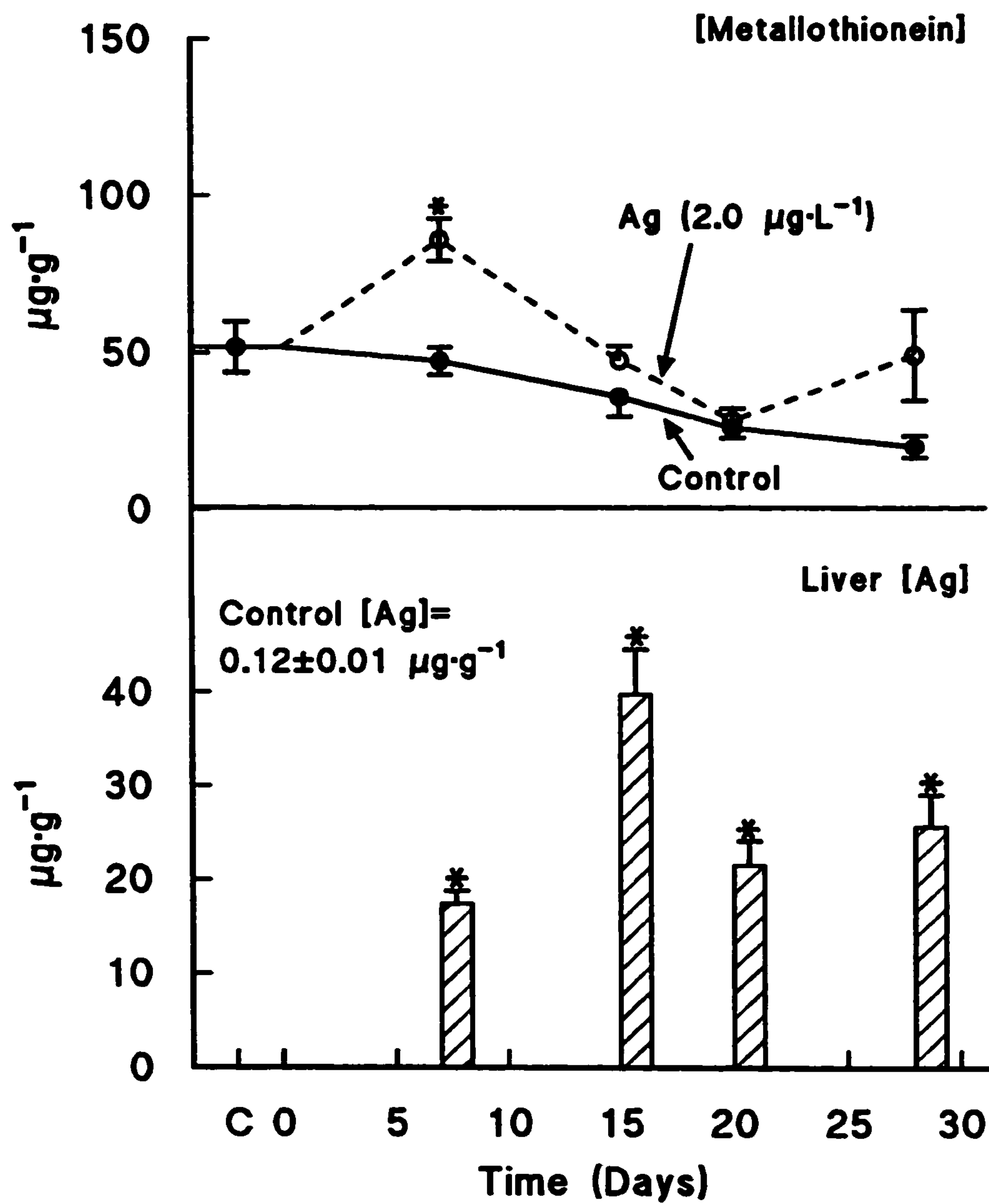

Feeding
A
Cumulative Food Eaten per Fish (g)
5
4
3
2
1
0
Control
Ag (2.0 µg·L⁻¹)
**
10
B
Growth
8
6
4
2
0
Mean Body Weight (g)
Control
Ag (2.0 µg·L⁻¹)
*
**
0
5
10
15
20
25
30
Time (Days)


Plasma [Na+]
Control
Ag (0.5 μg·L⁻¹)
mmol·L⁻¹
140
130
120
110
100
Plasma [Cl⁻]
Control
Ag (0.5 μg·L⁻¹)
mmol·L⁻¹
130
120
110
100
90
C 0 5 10 15 20 25 30
Time (Days)



Plasma [Na+]
Control
140
130
120
110
100
mmol·L-1
Ag (2.0 μg·L-1)
Plasma [Cl-]
Control
130
120
110
100
90
mmol·L-1
Ag (2.0 μg·L-1)
C 0 5 10 15 20 25 30
Time (Days)


[Metallothionein]
150
100
50
0
μg·g⁻¹
Ag (0.5 μg·L⁻¹)
Control
Liver [Ag]
40
30
Control [Ag]=
0.13±0.02 μg·g⁻¹
μg·g⁻¹
1.0
0.5
0
*
*
C 0
5
10
15
20
25
30
Time (Days)


[Metallothionein]
150
100
50
0
μg·g⁻¹
Ag (2.0 μg·L⁻¹)
Control
Liver [Ag]
Control [Ag]=
0.12±0.01 μg·g⁻¹
40
30
20
10
0
μg·g⁻¹
C 0 5 10 15 20 25 30
Time (Days)

CHAPTER 6

THE MECHANISMS AND COSTS OF PHYSIOLOGICAL AND TOXICOLOGICAL ACCLIMATION TO WATERBORNE SILVER IN JUVENILE RAINBOW TROUT (*Oncorhynchus mykiss*)

ABSTRACT

Juvenile rainbow trout were exposed to 0, 0.1, 1, 3 and 5 $\mu g \cdot L^{-1}$ silver (Ag, as $AgNO_3$; nominal values) in moderately hard water (120 ppm as $CaCO_3$) for 23 days. The measured total Ag concentrations were: <0.05 $\mu g \cdot L^{-1}$ for controls, 0.20 ± 0.02 $\mu g \cdot L^{-1}$, 1.04 ± 0.43 $\mu g \cdot L^{-1}$, 3.02 ± 0.10 $\mu g \cdot L^{-1}$, and 4.82 ± 0.28 $\mu g \cdot L^{-1}$ Ag, respectively. Specific growth rate, cumulative food consumption (but not food consumption per unit body weight), and food conversion efficiency were all significantly reduced in the 5 $\mu g \cdot L^{-1}$ Ag treatment alone. Critical swimming speed (U*crit*) was significantly decreased only during exposure to 5 $\mu g \cdot L^{-1}$ Ag suggesting that a physiological cost of toxicological acclimation existed. Only the 5 $\mu g \cdot L^{-1}$ Ag treatment had significant mortality (5.2 %) by the end of the exposure. Fish were especially susceptible to silver between days 5 and 15, manifested as a pronounced ionoregulatory disturbance in the plasma. Plasma Na^+ and Cl^- were significantly lower on days 5 and 10 of the 5 $\mu g \cdot L^{-1}$ Ag exposure but recovered thereafter. Unidirectional Na^+ uptake from the water and gill Na/K-ATPase activity were significantly inhibited by exposure to 3 and 5 $\mu g \cdot L^{-1}$ Ag. In the 3 μg/L Ag treatment, Na^+ uptake was inhibited at day 5 alone, whereas the effects at the highest Ag

exposure persisted up until day 15. Gill Na/K-ATPase was inhibited on day 5 in both the 3 and 5 $\mu g \cdot L^{-1}$ Ag treatment. Interestingly, the gill Na/K-ATPase concentrations of these Ag-treated fish were elevated approximately 1.5 times above controls at day 23. While physiological acclimation was shown to occur at all Ag concentrations tested here, only the 3 $\mu g \cdot L^{-1}$ and 5 $\mu g \cdot L^{-1}$ Ag treatment produced toxicological acclimation as evident by at least a 2-fold elevation in 168-hour LC50 at days 15 and 23. We conclude that physiological and possibly toxicological acclimation is produced by compensatory changes in Na^{+} transport at the gill epithelium.

Keywords: rainbow trout, silver nitrate, acclimation, sodium uptake, sodium-potassium ATPase.

INTRODUCTION

It is well established that the free ionic silver species, Ag^+, is extremely toxic to freshwater fish, with estimates of 96-hour LC50 between 5 and 65 $\mu g{\cdot}L^{-1}$ for total Ag when added as $AgNO_3$, the soluble salt strongly dissociates to produce Ag^+ (Coleman and Cearley, 1974; Davies *et al.*, 1978; Hogstrand *et al.*, 1996). This agrees with recent findings showing that acute silver toxicity in juvenile rainbow trout is predicted well by the ionic Ag^+ concentration of water as calculated by geochemical modelling (Hogstrand *et al.*, 1996; Wood *et al.*, 1996a; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury *et al.*, 1999a). The primary mechanism of acute toxicity in freshwater fish involves a blockade of gill Na^+ and Cl^- transports (Hogstrand and Wood, 1998; Wood *et al.*, 1999). Ionic Ag^+ acts by severely inhibiting Na/K-ATPase transporters on the basolateral surface of the gill epithelium (Morgan *et al.*, 1997; McGeer and Wood, 1998; Bury *et al.*, 1999b), specifically by competitive inhibition of the Mg^{2+} activation site (Ferguson *et al.*, 1996). The ensuing inactivation of the transporter inhibits active Na^+ uptake, causing severe reductions in plasma Na^+ and Cl^- concentration (Wood *et al.*, 1996a; Webb and Wood, 1998). These events lead to circulatory failure through alteration of plasma fluid volume, and ultimately death of the fish.

In contrast to acute toxicity, little is known about the physiological responses of freshwater fish to chronic low-level silver exposure. Previous studies have shown that silver at concentrations as low as 0.09-0.17 $\mu g{\cdot}L^{-1}$ Ag (as $AgNO_3$) impact fish by significantly reducing growth rates and increasing mortality during long term exposure (Davies *et al.*, 1978). Early life stage tests on trout found that 0.5 $\mu g{\cdot}L^{-1}$ Ag significantly

elevated the mortality in fish after 21 days of exposure (Nebeker *et al.*, 1983). Preliminary work has suggested that the physiological basis for chronic toxicity may be partly related to the effects of silver on Na^+ balance. Exposures to 0.5 $\mu g \cdot L^{-1}$ Ag or 2.0 $\mu g \cdot L^{-1}$ Ag for 28 days both resulted in significantly decreased plasma Na^+ and Cl^- concentrations in juvenile rainbow trout, as well as decreases in food conversion efficiency, and reduction in growth at the higher concentration (Galvez *et al.*, 1998). Interestingly, the effects of silver on ion balance were short-lived, as plasma Na^+ and Cl^- were restored to control concentrations by day 28. This recovery of ion balance, despite the continued presence of silver, is characteristic of adaptational responses (physiological acclimation) seen with other metals during chronic exposure (McDonald and Wood, 1993).

Acclimation to surface-acting metals is triggered by physiological and/or morphological disturbances at the gill epithelium, referred to as the 'shock phase' (McDonald and Wood, 1993). In order to compensate for the effects of the initial 'shock phase' (e.g., ion loss), specific biochemical and physiological alterations of the gill epithelium can be implemented. If the initial physiological disturbance is large enough, this damage-repair process may result in increased tolerance of higher metal concentrations (toxicological acclimation). The most reliable method for assessing whether toxicological acclimation has occurred is the acute toxicity test. At present, although physiological acclimation to silver is known to occur, nothing is known of the mechanism involved (Galvez *et al.*, 1998), and nothing is known about the possibility of toxicological acclimation. The present study addresses these issues.

Juvenile rainbow trout were exposed for 23 days to 0.1, 1, 3 or 5 $\mu g \cdot L^{-1}$ Ag (added as $AgNO_3$) in moderately hard water, during which time mortality, growth, appetite, and food-conversion efficiency were monitored. Critical swimming speed tests (c.f., Wilson and Wood, 1992) were used to provide additional information on the physiological costs, if any, of silver acclimation. Toxicological acclimation was evaluated using either 96-h or 168-h toxicity tests. The effects of silver on ion regulation in trout were evaluated throughout the exposures using various physiological parameters, which included plasma Na^+ and Cl^- concentrations, whole-body Na^+ uptake rates and gill Na/K-ATPase activities.

MATERIALS AND METHODS

EXPERIMENT 1:

Fish holding conditions

Approximately 1500 juvenile rainbow trout were purchased from Humber Springs Trout Hatchery (Orangeville, ON) on May 12, 1998. All fish were initially mixed in one 450-L tank and then randomly transferred to three separate 450-L holding tanks. Each holding tank contained approximately 500 fish. Holding tanks were supplied with ~2,400 mL/min of aerated, dechlorinated Hamilton tap water (from Lake Ontario). The measured water chemistry included: [Na^+] = 0.6; [Cl^-] = 0.7; [Ca^{2+}] = 1.0; [HCO_3^-] = 1.9 mM; titratable alkalinity to pH 4.0 = 1.9 mM; total hardness as $CaCO_3$ = 120 ppm; pH 8.0 and natural organic matter at 1.3 $mg{\cdot}L^{-1}$ measured as dissolved organic carbon; temperature = 14.5 to15.5 °C. Photoperiod was kept at 14 h light: 10 h dark. Fish were fed to satiation once daily with dry trout pellets (Martin Feed Mills, Elmira, ON).

Silver exposures

Trout were maintained for 5 weeks under laboratory holding conditions prior to experimentation. In order to distinguish between treatments, fish were assigned a unique marking with Alcian Blue dye using a Panjet injector (Wright Health Group; Dundee, Scotland). During the marking procedure fish were lightly anaesthetized with 0.1 $g{\cdot}L^{-1}$ MS-222 buffered with 0.2 $g{\cdot}L^{-1}$ sodium bicarbonate and allowed to recover in fresh water before being placed back in the holding tanks. All fish were marked two weeks before the

start of the experimental exposures. At the start of the experiment, concentrated silver stock was added to two of the holding tanks to immediately achieve nominal total Ag concentrations of 0.1 and 1 $\mu g \cdot L^{-1}$ Ag. One treatment of fish was maintained at control conditions. Silver, as $AgNO_3$ (Sigma; St.Louis, MO), was continually added to holding tanks via a Marriott bottle metering system. Each silver stock was made at 6,000-fold the concentration of the respective exposure tank and acidified to 0.5% (v/v) with trace metal grade HNO_3 acid (Fisher, Nepean, ON). Concentrated silver stocks were shielded from light and replenished every three days. Silver stocks were delivered at 0.5 $mL \cdot min^{-1}$ to header tanks, and diluted with 3000 $mL \cdot min^{-1}$ of dechlorinated Hamilton tap water. Each header tank was aerated with an air stone to adequately mix the silver before delivery to the experimental tanks. Silver-amended water was supplied to each experimental tank at 2400 $mL \cdot min^{-1}$ - the remainder of the water in the header tank was allowed to overflow to waste. Total Ag concentrations were measured daily. The measured Ag concentrations were control tank = below detection (0.05 $\mu g \cdot L^{-1}$); low silver exposure = 0.20 ± 0.02 $\mu g \cdot L^{-1}$ (n=22); high silver exposure = 1.04 ± 0.43 $\mu g \cdot L^{-1}$ (n=22).

EXPERIMENT 2:

Fish holding conditions

Approximately 1,350 juvenile rainbow trout were purchased from Humber Springs Trout Hatchery on February 21, 1999, and held under similar conditions as those of experiment 1. At the time of arrival, ambient water temperature was only 5 °C. In order to maintain consistency between the studies, water temperature was gradually

increased to 15 °C over a period of two weeks. Fish were held for seven weeks before the start of the experiment.

Silver exposure

Silver was added to tanks in a similar fashion to experiment 1, except that nominal total Ag concentrations were 3 and 5 $\mu g \cdot L^{-1}$ Ag. A simultaneous control treatment was also included in experiment 2. The measured Ag concentrations were: control tank = below detection (0.05 $\mu g \cdot L^{-1}$); low silver exposure = 3.02 ± 0.10 $\mu g \cdot L^{-1}$ (n=22); high silver exposure = 4.81 ± 0.28 $\mu g \cdot L^{-1}$ (n=22).

EXPERIMENTAL PROTOCOL:

Food consumption, indices of growth and mortality

Fish were hand-fed to satiation once a day at 17:00 using the same protocol as Galvez *et al.* (1998). The amount of food consumed daily was recorded and used to calculate cumulative food consumption · $fish^{-1}$. Mean fish weights ± S.E. were calculated from the recorded weights of fish sampled for tissues, Na^+ uptake fluxes, and swimming tests on days 0, 5, 10, 15 and 23. Specific growth rates (SGR) in % · day^{-1} were calculated for each treatment from the slope of the least-squares regression through the natural logarithm (ln) transformed weight versus time data (SPSS, 8.0). The amount of food consumed at satiation was expressed on a % of mean fish weight· day^{-1} basis. Estimates of average fish weight were obtained using the specific-growth-rate equations.

Food conversion efficiency (FCE) (in %) for each treatment is the ratio of mean SGR (% $\cdot$ day^{-1}) and daily food consumed (% $\cdot$ day^{-1}), multiplied by 100. Mortality was recorded daily, and dead fish were removed immediately. To correct for the removal of fish by sampling, cumulative mortality was expressed as a percentage of the total number of fish in the tank at any time.

Tissue sampling

Fish were sampled on days 0, 5, 10, 15 and 23. The day 0 tissue sampling was performed prior to spiking the tanks with Ag. Ten fish per treatment were analyzed for a variety of physiological parameters. Fish were randomly selected and immediately sacrificed by a quick cephalic blow. Blood was taken by caudal puncture using 1-mL syringes pre-rinsed with ammonium heparin (50 i.u.$\cdot$mL^{-1}). Blood was centrifuged at 10,000 g for two minutes, and plasma was collected for analysis of total Na^+ and Cl^-. Plasma Na^+ was analyzed by flame atomic absorption spectrophotometry (AAS) (Varian, 1275), whereas plasma Cl^- was measured using the mercuric thiocyanate method (Zall *et al.*, 1956), adapted for use on a microtitre plate reader. Entire gill baskets were excised from fish, rinsed in 18 MOhm double-deionized water, blotted dry, and stored at -70 °C until analyzed for Na/K-ATPase activity. Gill Na/K-ATPase analysis was performed using the UV detection method of McCormick (1993), run at 25 °C. Briefly, an enzymatically-coupled reaction was employed, in which each mole of ATP hydrolyzed by ATPase enzymes would result in conversion of NADH to NAD^+. Absorbance change due to NADH oxidation was measured at 340 nM at 15-second intervals over a 10-

minute period, or until the reaction substrates became depleted. Na/K-ATPase was calculated as the difference in ATP hydrolosis in the absence and presence of oubain (Sigma, St. Louis, MO). Gill Na/K-ATPase activity was normalized against total gill protein, as measured by the Bradford method (Bradford, 1976) using a commercial dye reagent (Sigma).

Unidirectional sodium fluxes

Unidirectional sodium fluxes were measured using radioisotopic ^{22}Na (Mandel Scientific, Guelph, ON) over a four-hour period on days 0, 5, 10, 15 and 23. For each treatment group, all fluxes (including day 0) were performed at the nominal Ag exposure concentrations (0, 0.1 or 1 $\mu g \cdot L^{-1}$ Ag for experiment 1, or 0, 3 and 5 $\mu g \cdot L^{-1}$ Ag for experiment 2). Ten fish per treatment were transferred from their experimental tanks to 11-L polyethylene containers containing 5-L of aerated water at the appropriate Ag concentration. Fish were placed in chambers one hour before the start of fluxes. At the start of each flux, 4.5 μCi of ^{22}Na (as NaCl in H_2O) were added to each chamber and allowed to equilibrate for 5 minutes. After equilibration, and once again 5 minutes before the end of the 4 hour period, 4 x 5 mL water samples were taken for analysis of total Na and Ag, and ^{22}Na radioactivity. Total Na and Ag were analyzed by flame AAS and graphite furnace AAS (Varian GTA-95), respectively, whereas ^{22}Na radioactivity was measured by δ-counting (Canberra-Packard, Meridan, CT; Minaxi δ). After the four-hour flux period, fish were given an overdose (~1 g/L) of MS-222 and rinsed for one minute in a 7 $mmol \cdot L^{-1}$ NaCl solution to displace superficially-bound radioactivity

from the fish. Fish were blotted dry, and their weights and lengths were recorded before being stored in scintillation vials for δ-counting. The unidirectional Na fluxes in nmol · $g^{-1} \cdot h^{-1}$ were calculated as:

$$J_{in} = \frac{\text{Fish CPM}}{\text{MSA} \cdot \text{W} \cdot \text{t}} \qquad \text{Eqn. 1}$$

where MSA in the mean specific activity of Na in the water; W is the weight of fish in grams, and t is time in hours. Note that the MSA can be calculated as:

$$\text{MSA} = \frac{\frac{\text{CPM}_i\text{Na}}{[\text{Na}]_i} + \frac{\text{CPM}_f\text{Na}}{[\text{Na}]_f}}{2} \qquad \text{Eqn. 2}$$

where cpm_i Na and cpm_f Na are the radioactivity of the initial and final water samples in counts · $min^{-1} \cdot mL^{-1}$ of ^{22}Na, and $[Na]_i$ and $[Na]_f$ are the total Na concentrations for the initial and final water samples, respectively, in nmol · mL^{-1}.

Toxicity testing

Toxicological acclimation to silver (i.e., increased tolerance to acute silver exposure) was assessed using either 96-hour or 168-hour toxicity tests. To allow for direct comparison of silver sensitivity between treatments, marked fish from all exposure groups in an experimental series were tested in the same container (i.e., one container per concentration).

In experiment 1, 96-hour toxicity tests were performed starting on days 0, 5, 10, 15 and 23. For experiment 2, it was necessary to extend the exposure period to 168-hours in order to observe incipient lethal limits for control treatments. Consequently,

hours in order to observe incipient lethal limits for control treatments. Consequently, 168-hour tests could only be performed starting on days 5, 15 and 23, so as to avoid overlap. For each toxicity test, five Ag concentrations plus a simultaneous control were used. At the start of the tests, 60 fish were removed from each exposure tank and randomly distributed into the 6 test containers. $AgNO_3$ stocks were delivered at 0.5 $mL{\cdot}min^{-1}$ using Marriott bottles. Ag stocks were diluted with ~500 $mL{\cdot}min^{-1}$ of Hamilton dechlorinated water and mixed by gentle aeration prior to being gravity fed to 19-L covered containers on a flow-through basis. The nominal Ag concentrations used during the lethality tests followed a geometric series, which included: 10, 18, 32, 56 and either 5.6 or 100 $\mu g{\cdot}L^{-1}$ (nominal values). Water samples were taken daily, acidified to 0.5 % (v/v) with HNO_3 acid, and analyzed for total Ag by graphite furnace AAS. Mortalities were monitored during the course of the 96 or 168 hours. Fish were not fed during acute toxicity testing. Cessation of opercular movement and lack of response to gentle prodding were the criteria for death. Dead fish were immediately removed and their treatment and time of death recorded. The 96-h and 168-h LC50s ± S.E. were calculated by log probit analysis using measured total aqueous Ag concentrations (SPSS, 8.0).

Critical swimming speed

In experiment 1, swimming tests were performed on days 0, 5, 10, 15 and 23 of exposures, whereas, for experiment 2, swimming tests were conducted only on days 5, 10 and 15. Fish were tested at their nominal Ag concentrations. An additional series of swimming tests was conducted on day 6 (experiment 2 only), using the same fish as on

day 5, except with no Ag present in the tunnel. Swimming tests were performed in a modified 150-L Beamish-style swimming tunnel. Water flow rate was calibrated prior to use with a Kent Miniflow Type 265 flow metre. Fish (n=10 per treatment) were placed in the swim tunnel and allowed to adjust to their surroundings for 45 minutes at a velocity of 10 $cm \cdot s^{-1}$. Swimming tests involved increasing the water velocity by 7.5 $cm \cdot s^{-1}$ every 40 minutes until the fish exhausted and would no longer swim when reintroduced into the current. At the completion of the test, fish were blotted dry, weighed, and measured for total length. Fish tested on day 5 of experiment 2 were lightly anaesthetized (0.1 $g \cdot L^{-1}$ MS-222, buffered with 0.2 $g \cdot L^{-1}$ $NaHCO_3$), and weights and lengths were recorded. These fish were compartmentalized in their respective holding tanks and tested again on day 6, with no Ag present. Critical swimming speed was calculated as follows (Brett, 1964):

$$U\mathit{crit}\ (cm \cdot s^{-1}) = V_f + [(T \div t)\ \times\ dV] \qquad \text{Eqn. 3}$$

where V_f is the final velocity in which the fish was able to swim for the complete time (t) of 40 minutes; T is the time for which the fish was able to swim at the velocity causing exhaustion; and dV is the velocity increment ($cm \cdot s^{-1}$). U*crit* was normalized to body lengths $\cdot$ s^{-1} by dividing the U*crit* in $cm \cdot s^{-1}$ by the total length of the fish in cm.

Statistics

Data have been expressed as mean ± standard error (N). Specific growth rate and LC50s for silver treatments were statistically compared to simultaneous controls using an unpaired, two-tailed *t*-test. The alpha level was modified using a Bonferroni correction to allow for multiple comparisons. All other data were tested using a one-way analysis of variance, followed by Student-Newman-Keuls test for multiple comparisons. All data sets were tested for normality and homogeneity of variances prior to performing a one-way analysis of variance. A p value of 0.05 was considered statistically significant throughout.

RESULTS

Effects of silver exposure

A cumulative mortality of 5.2 % was obtained following exposure for 23 days to 5 $\mu g \cdot L^{-1}$ Ag. Fish were most susceptible to the silver exposure between days 5 and 15, during which time death rates averaged 0.58 % $\cdot day^{-1}$. In comparison, less than 1.6 % cumulative mortality occurred in the other silver treatments after 23 days. Control mortalities were 0.6 % and 0.2 % during experiments 1 and 2, respectively.

Cumulative food consumption per fish was 23 % lower in the 5 $\mu g \cdot L^{-1}$ Ag treatment than in the controls (Fig. 1). These fish were significantly smaller than controls on days 10 and 23, a difference that reached 22.2 % by the latter day (Fig. 2). The SGR values tended to be slightly higher in experiment 1 than in experiment 2 (Table 1). In the latter, the 5 $\mu g \cdot L^{-1}$ Ag exposure significantly inhibited SGR by 70 %; there were no other significant differences. Daily ration consumed (when expressed as % body weight$\cdot$ day^{-1}) was not significantly different between treatments, whereas food-conversion efficiency in the 5 $\mu g \cdot L^{-1}$ treatment was 58 % lower relative to controls due to the significant reduction in SGR in this group (Table 1).

Lethal challenges with silver

Fish subjected to either 0.1 or 1 $\mu g \cdot L^{-1}$ Ag for 23 days did not develop increased tolerance to acutely lethal challenges with silver (Fig. 3a). The 96-hour LC50s for control fish ranged between 7.6 ± 0.6 and 15.1 ± 2.8 $\mu g \cdot L^{-1}$ Ag. The 168-hour LC50s for control fish ranged between 9.9 ± 1.6 and 22.4 ± 7.6 $\mu g \cdot L^{-1}$ Ag (Fig. 3b). Fish which had

been chronically exposed to 5 $\mu g \cdot L^{-1}$ Ag were more tolerant to acute silver exposure on days 15 and 23, by at least 2-fold, indicating that toxicological acclimation had occurred. Unfortunately, LC50s could not be estimated for this treatment because < 50 % of the fish died at the highest Ag concentrations (10 % mortality at 40.4 $\mu g \cdot L^{-1}$ Ag on day 15 and 30 % mortality at 53.6 $\mu g \cdot L^{-1}$ Ag on day 23). The LC50 of fish exposed to 3 $\mu g \cdot L^{-1}$ Ag was > 139 % higher than the controls on day 23. Because only 50 % mortality was produced at the highest silver concentration, an LC50 could not be estimated with confidence.

Effects of silver on ion regulation

Silver exposure up to 3 $\mu g \cdot L^{-1}$ produced no significant differences in plasma Na^+ compared with controls over 23 days, apart from a small reduction in plasma Na^+ concentration in the 0.1 $\mu g \cdot L^{-1}$ Ag treatment at day 15 (Fig. 4a). Nonetheless, plasma Na^+ concentrations in the 1 $\mu g \cdot L^{-1}$ and 3 $\mu g \cdot L^{-1}$ Ag treatments tended to decrease on days 5 or 10, with recovery thereafter. Exposure to 5 $\mu g \cdot L^{-1}$ Ag had the most marked influence on plasma Na^+ concentration (Fig.4b). Plasma Na^+ concentrations were 23 % and 18.3 % lower than controls ($p < 0.05$) on days 5 and 10, respectively. This ionoregulatory disturbance was transient in nature, and plasma Na^+ returned to control concentrations by day 15.

Silver exposure had similar transient effects on plasma Cl^- concentrations (Fig. 5a,b). The mean plasma Cl^- concentration was significantly reduced only in fish exposed to 0.1 $\mu g \cdot L^{-1}$ (on day 15) and 5 $\mu g \cdot L^{-1}$ Ag (on days 5 and 10). The most dramatic effects

on plasma Cl^- were noted in the 5 $\mu g \cdot L^{-1}$ Ag treatment, with a 21 % reduction by day 5 and a 17 % decrease in concentration by day 10 ($p < 0.05$). In all cases, plasma Cl^- was restored to control concentrations following an initial period of decrease. Physiological acclimation appeared to occur regardless of the time required to evoke the response or the magnitude of the disturbance.

Sodium influx rates of control fish ranged from 710 ± 58 to 920 ± 47 nmol · kg^{-1} · h^{-1} for experiment 1 (Fig. 6a), and 340 ± 33 to 490 ± 35 nmol · kg^{-1} · h^{-1} for experiment 2 (Fig. 6b). The higher influx rates in series 1 were likely associated with the lower concentrations of plasma Na^+ in these fish (Fig. 4), but the reason for this difference is unknown. Silver exposure at 0.1 and 1 $\mu g \cdot L^{-1}$ did not influence sodium uptake rates in trout (Fig. 6a). In contrast, Na^+ uptake was significantly inhibited on days 0 (in the presence of Ag), 5, 10 and 15 of the 5 $\mu g \cdot L^{-1}$ Ag treatment (Fig. 6b). The most pronounced effect of Ag exposure on Na^+ uptake was seen on day 5 when its concentration was only 74 % of the control. By day 23, Na^+ uptake rates in the 5 $\mu g \cdot L^{-1}$ Ag group returned to control concentrations. Exposure to 3 $\mu g \cdot L^{-1}$ Ag first slightly stimulated, and then inhibited Na^+ uptake by 30 % compared to controls at day 5. Na^+ uptake rates in the 3 $\mu g \cdot L^{-1}$ Ag treatment increased thereafter to concentrations either similar to or significantly higher than controls.

Mean Na/K-ATPase activity in crude gill homogenates of non-exposed control trout ranged between (in µmol P ·mg^{-1} · h^{-1}): 1.50 ± 0.10 and 2.50 ± 0.16 for experiment 1 and 1.06 ± 0.10 and 1.40 ± 0.20 for experiment 2 (Fig. 7a,b). Ag exposure at 0.1 and 1 $\mu g \cdot L^{-1}$ had no consistent effects on Na/K-ATPase activity, although concentrations were

significantly lower in the 0.1 $\mu g \cdot L^{-1}$ and 1 $\mu g \cdot L^{-1}$ Ag treatments relative to controls on either days 0 or 15 (Fig. 7a). Exposure to 3 and 5 $\mu g \cdot L^{-1}$ Ag resulted in a significant inhibition of the enzyme at day 5, followed by a recovery to control concentrations by day 10 (Fig. 7b). On day 15 and 23, Na/K-ATPase activities of both silver treatments were significantly elevated above controls.

Critical swimming speed

A critical swimming speed test was used to assess possible physiological costs associated with chronic exposure and acclimation to silver. Exposure to 0.1 and 1 $\mu g \cdot L^{-1}$ Ag produced no significant effects on critical swimming speed relative to simultaneous controls (Fig. 8a). In comparison, *Ucrit* was raised by 15 % on day 5 at 3 $\mu g \cdot L^{-1}$, and lowered by 14 % on days 5 and 15 ($p < 0.05$) in the 5 $\mu g \cdot L^{-1}$ Ag group. Fish tested on day 5, at their respective nominal Ag concentrations, were subjected to an additional swimming trial on day 6 in Ag-free water. No significant differences were seen in the swim performances of fish from each treatment (control, 3 and 5 $\mu g \cdot L^{-1}$) between days 5 and 6 (Table 2). Nonetheless, the significant differences on day 5 in *Ucrit* values of the 3 $\mu g \cdot L^{-1}$ and 5 $\mu g \cdot L^{-1}$ Ag treatments relative to controls, were no longer present on day 6 (when tested without Ag), although the general trends appeared to be similar.

Speciation analysis

In both experiments 1 and 2, geochemical speciation analysis for Ag was performed using measured water chemistry and the modelling program MINEQL+ (Version 4; Environmental Research Software); the results are outlined in Figure 9.

DISCUSSION

Environmental relevance of experiment

Silver concentrations of 0.1, 1, 3 and 5 $\mu g \cdot L^{-1}$ Ag were chosen in this study based on their environmental relevance and regulatory importance. The lowest silver concentration tested (0.1 $\mu g \cdot L^{-1}$ Ag) represents the upper range (0.01-0.10 $\mu g \cdot L^{-1}$ Ag) of silver concentrations recently monitored using "clean" techniques for surface water near U.S. urban centres (Shafer *et al.*, 1998). Moreover, a chronic guideline close to 0.1 $\mu g \cdot L^{-1}$ Ag has been proposed or implemented in Europe (RIVM, 1999), Australia (NWQMS, 1999) and Canada (CCME, 1995), and has been adopted as the water quality objective in the Canadian provinces of Manitoba and Ontario (CCME, 1995). The 1 $\mu g \cdot L^{-1}$ and 3 $\mu g \cdot L^{-1}$ Ag treatments were tested to address the hardness-based criterion recently promulgated in British Columbia, Canada (BC MOELP, 1995). This guideline stipulates that at hardness exceeding 100 $mg \cdot L^{-1}$, as $CaCO_3$ (i.e., comparable to the Lake Ontario water used in the present study; 120 $mg \cdot L^{-1}$), a 30-day average (chronic) limit of 1.5 $\mu g \cdot L^{-1}$ Ag, and a criterion maximum concentration (acute) of 3 $\mu g \cdot L^{-1}$ Ag is enforceable. Finally, the 5 $\mu g \cdot L^{-1}$ Ag treatment tests the effects of Ag at the hardness-based US EPA Ambient Water Quality Criterion for total recoverable silver in Lake Ontario water (i.e., ~5.5 $\mu g \cdot L^{-1}$ Ag at 120 $mg \cdot L^{-1}$ as $CaCO_3$) (US EPA, 1980).

Physiological acclimation response

Silver exposure to 5 $\mu g \cdot L^{-1}$ Ag produced equimolar reductions in both plasma Na^+ and Cl^-, with ion losses of ~22 % and 18 %, respectively on days 5 and 10 (Fig. 4, 5).

Nonetheless, impairment in ion balance was short-lived, and plasma Na^+ and Cl^- returned to control concentrations by day 15. The ionoregulatory disturbance produced in the 3 $\mu g \cdot L^{-1}$ Ag treatment was less pronounced than seen in the 5 $\mu g \cdot L^{-1}$ Ag treatment, although it followed the same general trend. These findings are consistent with the study of Galvez *et al.* (1998), which demonstrated physiological acclimation in response to 28-day exposures at 0.5 and 2 $\mu g \cdot L^{-1}$ Ag. Fish experienced reductions in plasma concentrations of ~ 18 % for Na^+ and 12 % for Cl^- by day 7 of exposure to 2 $\mu g \cdot L^{-1}$ (Galvez *et al.*, 1998). In the present study, a moderate degree of physiological acclimation (based on Na^+ and Cl^- effects) was seen with exposures as low as 0.1 $\mu g \cdot L^{-1}$ Ag. Interestingly, exposure to low concentrations of Ag such as 0.1 $\mu g \cdot L^{-1}$ Ag (in the present study) or 0.5 $\mu g \cdot L^{-1}$ Ag (as in Galvez *et al.*, 1998) produced ionoregulatory imbalances which required a longer time to develop and were less severe than at elevated concentrations. Accordingly, it appears that that physiological acclimation is only elicited after ion regulation is sufficiently impaired.

Toxicological acclimation response

The present study is the first to demonstrate that exposure to fish at low concentrations of silver enhances tolerance to subsequent lethal exposure to the metal. Notably, toxicological acclimation was elicited at a threshold of about 13 % of the control LC50 for Ag (i.e., at 3 $\mu g \cdot L^{-1}$ Ag). Toxicological tolerance was not evoked in pre-treatments below 3 $\mu g \cdot L^{-1}$ Ag, despite the fact physiological acclimation had occurred. Consequently, physiological acclimation to Ag (as seen below 3 $\mu g \cdot L^{-1}$ Ag)

does not necessarily produce toxicological acclimation, whereas toxicological acclimation cannot occur without the onset of physiological acclimation. The threshold required to elicit toxicological acclimation in terms of total Ag (as a percentage of the LC50) is consistent with that seen for other metals. In general, threshold concentrations ranged from 8 to18 % of control LC50s in Cu (Dixon and Sprague, 1980), Zn (Alsop *et al.*, 1999; Bradley and Sprague, 1985), and Cd (Hollis *et al.*, 1999). Acclimation to Cu is especially relevant to the discussion here, because Cu and Ag share similar mechanisms of acute toxicity in freshwater fish. Both metals impair branchial Na^+ uptake and reduce plasma Na^+ concentration due to inhibition of gill Na/K-ATPase (Laurén and McDonald, 1987a,b; Morgan *et al.*, 1997). Dixon and Sprague (1980) found that lethal tolerance to Cu was increased from 60-106 % in rainbow trout following pre-exposure to Cu at 18 % of control LC50s. Interestingly, acclimation to Cu did not result in increased resistance to acute Zn challenges. In fact, Cu-acclimated rainbow trout were approximately 56 % more sensitive to Zn than were controls, reinforcing the conclusion that Cu and Zn have different modes of acute toxicity in fish (i.e., Cu and Ag impair Na balance and Zn and Cd impair Ca balance; McDonald and Wood, 1993).

Silver tolerance in fish was increased at least 2-fold following exposure to either 3 or 5 $\mu g \cdot L^{-1}$ Ag for 23 days. Unfortunately, exact LC50s could not be estimated for either treatment, because only 50 % and 10 % mortality rates, respectively, were obtained at the highest total Ag concentrations (i.e., 40.4 - 53.6 $\mu g \cdot L^{-1}$), despite extending the toxicity tests to 168 hours. Nonetheless, the relative magnitude of the increase in LC50 appears to be in line with values reported for Cu (Dixon and Sprague, 1980) and Zn

(Alsop *et al.*, 1999), but about 5-fold less than was reported for Cd (Hollis *et al.*, 1999). Worth noting is that, because cumulative mortality did not exceed 5.2 % in any pretreatment during 23 days, the acclimation responses seen here likely represent real compensatory changes, rather then pre-selection of tolerant fish.

Silver speciation and its effect on silver acclimation

Figure 9 illustrates the modelled speciation of silver, as calculated using MINEQL+ (Schecher and McAvoy, 1992) at various total silver concentrations in our test waters. Silver has been shown to be a potent inhibitor of gill Na/K-ATPase (Ferguson *et al.*, 1996) during acute lethal exposures (Morgan *et al.*, 1997), mainly due to the toxic action of the free Ag^+ ion (McGeer and Wood, 1998; Bury *et al.*, 1999b). In the studies of Bury *et al.* (1999b) and McGeer and Wood (1998), the IC50 for Na/K-ATPase inhibition was at 1.7 $\mu g \cdot L^{-1}$ Ag^+, or 9.4 $\mu g \cdot L^{-1}$ total Ag in Hamilton tap water. In the present study, exposure to 5 $\mu g \cdot L^{-1}$ total Ag (i.e., 0.37 $\mu g \cdot L^{-1}$ Ag^+) inhibited gill Na/K-ATPase by as much as 36 % by day 5.

On the basis of total Ag, 168-hour LC50s for control fish in experiment 2 ranged from 9.9 to 22.4 $\mu g \cdot L^{-1}$ Ag. Using geochemical speciation analysis, 7.2 $\mu g \cdot L^{-1}$ Ag^+ (22.4 $\mu g \cdot L^{-1}$ total Ag) was the LC50 for control fish on day 23 (Fig. 9b). This is in close agreement with Bury *et al.* (1999b). Silver tolerance was increased at least 2-fold following 23 days of pre-exposure to 3 $\mu g \cdot L^{-1}$ total Ag. The Ag^+ concentration at the 168-hour LC50 for acclimated fish was increased to about 20 $\mu g \cdot L^{-1}$, or at least 2.7-fold above the control LC50. At 3 $\mu g \cdot L^{-1}$ total Ag, the Ag^+ concentration was only 0.15 $\mu g/L$.

Therefore, only 2 % of the simultaneous control LC50 (expressed as Ag^+) was required to elicit toxicological acclimation (Fig. 9b). The fact that the threshold for acute Ag toxicity in fish may be modified by pre-exposure to the metal has important implications to ambient water quality criteria. In addition, these results suggest that silver acclimation depend on the Ag^+ concentration rather than the total Ag level of the exposure, and therefore is expected to be highly sensitive to changes in water chemistry. Certainly, other recent studies have similarly suggested that metal speciation can greatly influence the acclimation responses to Zn (Alsop *et al.*, 1999) and Cd (Hollis *et al.*, 1999).

The mechanistic basis for silver acclimation

The present study is the first to demonstrate that the inhibitory action of Ag^+ on Na/K-ATPase activity *in vivo* is transient (Fig. 7), mirroring the changes in plasma Na^+ and Cl^- during Ag acclimation (Figs. 4, 5). A similar response has been reported during chronic sublethal exposure to Cu (Laurén and McDonald, 1987a,b). Surprisingly, the inhibition of Na^+ uptake in the 5 $\mu g \cdot L^{-1}$ Ag group persisted until day 15 (Fig. 6), whereas plasma Na^+ concentration (Fig. 4) and gill Na/K-ATPase were reduced only up to day 10 (Fig. 7). Previous studies showed that Na^+ uptake in freshwater fish is quickly and severely inhibited by acute silver exposure (Morgan *et al.*, 1997; Webb and Wood, 1998). Recently, Bury and Wood (1999) demonstrated that silver, likely as Ag^+, is taken up across the apical membrane of gill epithelia via a proton-coupled Na^+ channel, coupled to a V-type H^+-ATPase. Furthermore, silver taken up via this Na^+ transport mechanism was found to competitively inhibit Na^+ transport. Acclimation could

potentially involve an alteration in the affinity of the Na^+ channel in order to favour Na^+ transport over Ag^+. An analogous mechanism has been proposed for rainbow trout acclimated to low-concentrations of zinc (Hogstrand *et al.*, 1994, 1995). Zinc acclimation reduces the affinity (increases the K_m) of specific Ca^{2+} channels, known to co-transport calcium and zinc across the apical membrane. Hogstrand *et al.* (1994, 1995) have suggested that increasing the K_m of the Ca^{2+}/Zn^{2+} co-transporter would significantly decrease zinc uptake into the cell, while having little effect on Ca^{2+} transport. Although this model has not been experimentally tested for silver, it seems logical that preventing Ag^+ from entering the gill cells would help to protect Na/K-ATPase transporters. If not, any increase in Na/K-ATPase transporters for the purpose of alleviating the ionoregulatory impairment would be futile, since they too would be susceptible to Ag^+.

Increased gill Na/K-ATPase activity in Ag-acclimated fish likely represented a recovery from Ag^+ intoxication of the existing transporters, as well as an induction of new Na/K-ATPase molecules. Morgan *et al.* (1997) have demonstrated that the inhibition of Na/K-ATPase by Ag^+ is quickly reversed once fish are placed in Ag-free water. A physiological acclimation response involving a reduction in the apical entry of Ag^+ (as previously discussed), would be analogous to transferring the fish to Ag-free water. Induction of new Na/K-ATPase transporters could potentially be mediated by a number of different mechanisms. Certainly, it is expected that changes in intracellular Na^+ and plasma osmolarity will either directly or indirectly mediate this response. Furthermore, Webb and Wood (1998) found that cortisol was induced in rainbow trout during silver exposure at concentrations at or below the LC50. Plasma cortisol is believed to play a role in Na^+ regulation by inducing gill Na/K-ATPase (McCormick and

Bern, 1989), and by stimulating proton-activated Na^+ channels (reviewed by Lin and Randall, 1995). In addition to alleviating the dramatic ionoregulatory disturbance, stimulation of Na^+- linked H^+ excretion would counteract the metabolic acidosis produced during acute $AgNO_3$ exposure (Wood *et al.*, 1996a). Certainly, the role of cortisol in mediating the silver acclimation response is worthy of further investigation.

Up to this point, our discussion has revolved around the impact and changes of silver acclimation on branchial Na^+ influx mechanisms. Nonetheless, there is some evidence that reductions in Na^+ efflux may also contribute to the silver acclimation response. Morgan *et al.* (1997) demonstrated that Na^+ efflux was reduced during short-term silver exposure. They proposed that this response involved a progressive decrease in the diffusive gradient for Na^+ efflux as a result of the low osmolarity of the blood. As mentioned earlier, Na^+ influx rates in the 5 $\mu g \cdot L^{-1}$ Ag treatment were reduced up until day 15 (Fig. 7), whereas plasma Na^+ concentration was reduced only to day 10 (Fig. 4). These data suggest that at least part of the recovery in plasma electrolyte balance was elicited by a reduction in Na^+ efflux. In addition to a reduction in the diffusive gradient, either a general reduction in the permeability of the gill epithelium, or increased retention of Na^+ by the kidneys (as seen with Cu- e.g. Grosell *et al.*, 1998) could explain the decrease in Na^+ efflux from fish.

The physiological costs of silver acclimation

This study incorporated a controlled feeding regime in order to decipher the effects and costs of Ag acclimation on maximal food consumption rate (appetite) and

food-conversion efficiency. Although fish from all silver treatments underwent physiological and/or toxicological acclimation, only the 5 $\mu g \cdot L^{-1}$ Ag group showed any physiological cost of silver acclimation. Reductions in specific growth rate and food-conversion efficiency (Table 1), as well as increased mortality during acclimation were seen in the 5 $\mu g \cdot L^{-1}$ Ag treatment alone. The fact that food consumption, when expressed on a %/day basis, was not decreased during exposure to high Ag, suggests that the effect on appetite (Fig. 1) was due solely to the significant decrease in SGR in the 5 $\mu g \cdot L^{-1}$ Ag treatment (Table 1). This is contrary to the effects of sublethal exposure to Al (Wilson and Wood, 1992; Wilson *et al.*, 1996), in which appetite was suppressed to the extent that food-conversion efficiency was actually enhanced. SGR remained impaired throughout the entire 23-day period, in contrast to the lack of persistent SGR impairment seen during acclimation to other metals (Alsop *et al.*, 1999; Dixon and Sprague, 1980; Hollis *et al.*, 1999; Wilson *et al.*, 1996). It is generally believed that the greatest cost of metal acclimation is imposed during the first 7-15 days of exposure, during which time the acclimation response is typically established (McDonald and Wood, 1993). In the present study, it appears that the greatest cost of silver acclimation may be associated with maintaining the response over time, rather than its initial development.

Reduction in the U*crit* of fish acclimated to 5 $\mu g \cdot L^{-1}$ Ag suggests that aerobic capacity in these fish was affected (Fig. 8). Reduction in U*crit* appeared to be independent of the presence or absence of silver during testing (Table 2), suggesting that Ag acclimation represented a real physiological cost to these fish, and was not simply an irritant. Critical swimming speed was significantly lowered on day 5 and thereafter

recovered only slightly. A similar response has been noted for Al (Wilson and Wood, 1992), Zn (Alsop *et al.*, 1999) and Cu (at low pH) (Waiwood and Beamish, 1978), and may represent an inescapable cost of metal acclimation. U*crit* can be affected by a number of factors. Generally, factors that decrease the maximum rate of oxygen uptake are termed 'limiting stressors', whereas factors that decrease aerobic scope by increasing metabolic costs are termed 'loading stressors' (Brett, 1958). Typically, the diagnosis employs oxygen uptake measurements at rest and at different swimming speeds. Nonetheless, the fact that U*crit*, SGR and FCE were affected throughout the whole course of the exposure, would suggest that silver acted as a loading stressor. At present, there is no experimental evidence available to assess whether silver also acted as a limiting stressor. However, metals such as Al (Wilson *et al.*, 1994a) and Cu (Waiwood and Beamish, 1978) are known to be both loading and limiting stressors. McDonald and Wood (1993) have proposed that acclimation to metals involves a damage/repair process, and thus will only occur at concentrations high enough to elicit damage to the gills. According to Mallatt (1985), necrosis of the gill epithelium, mucus hypersecretion, and branchial epithelial cell hypertrophy were the most common alterations in gill morphology following metal exposure. These structural responses would certainly be expected to decrease oxygen transport across the gill epithelium and reduce the aerobic capacity in Ag-acclimated fish.

Acknowledgements

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Table 1. Specific growth rates, ration of food consumed daily, and food-conversion efficiencies for rainbow trout exposed to 0, 0.1 and 1 $\mu g \cdot L^{-1}$ Ag for experiment 1, and 0, 3 and 5 $\mu g \cdot L^{-1}$ Ag for experiment 2. SGR is the slope ± S.E. for the least-squares regression through the ln-transformed wet weight versus time data. Ration is the mean ± S.E.. Food-conversion efficiency is SGR ÷ ration. An asterisk (*) denotes a significant difference in SGR of the 5 $\mu g \cdot L^{-1}$ Ag treatment relative to its simultaneous control, $p < 0.05$. There were no significant differences in ration within each experiment.

	Control	*0.1 $\mu g \cdot L^{-1}$ Ag*	*1 $\mu g \cdot L^{-1}$ Ag*	*Control*	*3 $\mu g \cdot L^{-1}$ Ag*	*5 $\mu g \cdot L^{-1}$ Ag*
SGR (% $\cdot day^{-1}$)	2.65 ± 0.45	2.04 ± 0.18	2.29 ± 0.44	1.86 ± 0.30	1.99 ± 0.41	0.66 ± 0.33 *
Ration (% $\cdot day^{-1}$)	3.38 ± 0.49	4.05 ± 0.69	3.54 ± 0.58	4.02 ± 0.36	4.39 ± 0.24	3.37 ± 0.40
Food Conversion Efficiency (%)	78.4	50.4	64.7	46.3	45.3	19.6

Table 2. Critical swimming speeds (U*crit*) of juvenile rainbow trout on days 5 and 6 of exposure to 0, 3 or 5 µg/L Ag. On day 5, the swimming test was performed in the presence of Ag, and on day 6 in the absence of Ag. The same groups of 10 fish per treatment were used during both testing periods. Values are expressed as means ± S.E. (N = 10). An asterisk (*) denotes a significant difference in U*crit* values for Ag treatments relative to their simultaneous controls, $p<0.05$. No significant differences were noted between treatments on day 6, or between days 5 and 6 within treatments.

U*crit* (body lengths · $second^{-1}$)

	Control	*3 µg · L^{-1} Ag*	*5 µg · L^{-1} Ag*
Day 5 (Ag present)	4.71 ± 0.18	5.41 ± 0.18*	4.03 ± 0.26*
Day 6 (No Ag present)	4.68 ± 0.23	5.14 ± 0.11	4.21 ± 0.24

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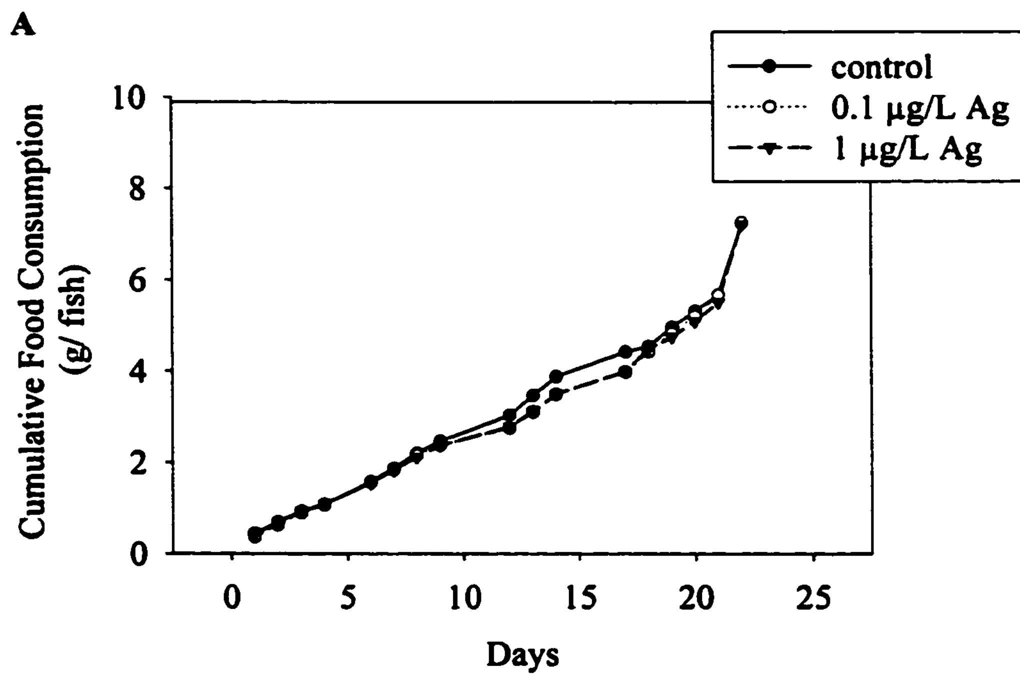

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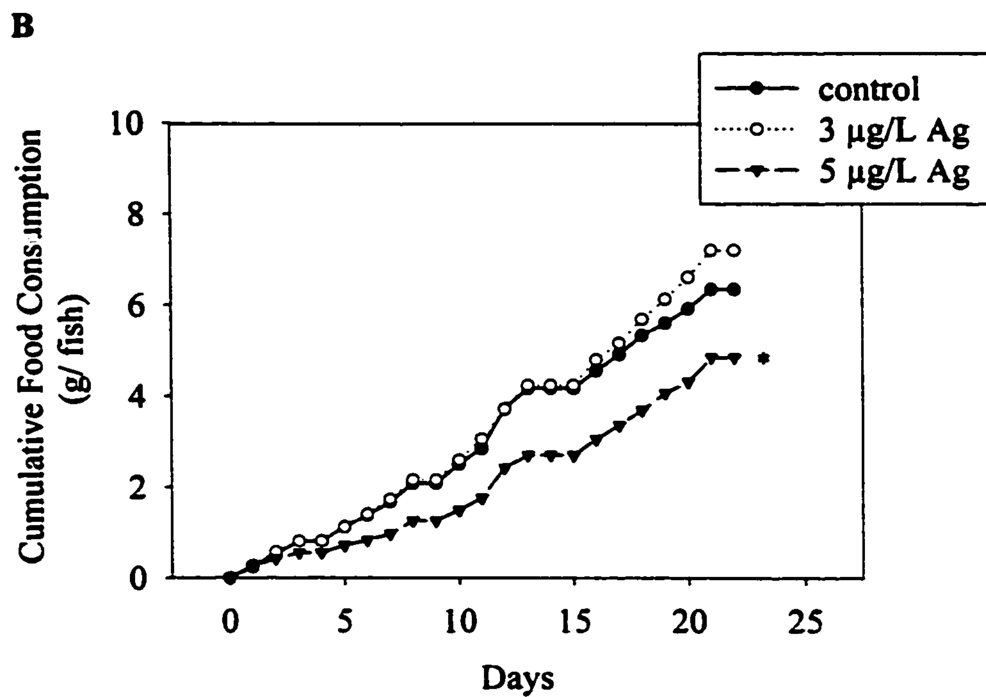

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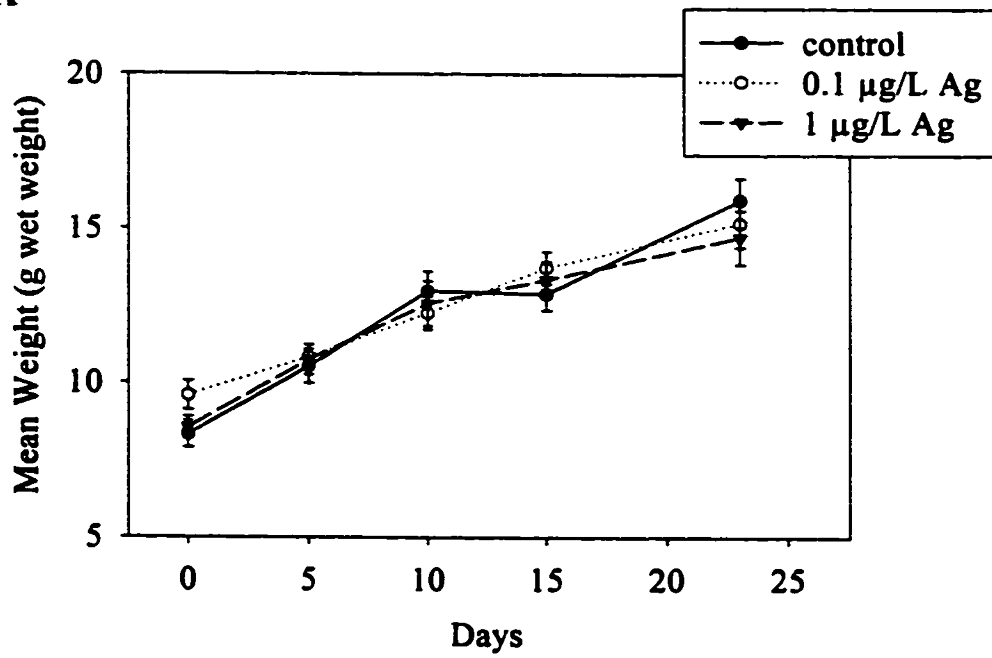

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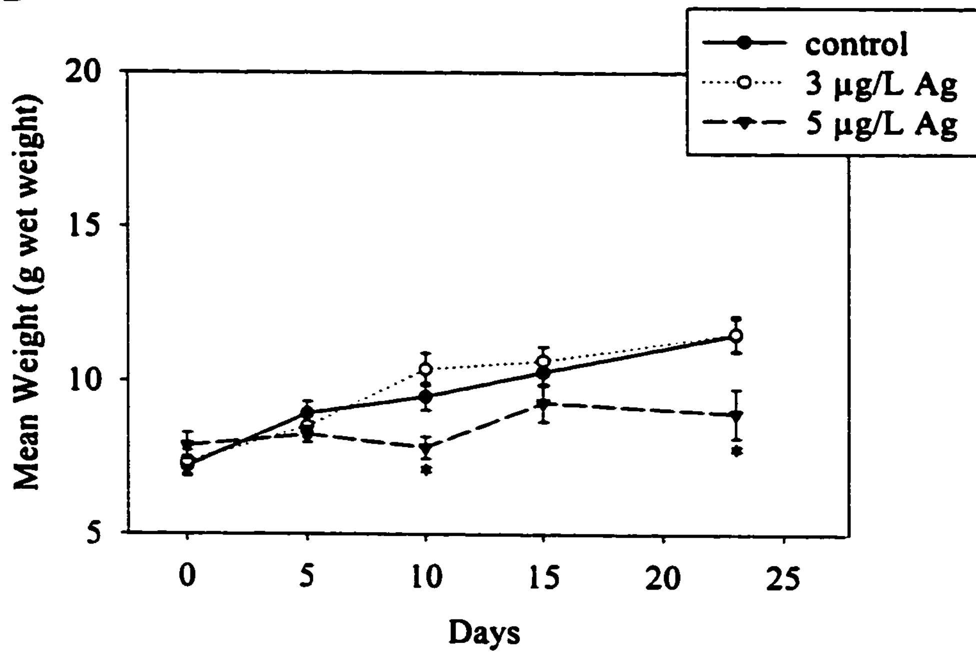

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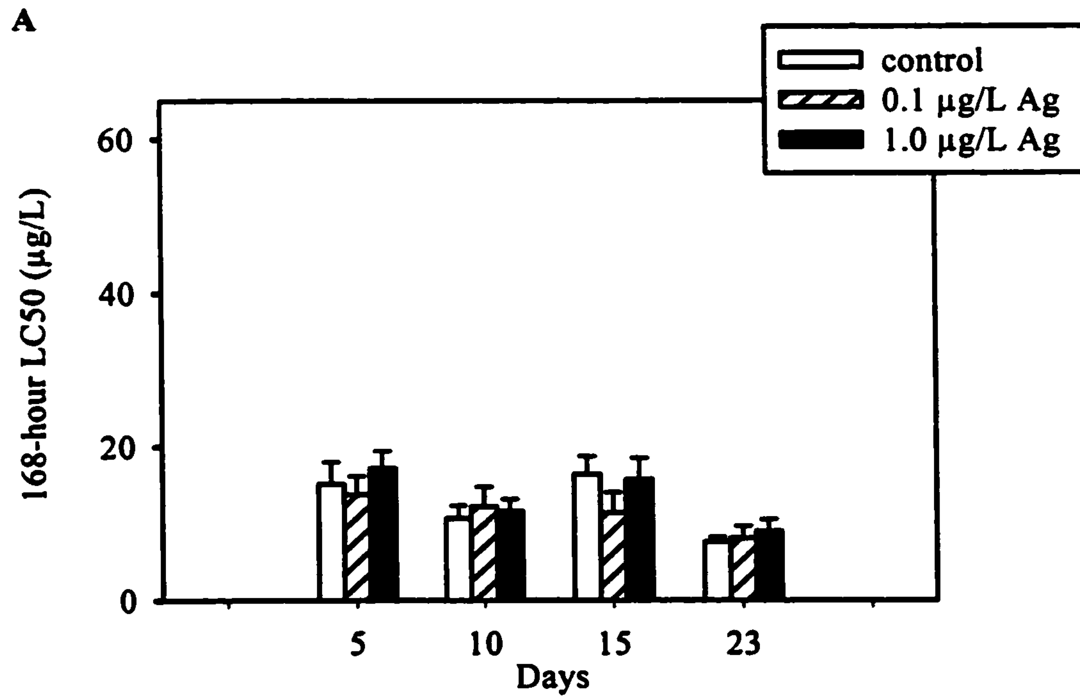

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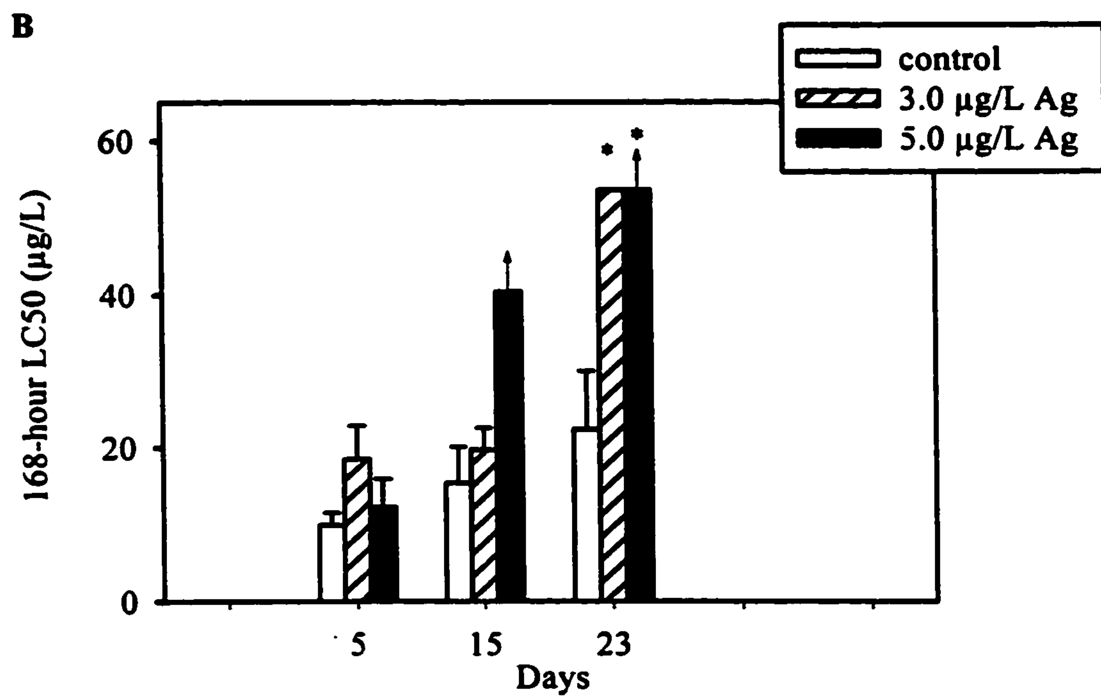

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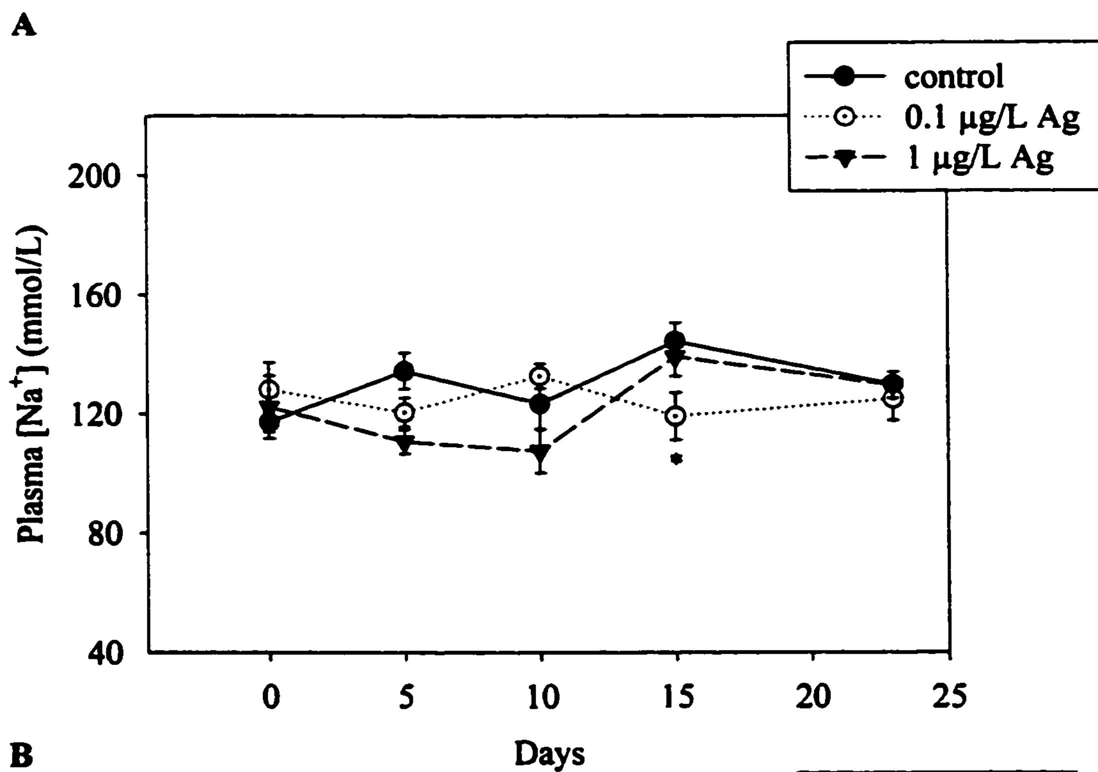

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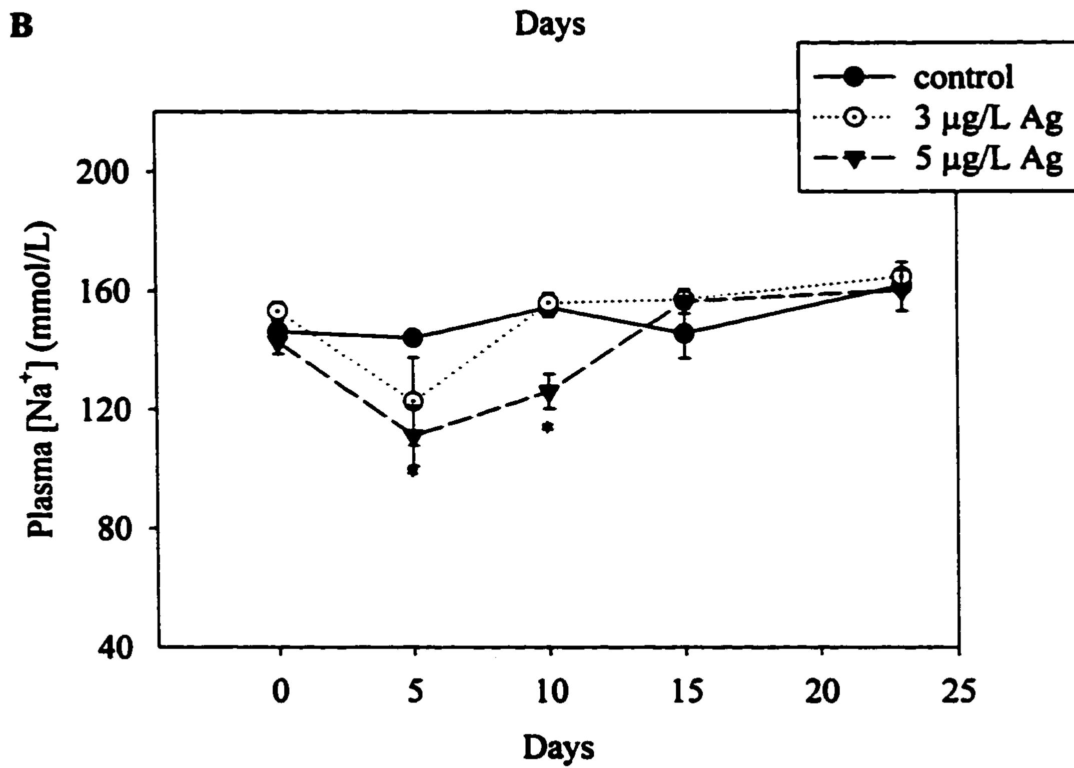

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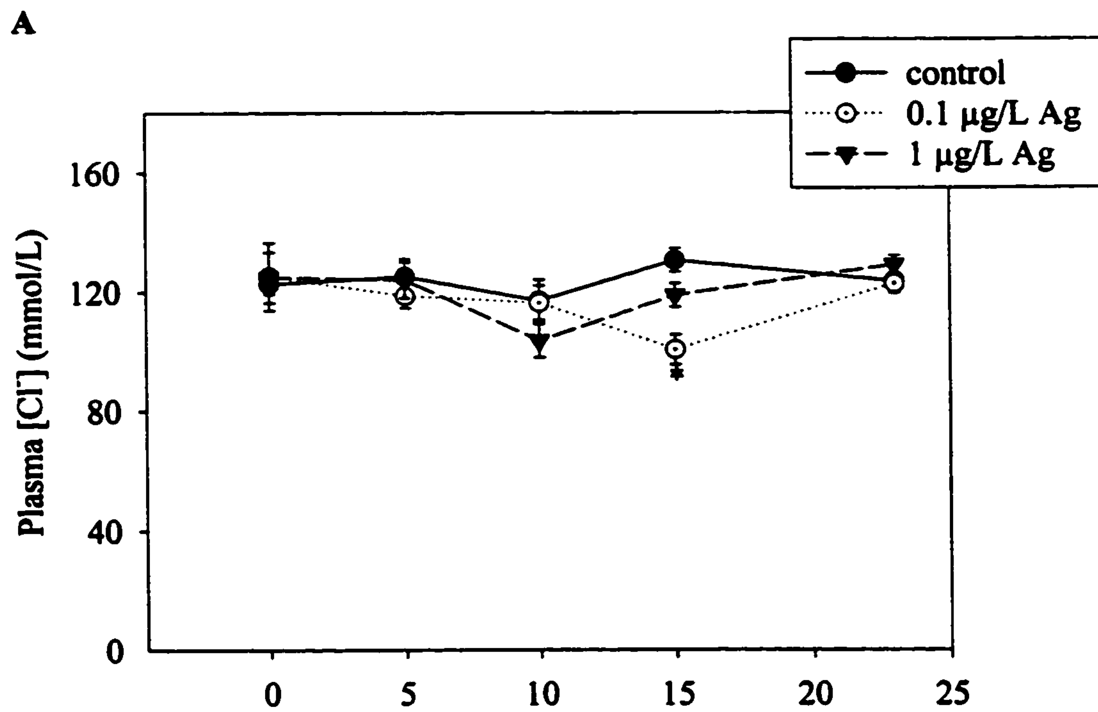

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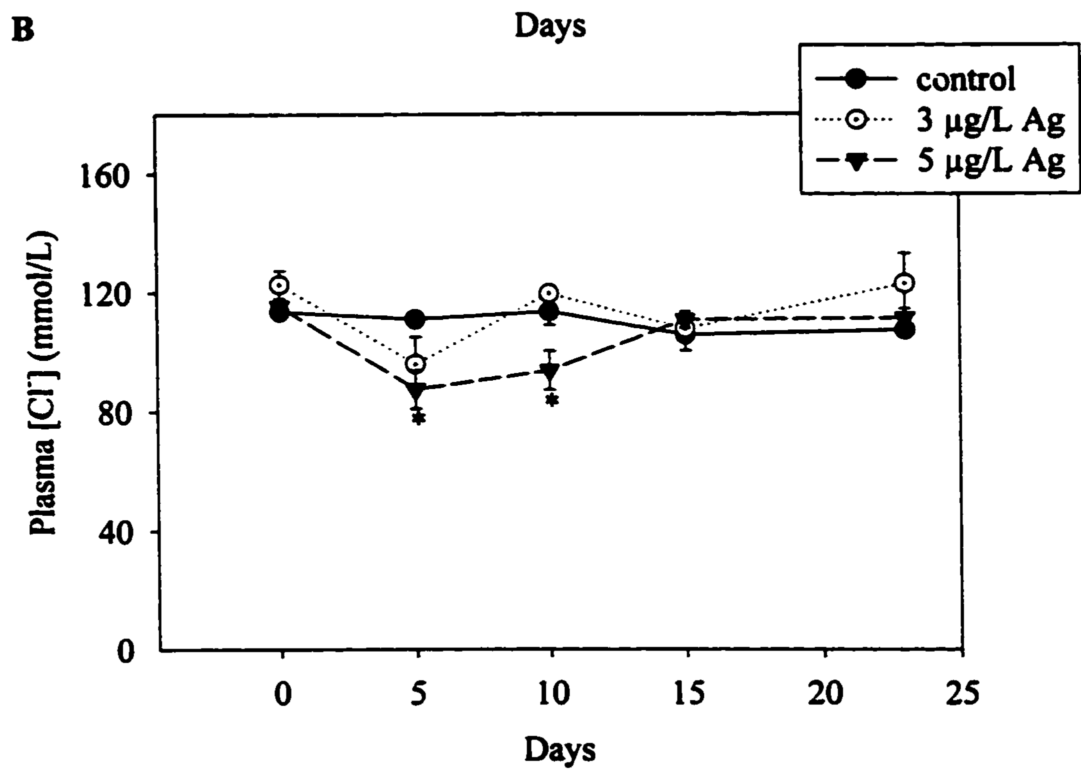

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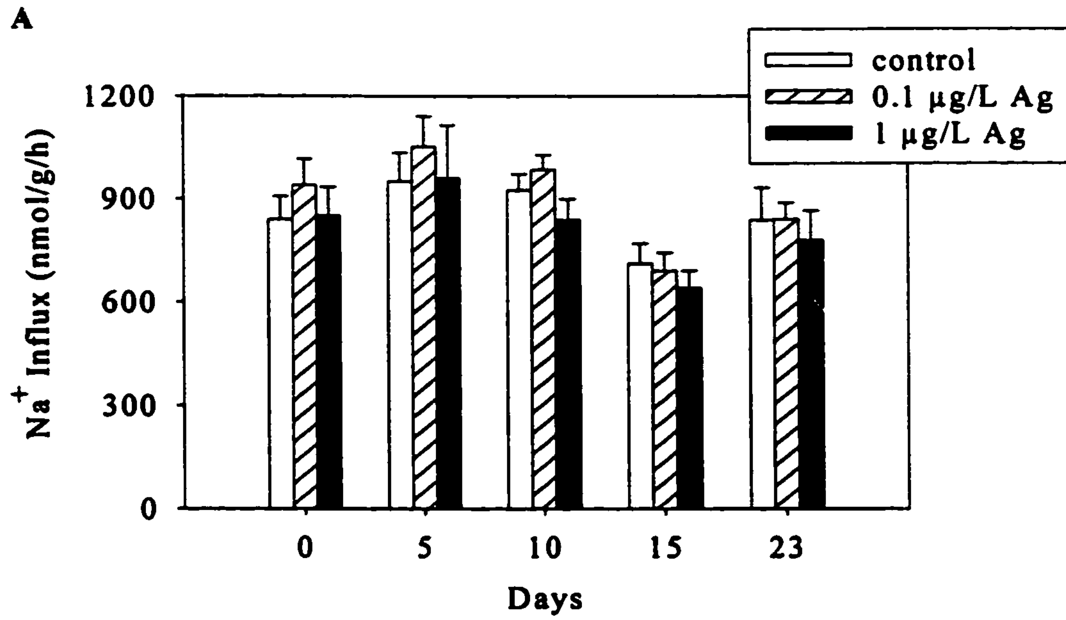

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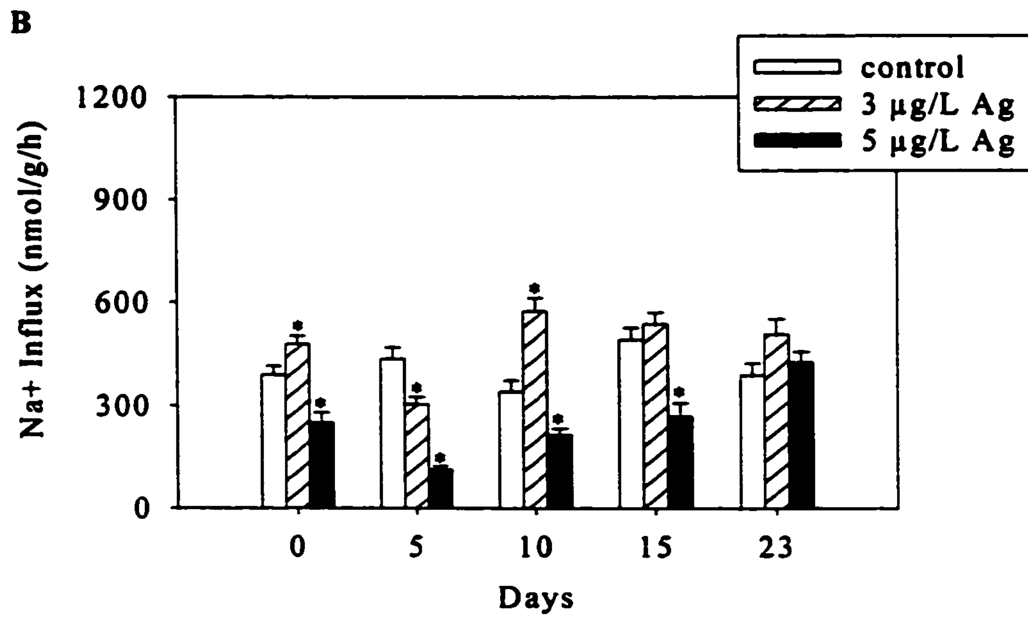

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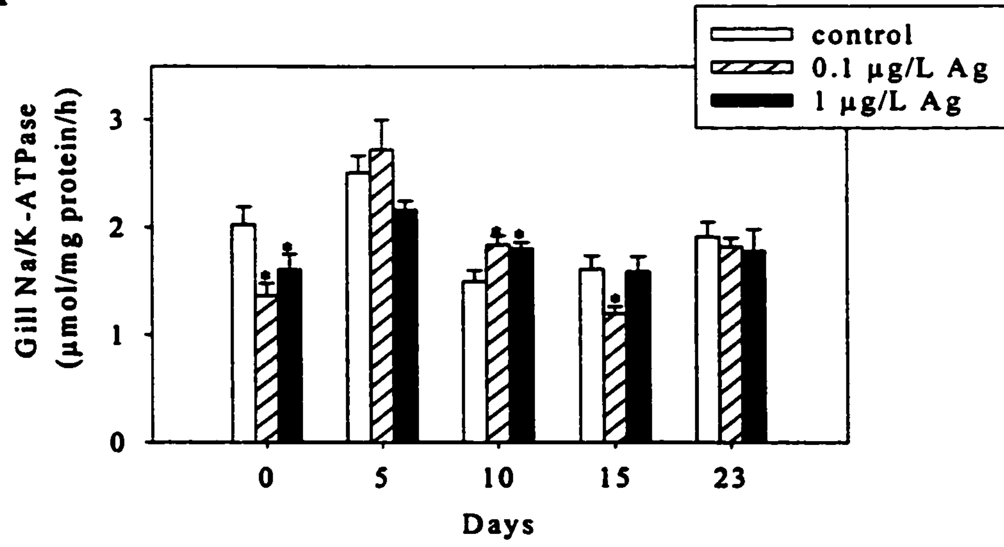

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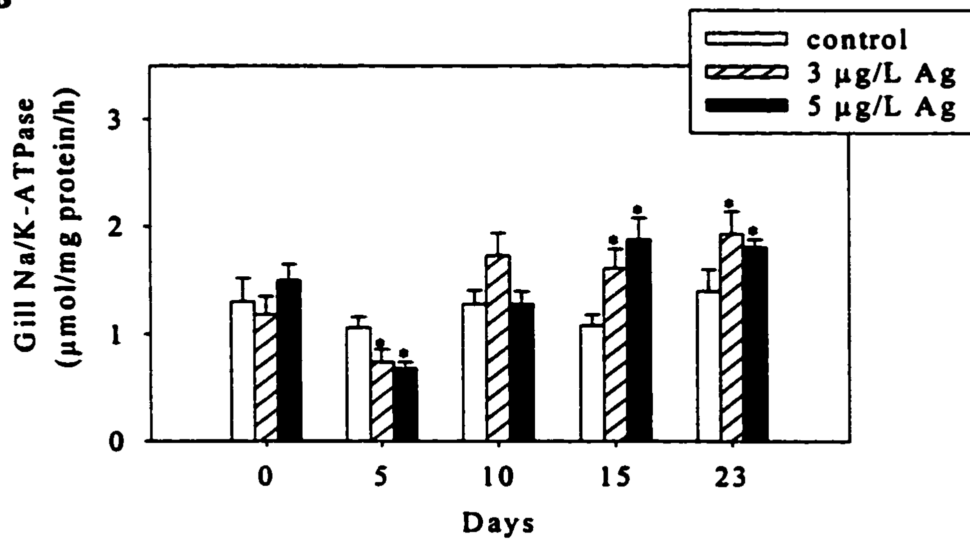

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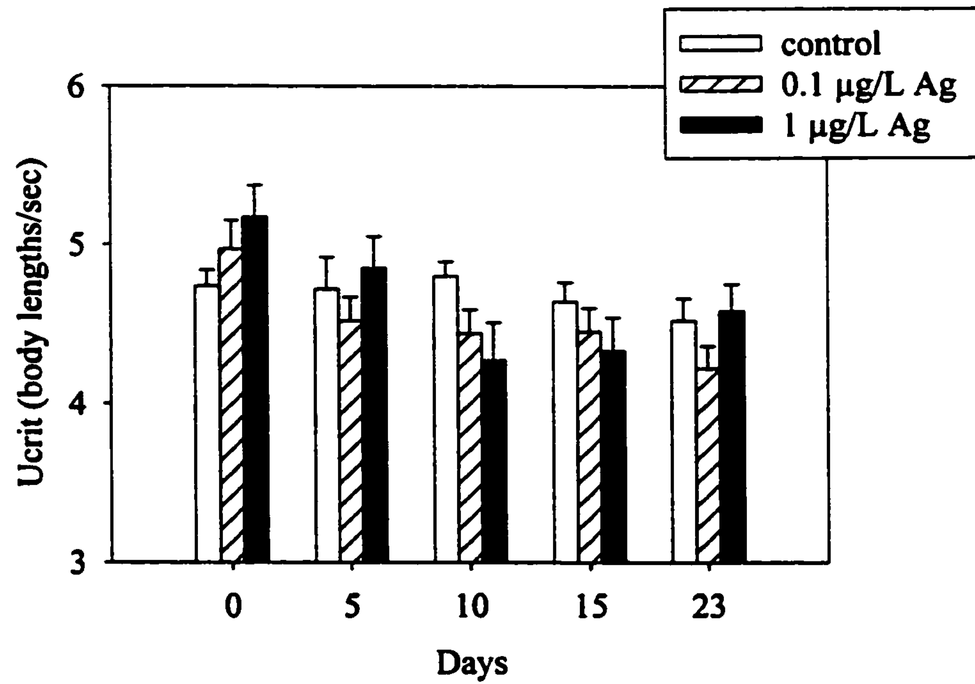

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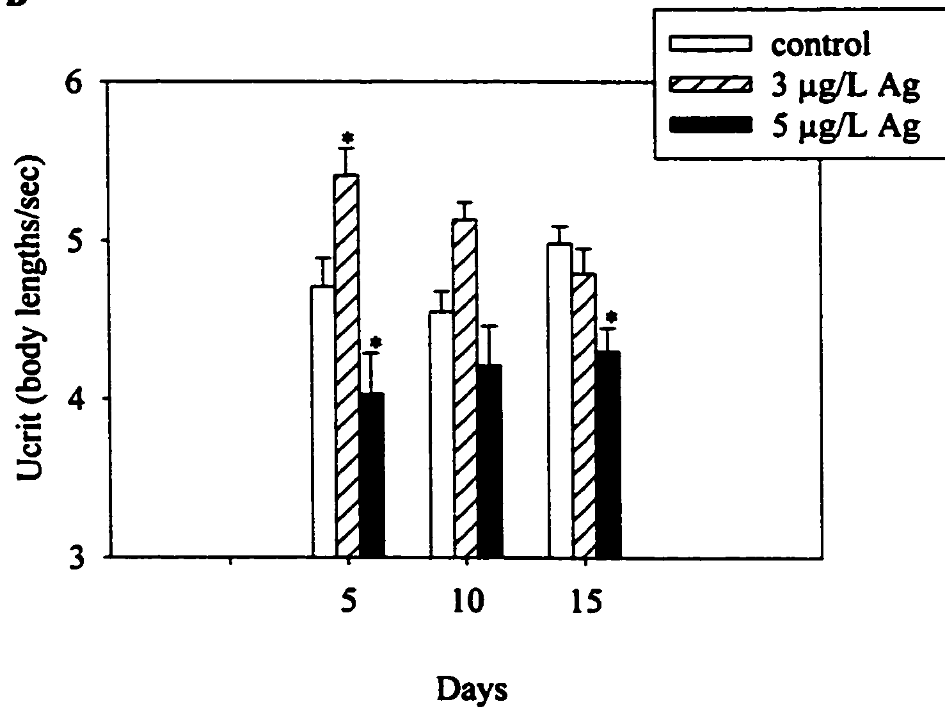

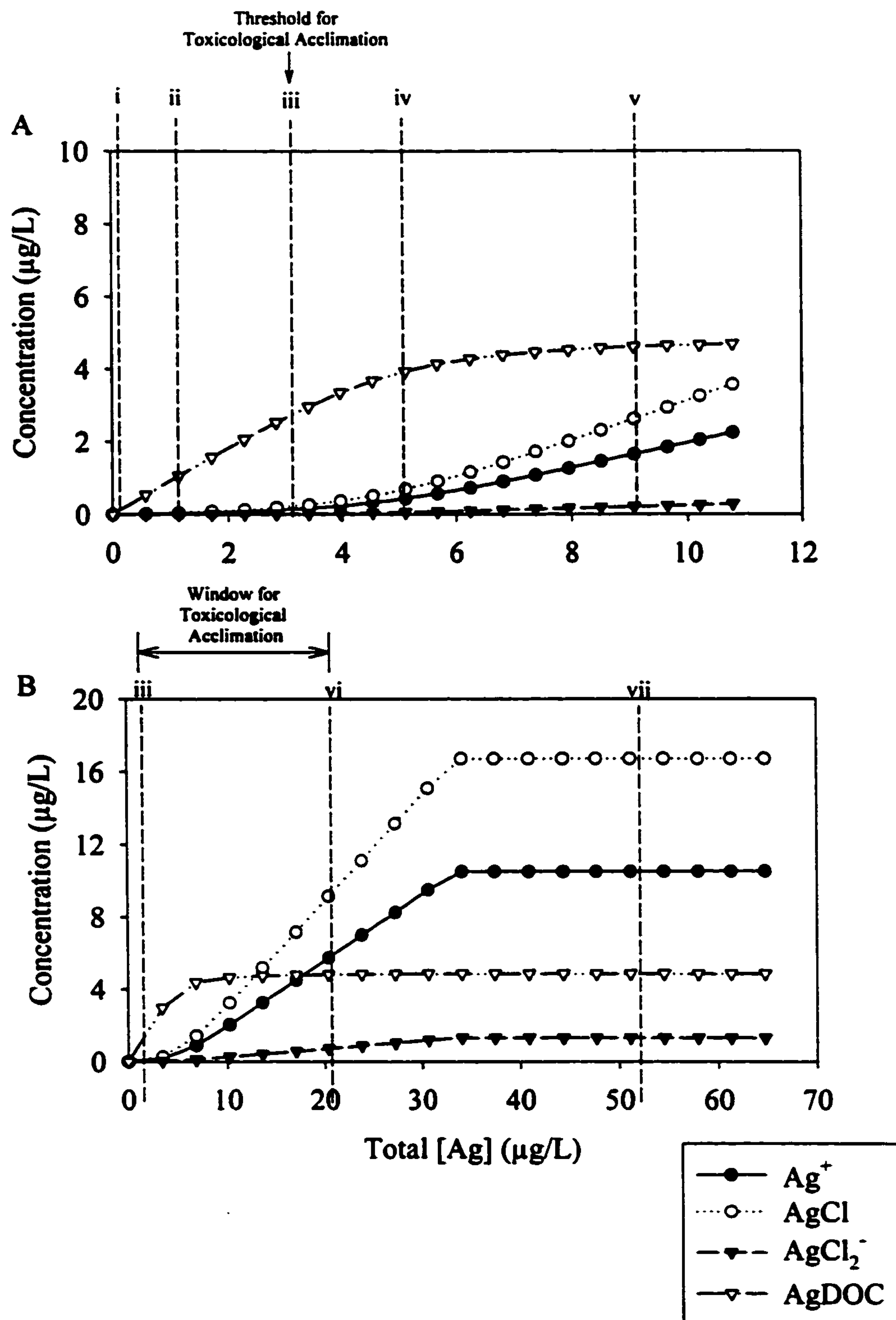

Threshold for Toxicological Acclimation
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ii
iii
iv
v
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Concentration (µg/L)
Window for Toxicological Acclimation
B
iii
vi
vii
Concentration (µg/L)
Total [Ag] (µg/L)
Ag+
AgCl
AgCl2-
AgDOC

CHAPTER 7

THE DISTRIBUTION KINETICS OF WATERBORNE SILVER-110m IN JUVENILE RAINBOW TROUT

ABSTRACT

Juvenile rainbow trout (*Oncorhynchus mykiss*) were subjected to a two-day radioactive pulse of ^{110m}Ag at 11.9 µg/L (as $AgNO_3$), followed by a 19-day post-tracer exposure of non-radioactive Ag (3.8 µg/L). The distribution of ^{110m}Ag in the gill, liver, intestine, kidney, brain and remaining carcass was investigated over the 19-day post tracer period. Initially, the intestine contained the highest proportion of the ^{110m}Ag (34%), however by day 8, less than 5% of the total radioactivity remained in this tissue. The majority of the ^{110m}Ag eliminated from the intestine was redistributed to the liver. Eventually, the ^{110m}Ag content in the liver accounted for as much as 65% of the total radioactivity of the fish. Once growth dilution was taken into account, there was no significant depuration of ^{110m}Ag from the fish over the 19-day post-tracer period. Apart from the liver and intestine, only the gills and carcass contained any appreciable amount (>5%) of the total body ^{110m}Ag content. Liver and gill samples were fractionated using differential centrifugation techniques to discern the subcellular distribution of ^{110m}Ag in these tissues. In the liver, the ^{110m}Ag levels in the cytosolic fraction increased from 35% to 72% of the total cellular burden between days 8 and 19, respectively. In contrast, the radioactive pulse in the gills was predominantly found in a relatively insoluble

compartment termed here as the nuclear fraction (~60% of the total). Little change in the subcellular distribution of Ag over time (day 8 to day 19) was observed in the gills. Using size-exclusion chromatography, most (~70%) of the ^{110m}Ag content in the liver cytosol eluted at a molecular weight characteristic of metallothionein. In comparison, the cytosolic distribution of ^{110m}Ag in gills was quite diffuse, occurring primarily in the heavy molecular weight fractions.

Key words: Silver, rainbow trout, metallothionein, subcellular fraction, distribution

INTRODUCTION

Silver nitrate is extremely toxic to freshwater fish, with 96-hour LC50s typically < 65 μg/L Ag (Coleman and Cearley, 1974; Davies *et al.*, 1978; Lemke, 1981; Hogstrand *et al.*, 1996). Only recently has progress been made towards understanding the physiological mechanisms of silver toxicity. Acute silver toxicity results from a dramatic impairment of Na^+ uptake processes due to non-competitive inhibition of the branchial Na/K-ATPase enzyme (Wood *et al.*, 1996a; Morgan *et al.*, 1997). The ensuing ionoregulatory disturbance produces a series of physiological perturbations that can eventually lead to cardiac failure and death (Hogstrand and Wood, 1998). It is believed that the free Ag^+ form of the metal actively binds to sites on the gill, namely the magnesium sites on the Na/K-ATPase (Ferguson *et al.*, 1996), thus causing toxicity (Terhaar *et al.*, 1977, Galvez and Wood, 1997; Bury and Wood, 1999). Conversely, Ag species such as the $AgCl_n^{(1-n)}$ ($n \geq 1$) and Ag thiosulphate complexes are much more benign to freshwater fish compared to $AgNO_3$ (Hogstrand *et al.*, 1996). Silver, when presented as these complexes, seems to pass through the branchial epithelium without causing an acute toxic response at the gills, and subsequently accumulates in other parts of the body (Hogstrand *et al.*, 1996; Hogstrand and Wood, 1998). The most notable example is a study in which rainbow trout were exposed for 6 days to extremely high concentrations of silver (30,000 μg/L) as Ag thiosulphate. Even though fish accumulated high burdens of Ag in the gills, plasma, kidney and liver, the exposure was not acutely toxic (Wood *et al.*, 1996b).

Bioaccumulated Ag can either directly or indirectly induce metallothionein in fish livers, suggesting that silver can affect biochemical processes (Cosson, 1994; Hogstrand

et al., 1996). Moreover, trout exposed to 2.0 μg/L silver added as $AgNO_3$ during a 28-day period exhibited a marked elevation in hepatic Ag levels, accompanied by a modest induction of metallothionein, depressed growth rates, and decreased food-conversion efficiencies (Galvez *et al.*, 1998). However, further studies are needed to identify whether chronic toxicity is eventually produced from elevated metal tissue burdens. One of the existing hurdles is the lack of information on the kinetics of uptake, internal distribution, fate and elimination of Ag in teleost fish.

The purpose of this study was to investigate the pharmacokinetics of a two-day radioactive Ag pulse during a 19-day post-tracer period, with continuing chronic exposure to silver at a sublethal level. The Ag isotope ^{110m}Ag was used to monitor Ag distribution and elimination in individual tissues, and silver was added as $AgNO_3$. Differential centrifugation techniques, as well as size-exclusion chromatography were used to elucidate the subcellular distribution of the ^{110m}Ag pulse in gill and liver.

MATERIALS AND METHODS

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing approximately 10 g were obtained from Wolf Creek Dam National Fish Hatchery (Jamestown, KY, USA), and transported to the University of Kentucky, Lexington. Fish were allocated to three separate 60-L polystyrene tanks (46 fish per tank), each supplied with 150 mL/min of dechlorinated Lexington tap water plus aeration. The chemical composition of the water was: (in mmol/L) [Na^+] 0.65; [Cl^-] 0.61; [Ca^{2+}] 0.22; (in mg/L as $CaCO_3$) total alkalinity of 68 and (total hardness of 126; and pH of 7.7. Although dissolved organic matter (DOM) was not measured, typical values for groundwater sources approximate 1.3 mg carbon/L. Water temperature was maintained at 15 °C. Fish were allowed to acclimate to laboratory conditions for two weeks prior to the start of Ag exposures. Fish were fed commercial trout pellets (Purina Trout Chow) to satiation once daily, except during the 2-day radioactive ^{110m}Ag pulsing period.

Silver exposures

Water flow to the tanks was stopped, vigorous aeration was instituted, and the fish were statically exposed for 2 days to a radioactive silver pulse of ^{110m}Ag (as $AgNO_3$) with a measured total [Ag] of 11.9 µg/L Ag. Silver concentrations were achieved by adding radiolabeled silver stock (2.2 g/L ^{110m}Ag as $AgNO_3$ at an activity of 303 MBq/mL) (Amersham, Oakville, ON, Canada) to each tank containing 40-L of water. Geochemical speciation analysis (MINEQL+ Version 4.0, Environmental Research Software) using measured water quality suggested that Ag during the pulsing period existed as: 24.9 %

Ag^+, 33.1% AgCl, 2.2% $AgCl_2^-$, and 39.8% AgDOM (expressed as % of total [Ag]). Silver-DOM interaction was modeled using a log K value of 9.0 for Ag-DOM and DOM binding site density of 35 nmol Ag/mg C as derived by Janes and Playle, 1995. The post-tracer phase of the experiment was commenced by removing ~85% of the radioactive water from each tank and replacing it with water containing 3.8 µg/L of non-radioactive Ag (as $AgNO_3$; Sigma, St.Louis, MI, USA). Fish were subsequently exposed for 19 days to 3.8 µg/L Ag delivered at a rate of 150 mL/min (post-tracer period). Efficient removal of the radioactive ^{110m}Ag from the water was verified by lack of any measurable gamma-radiation in water samples taken 12 hours after starting the post-tracer period (MINAXI Auto-Gamma 5000 series, Canberra-Packard). Water samples were taken daily from each exposure tank for analysis of total Ag. Samples were acidified with trace-metal grade HNO_3 acid (Fisher Scientific) to a concentration of 0.5 % (v/v). Total Ag was measured by atomic absorption spectroscopy (Varian SpectrAA-20) with a graphite furnace atomizer (Varian, GTA96).

Tissue sampling and analysis

On days 1, 4, 8, 14 and 19 of the post-tracer period, six fish were randomly selected and placed in 20 L of dechlorinated tap water. Each fish was subsequently rinsed in 250 µg/L Ag (as $AgNO_3$) solution for one minute to displace any superficially bound ^{110m}Ag. This was followed by another one-minute rinse in a solution containing 150 mg/L sodium thiosulfate and 0.3 g/L MS-222 anaesthetic. This rinsing protocol was found to be effective at removing superficially bound Ag from the outer surface of fish. Sacrificed fish were blotted dry and weighed; blood samples were collected using a

sodium-heparinized Hamilton syringe; and plasma aliquots were obtained by centrifuging the blood at 10,000 g for 2 minutes. The liver, intestine (from the pyloric caeca to the anus), entire gill basket, kidney and brain were then dissected out of each fish. Each tissue, in addition to the remnant carcass, was blotted dry, weighed and quick-frozen in liquid nitrogen. Samples were stored at -80 °C until analyzed for ^{110m}Ag and total Ag burdens. The contents of the intestinal tract were carefully removed prior to cold storage of the tissue.

Subcellular fractionation

On days 8 and 19 of the post-tracer period, ten additional fish were sacrificed according to the above methods. Gills and livers were freeze-clamped in liquid nitrogen for subcellular fractionation using a protocol modified from Julshamn *et al.* (1988). In short, tissues were thawed, and immediately homogenized at 0 to 2 °C in either a hypertonic (35 mM Tris-HCl, 250 mM sucrose, 200 mM KCl, pH 7.4) or hypotonic (10 mM Tris-HCl, pH 7.6) buffer using a glass-Teflon homogenizer. The two different homogenization media were used to assess the affinity of ^{110m}Ag to each of these fractions. Subcellular fractions were obtained by a series of centrifugations whereby the pellet of each spin was collected, and the supernatant re-centrifuged at a higher centrifugal force. The original homogenate was first centrifuged at 370 g for 5 minutes, and the pellet from this spin was labeled the nuclear (*nuc*) fraction. In addition to nuclear material, this fraction would contain relatively insoluble cellular debris. Likewise, pellets produced from 9,200 g (5 minutes) and 130,000 g (1 hour) spins were termed the mitochondrial and lysosomal (*m&l*) and microsomal (*mic*) fractions, respectively. The

remaining supernatant was labeled the cytosolic fraction (*cyt*). Individual subcellular fractions were analyzed for ^{110m}Ag, and the data were expressed as radioactive counts per minute (CPM) per gram tissue. Cytosolic fractions were analyzed frozen and then returned to liquid nitrogen, and stored at -80 °C. Further fractionation of cytosolic fractions was performed by size-exclusion liquid chromatography.

Chromatography of cytosolic fractions

Cytosolic fractions obtained from differential centrifugation were thawed individually and fractionated by size-exclusion liquid chromatography using Superdex-75 filtration media (Pharmacia, Uppsala, Sweden) in a 60cm x 1.6cm gel filtration column (xK 16/70, Pharmacia). The column was calibrated for molecular weight with bovine albumin (mol. Wt.= M^r= 66,000), cytochrome-c Type III (lung tissue; M^r= 12,400), rabbit metallothionein (MT; apparent mol. Wt = 10,000) and glutathione (M^r= 310). A liver homogenate from Cd-injected rainbow trout was fractionated and the MT fractions (eluted at 9.4 kDa) were partially verified by the presence of Cd, as analyzed by graphite furnace atomic absorption spectroscopy. All gill and liver cytosols (100 µL) were chromatographed at a rate of 1 mL/min with 250 mL of 10 mM Tris-HCl (pH 7.6) at 2 °C. The elutant was collected in 3-mL fractions, which were then analyzed for radioactivity, and for absorbance at 280 nM. The elution profile was divided into three parts: void volume to 93 mL was termed the heavy molecular weight fractions (HMW), 93 to 114 mL elution volume (i.e., M^r ranging from 5,800-22,900 KDa) was referred to as the metallothionein fractions (MT) and >114 mL was termed the low molecular weight fractions (LMW). The MT region corresponded to the elution of MT in the liver

reference, as well as purified rabbit MT.

Calculations and statistical analysis

The distribution of ^{110m}Ag radioisotope in each tissue was expressed either as CPM per gram tissue or as a percentage of the total body radioactivity. Total radioactivity per gram fish was used to calculate the exponential rate of Ag elimination (SPSS 8.0). A specific growth rate (SGR) (%/day) was calculated using the mean weights of fish sampled during the experiment. SGR was determined by fitting an exponential growth curve to the mean weight versus time data. To account for the effects of growth dilution on apparent elimination of ^{110m}Ag from the fish, CPM per gram was converted to total by multiplying the CPM per g by the nominal fish weight (grams). Since subcellular distributions were expressed as a % of total tissue radioactivity, data were arcsine transformed prior to statistical analysis. The effects of time and homogenization buffer on the ^{110m}Ag distribution in each subcellular fraction were statistically compared using an unpaired two-tailed t-test. The alpha level was modified using a Bonferroni correction to allow for multiple comparisons.

RESULTS

Total Ag burdens of tissues sampled during the 19-day post-tracer period are outlined in Table 1. Silver concentrations in the liver were at least 10-fold higher than the Ag concentrations of all other tissues. Hepatic Ag concentrations remained constant at approximately 9 µg/g. Similarly, there were no clear trends in the levels of Ag in tissues over time, except for the brain, where it tended to increase with time. Nevertheless, the final Ag concentration in the brain was only a few percent of the concentrations found in the liver.

Fish growth rate was 1.46 %•day^{-1} ($r^2 = 0.88$; $p<0.05$), with a mass doubling time of 34.2 days. The ^{110m}Ag whole-body concentration on day 1 was 2,967 cpm/g body weight, eventually decreasing to 1,782 cpm/g body weight by day 19. This represented a 40% reduction in whole-body ^{110m}Ag concentration. The first-order elimination half time was 36 days ($r^2= 0.27$; $p=0.37$; n.s.). However, total ^{110m}Ag content (CPM) over time was used to assess the elimination kinetics of ^{110m}Ag independent of growth dilution effects. There was no significant elimination of ^{110m}Ag fish during the post-tracer period based on evaluation of normalized CPM burdens over time.

The internal distribution of ^{110m}Ag was also monitored in various tissues over the 19-day post-tracer period. Figure 1 illustrates ^{110m}Ag content in each tissue as a percentage of whole-body radioactivity levels (Table 2). Initially, the intestine contained the highest proportion of the ^{110m}Ag (34%), although by day 8 less than 5% of the total radioactivity remained in this tissue. Interestingly, the ^{110m}Ag proportion of the liver increased steadily from 24% (day 1) to ~61% (day 19). Apart from the liver and intestine, only the gills and carcass contained any appreciable amount (>5%) of the

whole body ^{110m}Ag content at any time during the post-tracer period. By expressing the data from Figure 1 on a per gram basis, the affinity of the liver for Ag becomes much more apparent (Figure 2). The mean BCF value for the liver (~84) is at least 10-fold higher than the estimates for all other tissues, except the intestine (Table 3).

Subcellular fractionation was performed on livers and gills sampled on days 8 and 19. The effects of homogenization buffer osmolarity on the subcellular distribution of ^{110m}Ag were also investigated. There were fundamental differences in the relative distribution of Ag between the gills, where ^{110m}Ag was mainly found in the nuclear fraction "*nuc*", versus the liver, where it was mainly in the cytosolic fraction "*cyt*" (Figs. 3, 4). There was no significant influence of buffer osmolarity on the distribution of ^{110m}Ag in gills (Fig. 3). Furthermore, the proportion of Ag in each fraction was constant between days 8 and 19, regardless of buffer used. In comparison, significant reductions in relative ^{110m}Ag content in the mitochondrial and lysosomal fractions "*m&l*" ($p<0.05$) and *nuc* ($p<0.005$) fractions of the liver were noted from day 8 to day 19 (Fig. 4). Interestingly, these effects were only seen when livers were homogenized with hypertonic buffer. The relative content of ^{110m}Ag in the microsomal fraction "*mic*" also tended to decrease with time, although this effect was not significant. Silver from the previously mentioned subcellular fractions appeared to redistribute to the *cyt* fraction between days 8 and 19 ($p<0.05$). Within any given time (day 8 and day 19 of the post tracer period), homogenization buffer type did not greatly affect the subcellular distribution of ^{110m} Ag. The one significant effect, seen only on day 8 of the post-tracer period, was a 2-fold higher Ag content of the *m&l* fraction of livers homogenized with hypertonic buffer, relative to the hypertonic buffer ($p<0.05$).

There were clear differences in the cytosolic distribution of ^{110m}Ag between the gills and liver (Figs. 5, 6, 7). Figures 5 (A,B) are representative gel elution profiles of *cyt* gill samples. The cytosolic distribution of ^{110m}Ag in gills was quite diffuse, with binding of the metal to several fractions, including the MT region. The proportions of total ^{110m}Ag in the MT region of gills were only 17.8 % and 8.0 % for day 8 and day 19, respectively. In comparison, Ag content in the HMW and LMW regions was 60.7 % to 63.0 % and 21.5 % to 28.3 %, respectively (Fig. 7A). In the HMW fractions of gills, the major ^{110m}Ag peak was found at 216 KDa, whereas the main ^{110m}Ag binding region in the LMW fractions was at 1.2 KDa. Figures 6 (A, B) are representative elution profiles of liver *cyt* fractions following size-exclusion chromatography. Fractionation of liver cytosols from day 8 produced one major ^{110m}Ag peak eluting at a molecular weight characteristic of MT (Fig. 6A). Approximately 70% of the ^{110m}Ag fractionated within the MT region, whereas only 11% and 14% of the radioisotope eluted at the HMW and LMW regions, respectively (Fig. 7B).). In the HMW fractions of the liver, ^{110m}Ag was mainly found in the 216 KDa region (same as in the gills), whereas the main ^{110m}Ag binding region in the LMW fractions was at 2.2 KDa. The elution profiles of livers varied slightly with time (Fig. 6B). Although over 65% of the ^{110m}Ag continued to elute at the MT region, a slight spill over of Ag to HMW fractions (2-fold increase) occurred by day 19 (Fig. 7B).

DISCUSSION

This study assessed the uptake of Ag from water and its subsequent internal distribution in freshwater rainbow trout. Metal uptake in teleost fish occurs primarily at the gill surface and in the gastrointestinal tract. Although waterborne metals are predominantly taken up via the gills in freshwater fish, the inadvertent consumption of water during feeding may lead to increased gastrointestinal metal uptake. Drinking rates in starved seawater fish are around 3.0 mL/kg/h, but only 0.25 mL/kg/h in starved freshwater fish (Fuentes and Eddy, 1997). The present study attempted to look at Ag accumulation across the gills alone. As a result, feeding was suspended during the two day ^{110m}Ag pulsing period to reduce gastrointestinal uptake of the metal. Consequently, in these starved, freshwater rainbow trout, Ag uptake from water probably occurred almost exclusively across the gill epithelium.

Radioisotopic ^{110m}Ag was used to distinguish between newly accumulated Ag and background Ag levels in the fish. ^{110m}Ag was completely removed from the water by the start of the post-tracer period (Day 0). Therefore, any radioactivity in the fish would have accumulated solely during ^{110m}Ag pulsing (Days –2 to 0). Based on Day 1 whole body CPM levels (Table 2), the accumulation rate (AR) of Ag into rainbow trout from water was 15.8 ng/(g • day). To further assess the bioavailability of waterborne Ag, a Concentration Specific Accumulation Rate (CSAR) was determined (see Hogstrand *et al.*, 1998). The CSAR value, calculated by normalizing the Accumulation Rate (AR) to the exposure concentration in parts per billion, was 1.3 ng/(g • day • ppb). This is in contrast with the AR of 72.3 ng/(g • day) and CSAR of 9.0 ng/(g • day • ppb) reported for rainbow trout subjected to 4-hour fluxes of 8 µg/L Ag (as $^{110m}AgNO_3$) in synthetic

soft water (Bury *et al.*, 1999a). The discrepancy in AR and CSAR values between studies is likely due to variations in water chemistry. Water from the present study likely contained 3 X higher DOC and over 2 X higher sodium than Bury *et al.* (1999). The combined effect of increased levels of a complexing ligand and a competing ion in water is expected to decrease the bioavailability of Ag to whole fish. Notwithstanding, differences in aqueous chloride are likely inconsequential, since Ag^+ and AgCl have been shown to be equally bioavailable to trout (Bury *et al.*, 1999a,b). Interestingly, only Ag^+ is of acute toxicological concern (Galvez and Wood, 1997; Bury *et al.*, 1999a; Wood *et al.*, 1999).

Any apparent loss of whole-body ^{110m}Ag on a per gram basis was in fact dilution resulting from fish growth (Table 2). Thus, Ag is not easily eliminated from rainbow trout. The only other published study on Ag depuration in salmonids reported a 23% reduction in radioactivity after 28 days in clean water (Garnier *et al.*, 1990), but attributed much of the apparent elimination of Ag to growth dilution by the fish. The carcass is a relatively important sink for silver in fish, accounting for between 30 to 35% of the total body radioisotope burden (Fig. 1). Nonetheless, because of the relatively large mass of the carcass, the concentration of silver in the carcass was low when expressed on a per gram basis (Fig. 2), and therefore probably was not toxicologically relevant.

High amounts of ^{110m}Ag were accumulated in the intestinal tract up to 4 days following the radioactive exposure (Figs 1, 2). Based on the normal drinking rate of starved, freshwater fish (Fuentes and Eddy, 1997), only ~0.12 mL of water would have entered the gastrointestinal tract during the 48-hour radioactive pulsing period.

Consequently, only 140 CPM or about 1% of the intestinal ^{110m}Ag burden at day 1 can be explained by inadvertent drinking of water. It is therefore expected that Ag accumulated within the intestines was actually taken up at the gills and transported via the bloodstream. The high accumulation potential for Ag in the intestine could be due to the tissue's abundance of basement membranes (Baudin *et al.*, 1994). This is consistent with studies by Martoja *et al.* (1988) and Baudin *et al.* (1994), which also observed high concentrations of Ag in the digestive tract of other aquatic organisms.

Most of the intestinal radioactivity observed during the early portions of the study was quickly redistributed to the liver, probably via the hepatic portal vein. By day 8, the liver contained approximately 65% of the ^{110m}Ag. Similarly, Garnier *et al.* (1990) found that the liver contained from 62% to 70% of the whole body content in brown trout after waterborne ^{110m}Ag exposure. Clearly, the liver appears to be the primary site for silver accumulation in most aquatic (Hibiya and Oguri, 1961; Pentreath, 1977; Hogstrand *et al.*, 1996; Wood *et al.*, 1996b) and terrestrial vertebrates (reviewed by Eisler, 1996). According to Pentreath (1977), the accumulation potential of the liver for Ag will subsequently determine the biological turnover rate of Ag from the whole organism. In seawater, fish such as *Pleuronectes platessa,* which have low hepatic accumulation potentials for Ag, will tend to have relatively fast whole-body Ag elimination rates (e.g., $t½ = 12$ days). In comparison, *Raja clavata* accumulated high concentrations of Ag in the liver and had a correspondingly long half-time for Ag elimination ($t½ = 315$ days) (Pentreath, 1977). Our study is consistent with Pentreath's (1977) hypothesis.

Bioconcentration factors (BCF) have also been used to assess the relative bioavailabity of waterborne Ag exposures in aquatic organisms. In this study, the BCF

(as calculated from the whole-body radioactivity at day 1) was only 2.5 ± 0.3. This value approximates the whole-body BCF value of 2.7 for rainbow trout following a 57-day exposure to waterborne $AgNO_3$ (Gaudin *et al.*, 1990), and 1.8 for fathead minnows exposed to Ag thiosulphate (Ewell *et al.*, 1993). The highest BCF of any tissue in the trout was observed in the liver, with a mean of 84.2 over the entire post-tracer period, and a maximum on day 8 of 148 (Table 3). This is consistent with the BCF value of 282 for liver of freshwater brown trout after 57 days of exposure (Garnier *et al.*, 1990). Apart from the intestine, only the gills and kidney accumulated significant amounts of ^{110m}Ag.

Subcellular fractionation of liver and gills of Ag-exposed fish suggests that clear differences exist in the way that silver is handled between these tissues. The gill Ag load was primarily distributed to the *nuc* (~60 %) and *cyt* (~20%) fractions (Fig. 3). In bivalves, Ag in the gills was primarily associated with insoluble fractions, including basement membranes (Berthet *et al.*, 1992; Truchet *et al.*, 1990). Nonetheless, this does not preclude the possibility that Ag in the *nuc* fraction was bound to mucus and/or nucleic acids. The distribution pattern of Ag in the gills was not significantly different between days 8 and 19, suggesting that Ag was bound in a stable form, likely to sulphides. Certainly, work on bivalves has shown that Ag primarily exists as a sulphide in the gills of these organisms. Moreover, this insoluble fraction was found to depurate extremely slowly from the gills, in comparison to soluble protein-bound Ag (Berthet et al., 1992). Of the 20% of ^{110m}Ag distributed to the *cyt* fraction, less than 20% was found in the MT fraction (Fig. 7A). The fact that Ag is not readily sequestered in the gills may, in part, be due to the low background levels of MT in the tissue as a whole. In general,

the concentration of MT in the gills is at least 3-fold lower than hepatic levels following waterborne Ag exposure (Hogstrand *et al.*, 1996). Most of the cytosolic ^{110m}Ag burden in the gills was found associated with HMW proteins. Many studies have related toxic effects of metals with the amount of metal eluted with the HMW fraction, as well as other non-MT metal ligands (e.g., Hogstrand and Haux, 1991).

The subcellular distribution of Ag in the liver was quite different from the distribution observed in the gills. The proportion of ^{110m}Ag in the cytosolic fraction was increased from ~40% to ~65% between days 8 and 19 ($p<0.001$), likely due to the redistribution of ^{110m}Ag from the other three fractions. The distribution of Ag in the livers of rainbow trout was similar to that of squid, in which approximately 60% of the Ag was compartmentalized within the cytosol (Tanaka *et al.*, 1983). This is in stark contrast to the distribution of Ag seen in some mammalian studies that suggest microsomal compartmentalization of hepatic Ag. Incorporation of Ag in the microsomal fraction is associated with decreased activity of copper enzymes such as ceruloplasmin (Hirasawa *et al.*, 1994; Sugarawa and Sugarawa, 1984). In the present study, over 70% of the Ag in the liver cytosol eluted in the MT fractions. Consequently, cytosolic MT would bind approximately 24 % and 46 %, of the total ^{110m}Ag burden on days 8 and 19, respectively. This may explain why Ag accumulated in the liver from waterborne sources appears to be non-toxic in fish (Hogstrand *et al.*, 1996; Wood *et al.*, 1996b). Silver that eluted with the HMW and LMW fractions was primarily found in the 216 and 2.2 KDa fractions. Interestingly, Ag was associated with the same 216 KDa HMW fraction in both the liver and gills, but appeared to elute at different molecular weights (gills: 1.2 KDa; liver: 2.2 KDa) in the LMW region. Thus, in the present study, we show

evidence of a lower capacity of the gills than the liver to detoxify ^{110m}Ag. These differences may partly explain why the gill is the primary site for acute Ag toxicity in fish during exposure via the water.

Recent work suggests that Ag is an extremely powerful inducer of hepatic metallothionein in fish (Cosson, 1994; Hogstrand *et al.*, 1996). Silver has been shown to accumulate in the liver to concentrations of 900 µg/g wet weight following exposure to Ag thiosulphate (with Ag levels approximately 2000-fold above controls), without any discernable toxic effects (Hogstrand *et al.*, 1996; Wood *et al.*, 1996b). This ability to induce MT up to 400% above control concentrations may explain why bioaccumulated Ag is not acutely toxic. Once Ag accumulates in the tissue it is believed to directly interact with the transcriptory machinery of the rainbow trout MT-A gene to induce MT production (Mayer *et al.*, 1996). Exposure of juvenile rainbow trout for 7 days to waterborne Ag at 9.3 µg/L (as $AgNO_3$) increased the hepatic MT level by 80%, or about 40 µg/g (Hogstrand *et al.*, 1996). The present study is the first to demonstrate that almost all of the cytosolic ^{110m}Ag and 25-50 % of the ^{110m}Ag in the liver is bound to MT *in vivo*. This relatively high capacity of the liver to sequester Ag in MT likely offers protection against silver-induced hepatotoxicity. On the other hand, our results suggest that up to 75 % of the hepatic ^{110m}Ag was present in other fractions of the liver, known to contain less MT than the cytosol. The possibility exists that Ag is toxic in subcellular compartments other than the cytosol. In the gill, for example, Ag acts by blocking the Na/K-ATPase that sits in the basolateral membrane, and would be expected to separate to the microsomal "mic" fraction.

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Table 1. Total silver concentration in tissues of juvenile rainbow trout (n = 10) on days 1, 4, 8, 14 and 19 of the post-tracer period. Total Ag concentrations are expressed as mean values of µg Ag per gram wet weight ± S.E.

	Days				
Tissue	***1***	***4***	***8***	***14***	***19***
Gill	0.30 ± 0.07	0.20 ± 0.04	1.31 ± 0.42	0.17 ± 0.06	N.A.
Intestine	0.57 ± 0.13	0.73 ± 0.60	6.71 ± 2.94	0.35 ± 0.06	2.23 ± 1.18
Liver	8.88 ± 2.09	9.06 ± 3.27	9.28 ± 1.34	8.55 ± 0.74	N.A.
Kidney	0.92 ± 0.18	0.49 ± 0.15	0.58 ± 0.10	0.75 ± 0.17	1.01 ± 0.20
Brain	0.08 ± 0.06	0.10 ± 0.03	0.10 ± 0.05	0.20 ± 0.14	0.25 ± 0.01
Plasma	0.02 ± <0.01	0.03 ±0.01	0.12 ±0.02	0.04 ± 0.02	N.A.

Table 2. Whole body ^{110m}Ag concentrations and contents of rainbow trout on days 1, 4, 8, 14 and 19 of the post-tracer period. Values are expressed as means ± S.E. (n = 6). An exponential growth curve was used to obtain nominal fish weights[1]. The nominal fish weight derived for each sampling day was multiplied with its respective measured CPM per gram value[2] to calculate normalized total ^{110m}Ag contents (in counts per minute per whole fish)[3] in order to compensate for growth and size variability. There were no significant differences in normalized ^{110m}Ag content in the rainbow trout over time.

Days	*Nominal fish weight (g)*[1]	*Fish [^{110m}Ag] (CPM/g)*[2]	*Normalized total ^{110m}Ag Content (CPM)*[3]
1	9.5	2967 ± 380	30178 ± 3866
4	10.1	1811 ± 386	19121 ± 4072
8	10.7	3056 ± 328	33983 ± 3646
14	11.8	2126 ± 292	25868 ± 3554
19	12.7	1782 ± 482	23547 ± 5653

Table 3. Bioconcentration factors (BCF) of tissues from juvenile rainbow trout (n =10) and whole fish (n = 6) on days 1, 4, 8, 14 and 19 of the post-tracer period. BCF values are expressed as the ratio of CPM/g tissue (See Fig. 2) to the measured radioactivity of the water in the two-day ^{110m}Ag pulse (1,200 CPM/mL).

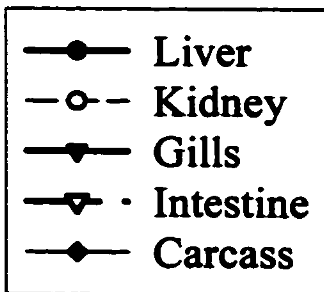
	Days					
Tissue	***1***	***4***	***8***	***14***	***19***	***Mean ± S.E.***
Gill	5.5	3.5	5.3	2.5	N.A.	4.2 ± 0.7
Intestine	102.0	28.8	13.1	2.7	3.0	29.9 ± 18.6
Liver	57.4	56.3	142.6	88.3	76.5	84.2 ± 15.8
Kidney	10.5	7.1	6.8	11.5	6.2	8.4 ± 1.1
Brain	0.6	0.2	1.1	0.8	0.8	0.7 ± 0.1
Plasma	0.9	0.4	1.1	0.4	N.A.	0.7 ± 0.2
Carcass	1.0	0.4	0.6	0.5	0.5	0.6 ± 0.1
Whole Body	2.5	1.5	2.5	1.8	1.5	2.0 ± 0.2

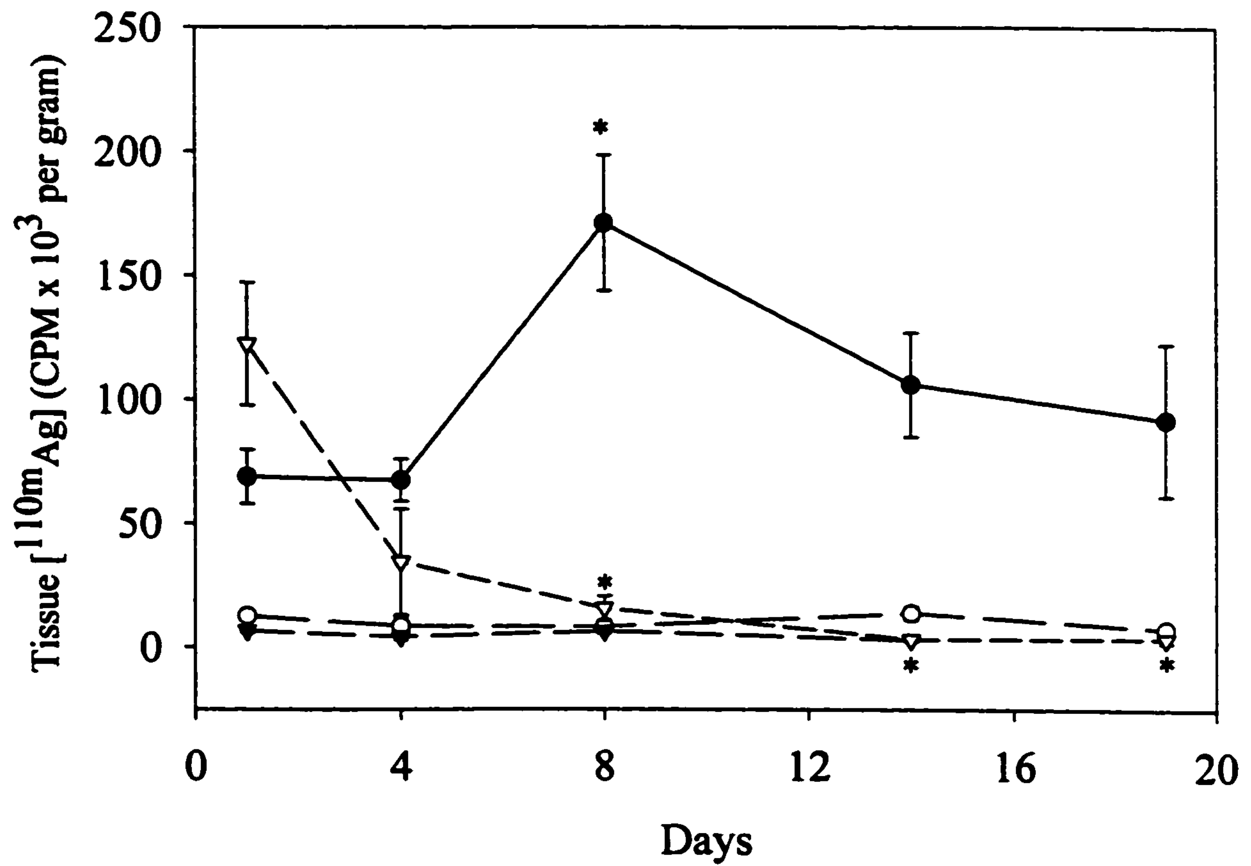
100
80
60
40
20
0

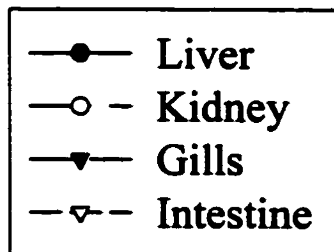
% of total ^{110m}Ag burden

0 4 8 12 16 20

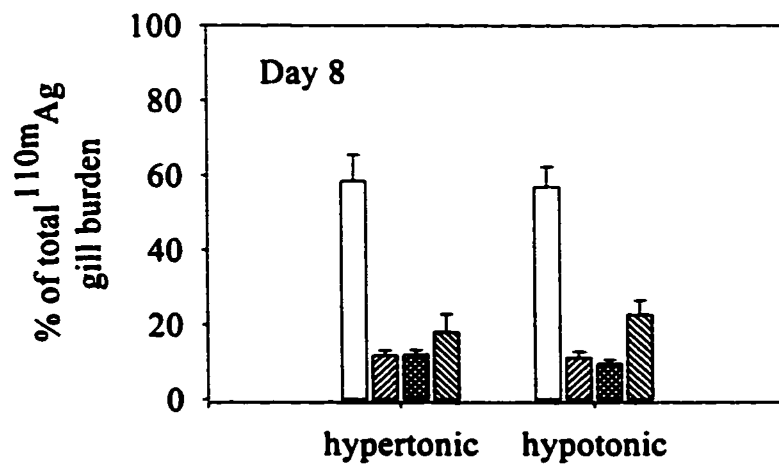
Days



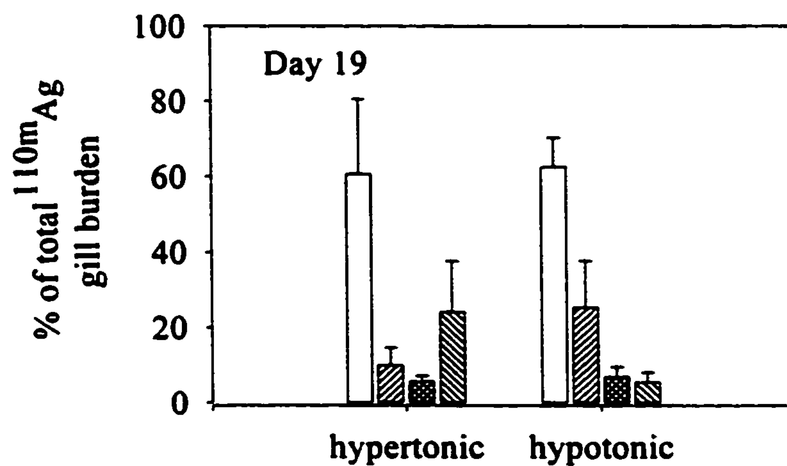

250
200
150
100
50
0
Tissue [^{110m}Ag] (CPM x 10^3 per gram)
0
4
8
12
16
20
Days
*
*
*
*

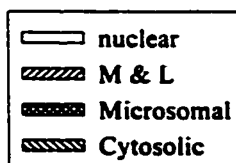

Liver
Kidney
Gills
Intestine

A

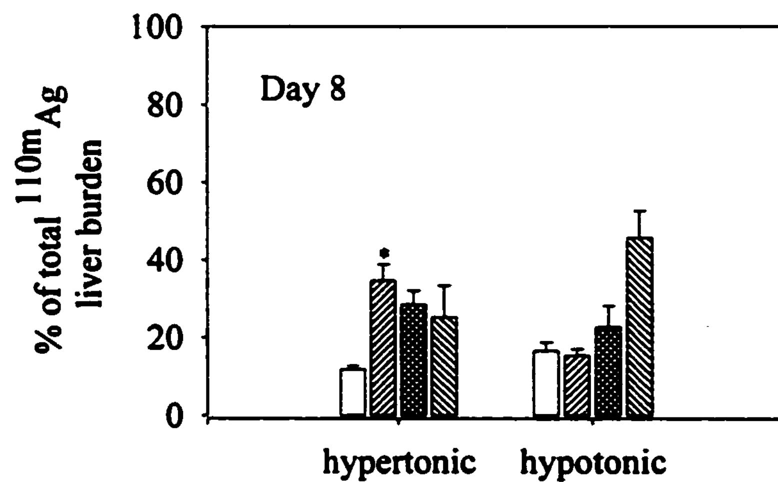


B

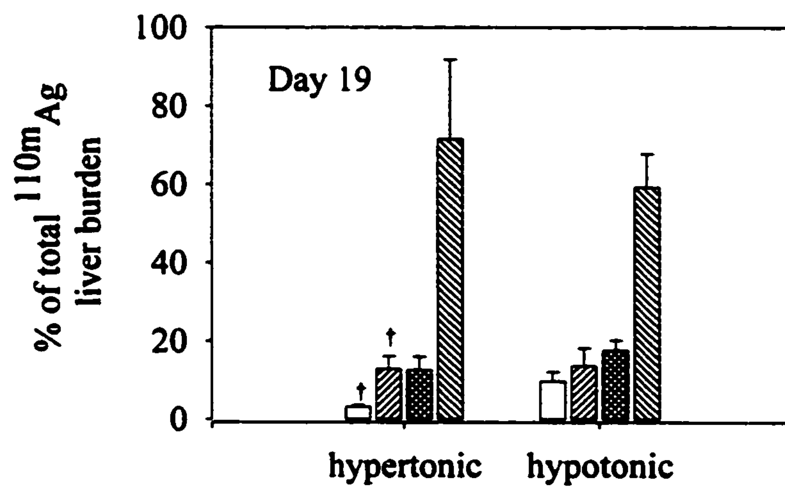


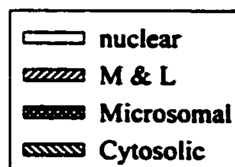


A

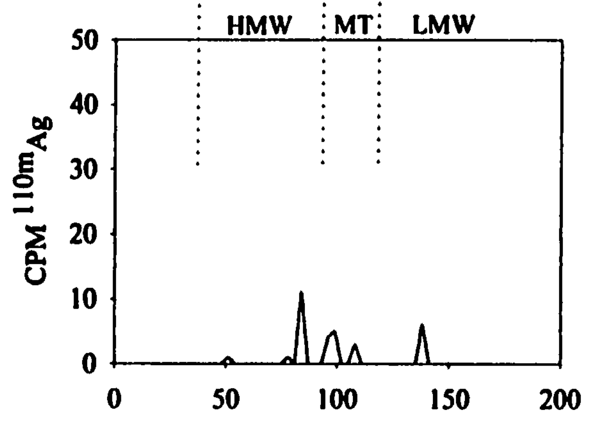


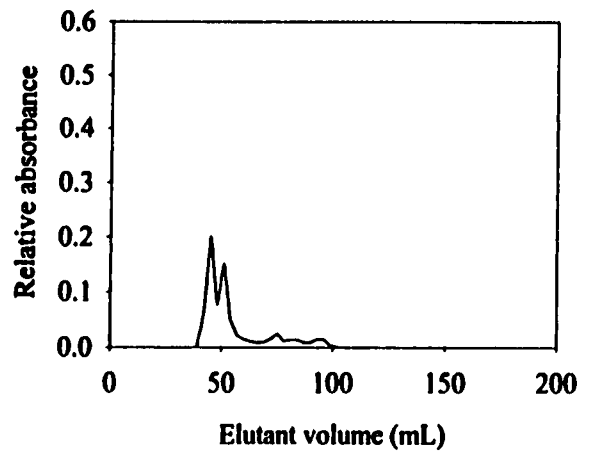
B



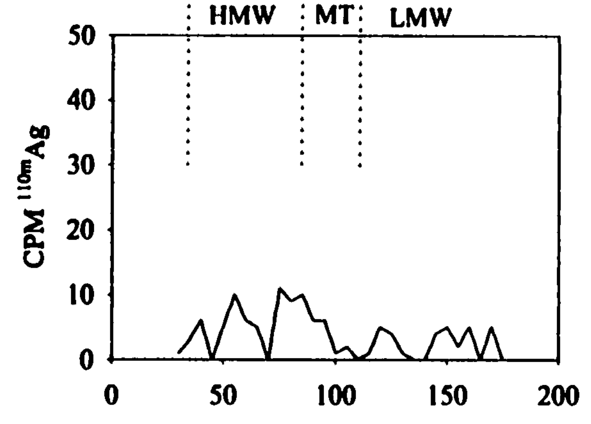


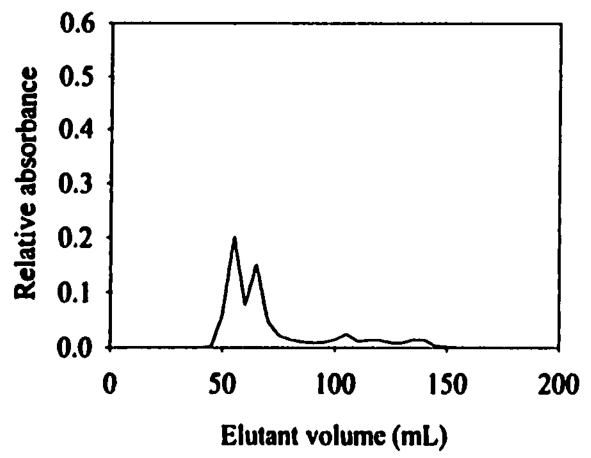
A (Day 8)

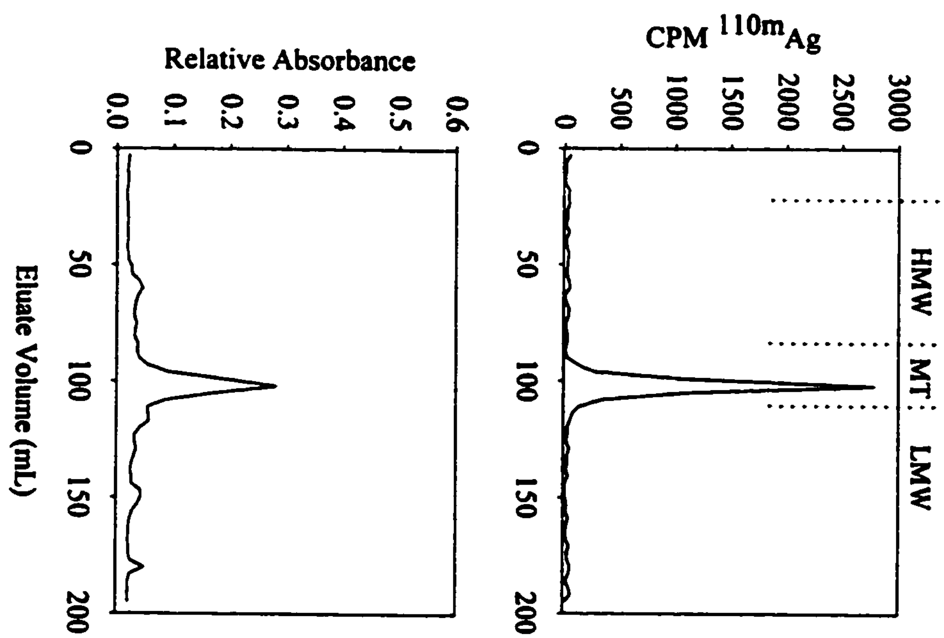


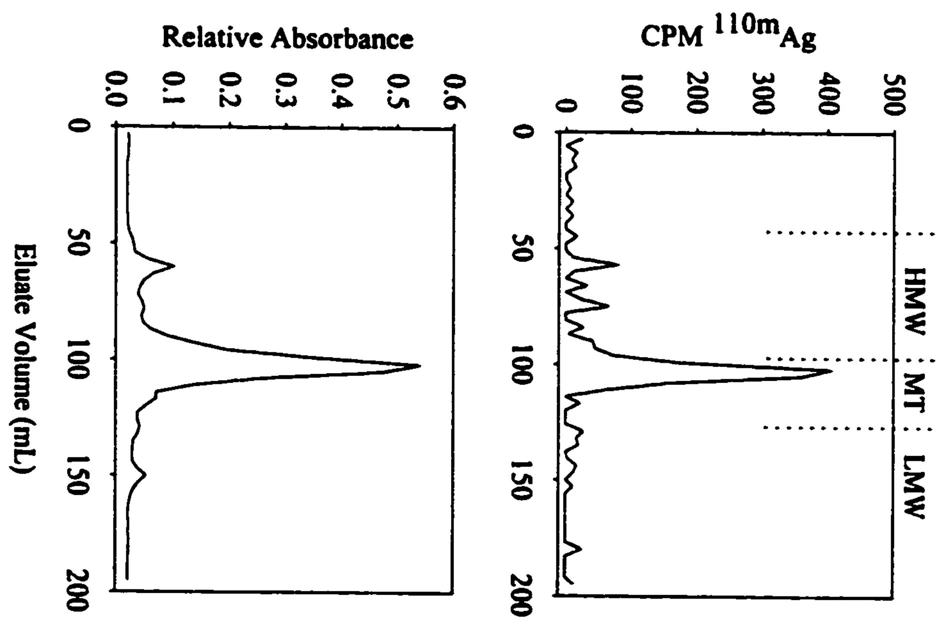


B (Day 19)

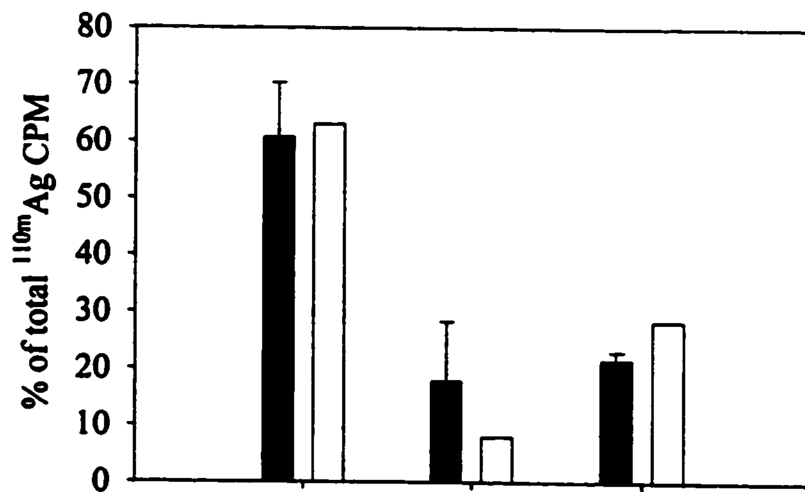




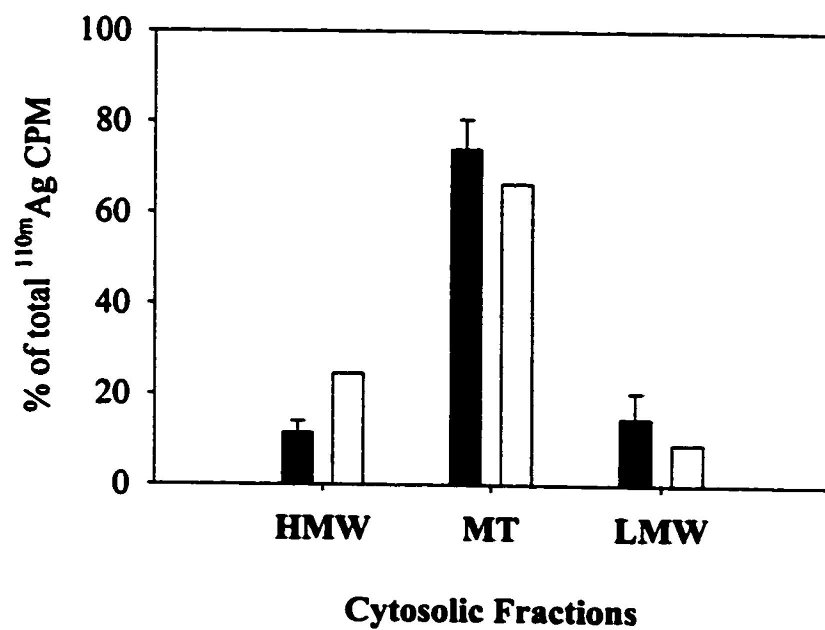

A (Day 8)
CPM 110mAg
0
500
1000
1500
2000
2500
3000
HMW
MT
LMW
0
50
100
150
200
Relative Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Eluate Volume (mL)

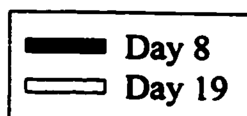

B (Day 19)
CPM 110mAg
0
100
200
300
400
500
HMW
MT
LMW
0
50
100
150
200
Relative Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Eluate Volume (mL)

A. Gills



B. Liver





CHAPTER 8

THE PHYSIOLOGICAL EFFECTS OF DIETARY SILVER SULFIDE EXPOSURE IN RAINBOW TROUT

ABSTRACT

Silver accumulation was examined in juvenile rainbow trout during a 58-day feeding study with silver sulfide (Ag_2S) added to the diet at concentrations ranging from 0 to 3,000 μg/g Ag. Silver in the livers of fish fed the 3,000 μg/g diet reached a level approximately fourfold higher than the control at day 43, representing an initial accumulation rate of 9.5 ng/(g·day). Despite this increase in silver levels in the liver, no influence of liver copper levels was observed. In comparison, there were no significant elevations in silver burdens in the kidneys, gills or intestines, apart from a transient increase in the gills of fish fed the 3,000 μg/g Ag diet ($p < 0.05$), on day 24 only. Daily food consumption rates were lowered by 14 to 22% in all the Ag_2S treatments relative to control levels, possibly because of the decreased palatability of the silver-laden diets. However, there were no significant differences in growth rates between any of the treatments for the duration of the study suggesting lack of any physiological perturbation by dietary Ag_2S exposure. The results of this study suggest that dietary silver sulfide exposure at or below 3,000 μg/g Ag is physiologically benign over a 58-day period.

INTRODUCTION

Approximately 700,000 kg of silver enters aquatic environments in the United States on a yearly basis. Of this amount, 267,000 kg of silver (38%) originates from anthropogenic activities, with photographic processing and photographic manufacturing facilities contributing at least 17% (see Purcell and Peters, 1998). Reportedly, more than 99% of photographic facilities in the United States discharge their effluents into municipal sewers leading to publicly owned treatment works (POTWs) (Versar, 1981). Shafer *et al.* (1998) recently showed that more than 94% of the silver entering POTWs is removed from the effluent before leaving the facilities, a finding consistent with results of previous studies (Lytle, 1984). Silver in photographic industrial effluents is predominantly discharged as soluble, undissociated silver thiosulfate complexes (Lytle, 1984); however, during secondary waste treatment at POTWs, silver thiosulfate is converted to chemically inert silver sulfide (log $K = 19.2$). Because of the low solubility of Ag_2S in water (solubility coefficient of 3×10^{-10} mg/L in natural waters), most of the silver entering POTWs is incorporated into sludge which is later shipped away as solid waste. Discharged silver is largely in the form of colloidal silver sulfide and silver chloride compounds, soluble organic silver complexes, and silver bound to organic particles (Purcell and Peters, 1998). In any case, once in the natural environment, silver is expected to exist in either a colloidal or particulate phase (Wen *et al.*, 1997), and is quickly scavenged by suspended sediments (Adams and Kramer, 1998), ending up in the benthic portion of receiving waters.

In comparison to our considerable knowledge of the magnitude and effects of waterborne silver uptake, relatively little is known about the extent of uptake and toxicity

of silver by means of food in aquatic organisms (Hogstrand and Wood, 1998). Evidence exists that carnivorous fish, fed silver contaminated fish, may accumulate silver via their food (Garnier and Baudin, 1990). In addition, teleost fish may inadvertently ingest silver-laden sediments while feeding on benthic organisms, another source of dietary silver. Although bioaccumulated silver is expected to be bound to sulfhydryl groups of proteins, silver may be further reduced to Ag_2S once internalized. Studies on bivalves suggest that up to 80% of their bioaccumulated silver is associated with sulfides and that only 20% is bound to protein (George *et al.*, 1986). Consequently, either direct or indirect exposure of fish to dietary Ag_2S is of environmental relevance and warrants investigation.

This article presents results obtained during 58-day dietary exposures of juvenile rainbow trout to diets enriched in Ag_2S over a broad range of concentrations. The main objectives were to assess the bioavailability of dietary Ag_2S by measuring tissue burdens of silver in gills, kidney, intestine, and liver and to use growth and feeding rates as indicators of possible physiological effects of these exposures. In addition, copper concentrations in the liver were monitored to determine whether bioaccumulated silver was able to impair the homeostatic control of this biologically essential metal. Previous studies have shown that silver (as $AgNO_3$) can interfere with copper metabolism in rats (Hirasawa *et al.*, 1994). Dietary concentrations used in this study ranged from 3 to 3,000 µg/g Ag (as Ag_2S). Sediment concentrations for rivers receiving industrial effluents (e.g., Genesee River, New York, USA) may be as high as 55.4 µg/g Ag (NYSDEC, 1995); the 3,000 µg/g Ag diet was chosen to represent a worst-case scenario because the concentration is 100 times higher than that found at contaminated sites. Furthermore, the

two lowest dietary concentrations (3 and 30 µg/g Ag) represent levels typically found in many species of freshwater bivalves near sites of sewage outflow (Reviewed in Eisler, 1996).

MATERIALS AND METHODS

Diet preparation

Silver-enriched trout diets were prepared from commercial trout food (Martin's Feed, Tavistock, ON, Canada) pulverized into a fine powder using a household blender. Silver sulfide (99.9%) (Ag_2S, Lot JN 02006 BN, Aldrich Chemical, Milwaukee, WI, USA) oven-dried to a constant weight, was added to the commercial feed to achieve nominal concentrations of 3, 30, 300 or 3,000 mg Ag/kg. This formulation was mixed thoroughly for ~30 min then extruded using a pasta maker and shaped into pellets (average wt = 0.018 g per pellet). The extruded feed was dried at 50°C for ~36 h, sieved using a fine mesh to remove small fragments and refrigerated until use. A control diet was also formulated in the same manner except that no silver diet was added.

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) (n≈500) weighing 2 to 3 g were obtained from Humber Springs Hatchery (Orangeville, ON, Canada). Fish were held for at least 2 weeks in a circular polyethylene tank (~400 L capacity). The tank was supplied with aerated, dechlorinated Hamilton tap water at a flow rate of 1,000 mL/min. The chemical composition of the water was as follows (in mM): Na^+, 0.6; Cl^-, 0.7; Ca^{2+}, 1.0; Mg^{2+}, 0.2; K^+, 0.05; titratable alkalinity to pH 4.0 of 1.0 and pH was 7.8 to 8.0. Water temperature was kept at ambient conditions, which varied between 8 and 13°C. The fish were fed to satiation once daily with commercial trout pellets (Martin's Feed). Food consumption was monitored visually to ensure that all food offered to the fish was consumed; daily consumption in each treatment was recorded.

Experimental design

After acclimation, 90 fish were transferred to each of five rectangular self-cleaning tanks. Each 60-L tank was aerated and supplied with a continuous flow of water at a rate of 500 mL/min. Fish were fed the commercial diet for an additional week, after which the study was commenced by switching to one of the experimental diets. The feeding regime during the experimental phase was identical to that of the acclimation period. Although the tanks were self-cleaning, feces were siphoned daily from each tank after feeding to minimize copraphagy. The daily food consumption (FC) was calculated for each treatment on the basis of the percentage of weight consumed per day. Tank biomass was determined approximately every 2 weeks by placing fish in a large plastic colander, and weighing the colander with and without the fish. Tank biomass (in g) was calculated as the difference between these weights. These data were used to calculate specific growth rates (SGR, in % wet weight). Specific growth rate values were obtained from the slopes of regression lines through the natural logarithm of fish weight versus time. Gross food conversion efficiencies (CEs) were calculated for each treatment using the formula:

$$CE = 100 * (SGR / FC) \qquad \text{eqn. 1.}$$

On days 24, 43 and 58, eight fish per treatment were randomly selected. Fish were killed with a quick blow to the head, dried and weighed, and then the liver, intestine (from the pyloric caeca to the anus), entire gill basket, and kidney were dissected from each fish for silver analysis. Gills were rinsed in 18 MΩ deionized water to remove any loosely bound particulate matter. The intestinal tract was flushed with Cortland saline (Wolf, 1963) and scraped with a fine spatula to remove any undigested feed or feces. All

tissues were blotted dry, weighed, placed in centrifuge tubes, frozen in liquid nitrogen, and then stored at -70°C until further processing.

Gills (0.06-0.37 g), kidney (0.01-0.33 g), intestine (0.01-0.07 g) and liver (0.02-0.33 g) were digested in acid-washed glass test tubes with 2 mL of concentrated trace-metal grade $HN0_3$ (Baker, Toronto, ON, Canada) and 400 μL H_2O_2 (Caledon Chemicals, Georgetown, ON, Canada) at 120 °C. Digests were evaporated without allowing the digested residue to become ashed. Tissues were then reconstituted with 2 to 5 mL of 0.5% HNO_3 (exact volume noted) and analyzed for silver by graphite furnace atomic absorption (Varian GTA-95). In addition, a subsample of the reconstituted liver digest was used for analysis of copper in liver by flame atomic absorption spectrophotometry (Varian AA 1275).

Specific growth rates are expressed as the percentage of weight per day. Values between treatments were statistically compared using an analysis of covariance in JMP 3.1 (SAS Institute, Cary, NC, USA). Tissue metal burden levels and food consumption rates for each treatment are expressed as mean ± SE. Mean values were statistically compared using a one-way analysis of variance in the SPSS (Version 8) statistical package. This was followed by the Tukey-Kramer post hoc test which allow for multiple comparisons between treatments. The level of statistical significance for all analyses was $p < 0.05$.

RESULTS

Food consumption rates were monitored in each dietary Ag_2S treatment and the relevant control group. Food consumption rates decreased between 14 and 22% in fish fed the silver-laden diets when compared to the control. However, apart from the 3 µg/g Ag group, these reductions in food consumption were not statistically significant (Table 1). No significant differences in SGR were observed among groups, and no clear trend was noted between the level of Ag_2S in the diet and mean growth rates. Similarly, food conversion efficiencies were not affected in a dose-dependent manner (Table 1).

No significant differences were observed in the concentrations of silver accumulated in the intestinal tissue among any of the fish fed the silver-enriched diets and control. Intestinal silver concentrations typically ranged between 0.10 and 0.30 µg/g Ag in all of the treatments, except in fish fed the 3,000 µg/g Ag diet, which had a mean measured value of 1.80 µg/g Ag by day 58 (Table 2). Despite a sixfold increase in intestinal silver concentration for this group (above control levels), the elevation was not statistically significant because of large variability among individuals.

In comparison, control silver levels in gills were one order of magnitude lower than in the intestine. Silver burdens in gill in the 3,000 µg/g Ag group were significantly elevated ($p<0.05$) compared to all other treatments (except the 300 µg/g Ag treatment) on day 24, but not on day 43 of the experiment (Table 3). Fish fed the 3,000 µg/g Ag diet exhibited moderate yet significant ($p<0.05$) accumulation of silver in their livers only at day 43. Hepatic silver concentrations for this treatment (0.52 ± 0.09 µg/g Ag) were approximately fourfold higher than control levels (Fig. 1). However, by day 58, silver levels in the livers of fish fed the control and 3,000 µg/g Ag diets were not significantly

different. Hepatic copper levels were also monitored to assess whether accumulated silver disrupted regulation of this essential metal. Figure 2 shows steady accumulation of hepatic copper between days 24 and 58 in all treatments, regardless of the silver concentration in the diet. Apart from a twofold elevation on day 43 in hepatic copper levels in fish fed the 300 µg/g Ag diet relative to controls, there appears to be no significant effect of dietary silver on copper levels in the liver. In addition, silver concentrations in the kidney showed no clear trend throughout the 58-d exposure, although silver levels in the kidneys of fish fed the 3,000 µg/g Ag diet tended to be higher (0.49 ± 0.24 versus 0.08 ± 0.01 µg/g in control) (Fig. 3). Kidney silver concentrations were elevated in all treatments above 30 µg/g Ag, (compared to controls) on day 24, although this increase was not significant. However, kidney silver levels for fish fed the 30 and 300 µg/g Ag diets decreased thereafter.

DISCUSSION

Previous studies have shown that various species of waterborne silver are able to accumulate in tissues, especially the liver, at extremely high rates. In a recent review, Hogstrand and Wood (1998) tabulated silver concentration specific accumulation rates (CSAR) (in ng/(g · d · ppb)) in the livers of juvenile trout during a variety of waterborne silver exposures. By normalizing for both silver concentration and duration of exposure, they were able to rank the bioavailability of different waterborne silver species. They found that juvenile trout had CSARs as high as 2,800 ng/(g · d · ppb) for $AgNO_3$ exposures in freshwater. However, if acutely exposed to elevated concentrations of negatively charged $AgCl_n^{1-n}$ (n > 2) or $Ag(S_2O_3)_n^{n-}$ (n > 1) complexes, CSAR values decreased significantly to between 0.34 to 0.38 ng/(g·d·ppb). They suggested that both negatively charged silver species and highly complexed silver (i.e., thiosulfates; log K = 8-14) were less bioavailable in water than easily dissociable forms of silver (i.e., $AgNO_3$).

The present study was designed to address whether silver, via dietary Ag_2S exposure, is bioavailable to freshwater teleost fish. It was observed that silver levels in the livers of fish fed the 3,000 μg/g diet were approximately fourfold higher than control levels by day 43, representing an accumulation rate of 9.5 ng/(g·d). However, if normalized for silver level in the diet, the hepatic CSAR value for dietary Ag_2S exposure was only $3.2 \cdot 10^{-6}$ ng/(g·d·ppb). In comparison, we have recently showed that juvenile trout exposed to a dietary exposure of biologically incorporated silver (~3 μg/g Ag) exhibited liver silver concentrations 12-fold higher than those of controls after 3 months (Galvez *et al.*, 1996). This represents a CSAR of 0.18 ng/(g·d·ppb). It can therefore be

concluded that the bioavailability of dietary silver is dependent on speciation, similar to the situation seen with waterborne silver exposures. Ag_2S, with a log K of 19.2, is probably not dissociated within the gut lumen during digestion, thus preventing internalization of the metal. This is further substantiated considering the low rates of accumulation of silver in the kidney, in response to dietary Ag_2S exposure. Recently, a 28-d bioaccumulation study was performed in the oligochaete *Lumbriculus variegatus* exposed to laboratory-spiked sediments containing approx. 444 mg Ag/kg dry weight (as Ag_2S) (Hirsch, 1998). Hirsch reported tissue silver levels of 80.3 µg/g dry weight at the end of the test, representing a CSAR of only $6.4 \cdot 10^{-3}$ ng/(g·d·ppb).

The elevated levels of silver in the intestine (3,000 µg/g Ag diet only) are likely due to adsorption of Ag_2S at the apical membrane of the gut epithelium. This outer integument is continually sloughed off as a result of the movement of food through the gut lumen. Consequently, adsorbed silver at the gut epithelium, is not expected to be of physiological relevance to the fish. In contrast, biologically incorporated silver is likely bound to proteins. During digestion, proteases should break down these proteins to form amino acid-silver complexes, which may be absorbed via specific amino acid transporters (Storelli *et al.*, 1989). This uptake route may explain the higher accumulation rate of biologically incorporated silver seen by Galvez *et al.* (1996), compared with dietary Ag_2S.

No significant differences were observed in growth rates of fish fed either the control diet or diets with Ag_2S levels as high as 3,000 µg/g Ag suggesting lack of any physiological perturbation, which is consistent with results of previous studies (Galvez *et al.*, 1996; Hirsch, 1998). The 14 to 22% decrease in food consumption between the

control and Ag_2S dietary treatments may have been due to a decreased palatability of the silver-laden diet. However, despite these slight decreases in food consumption no significant effects on SGR values were seen. As a result, the gross conversion efficiencies of fish fed the 30 and 3,000 μg/g Ag diets increased slightly compared with the control treatment. This is consistent with growth-ration curves, which typically show that SGRs begin to plateau before maximal ration levels are reached. The sum of these effects (further elevation in food consumption with no additional increase in specific growth rate) explains why gross conversion efficiencies tend to decrease as daily food consumption rates approach satiation (Brett, 1979). In a study in which brown trout (*Salmo trutta*) were fed a daily ration (5% daily) of juvenile carp pre-exposed to 4.3 μg/L ^{110m}Ag (as AgCN) for 3 d, no significant effects on growth rates were observed (Garnier and Baudin, 1990). Similarly, preliminary results from a 4-month study in which juvenile trout were fed biologically incorporated silver (Galvez *et al.*, 1996) suggest that dietary silver exposure is physiologically benign.

Acknowledgement

This work has been funded by Kodak Canada and the Natural Sciences and Engineering Research of Canada Industrially-Oriented Research Program.

Table 1. Mean daily food consumption, specific growth rates, and food conversion efficiencies for juvenile trout fed diets with silver concentrations ranging from 0 to 3,000 μg/g (as Ag_2S)[a].

	Silver concentration (μg/g wet weight)				
	Control	*3*	*30*	*300*	*3,000*
Food consumption,[b]	2.82 ± 0.16 AB	2.22 ± 0.13 B	2.42 ± 0.14 AB	2.26 ± 0.18 AB	2.33 ± 0.16 AB
Specific growth rate,[c] *% wet weight/d*	1.37 ± 0.35	1.05 ± 0.31	1.81 ± 0.39	1.05 ± 0.30	1.47 ± 0.38
Food conversion efficiency %	48.6	47.3	74.8	46.5	63.1

[a]Sample size used to derive these data decreased throughout the experiment because of fish sampling.

[b]Significant differences ($p<0.05$) in food consumption among treatment means exist wherever common letters are not shared.

[c]No significant differences in specific growth rate were observed among treatments.

Table 2. Mean intestinal silver concentrations silver concentrations (± SE) ($n = 8$) of juvenile trout on days 24, 43 and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 µg/g (as Ag_2S).[a]

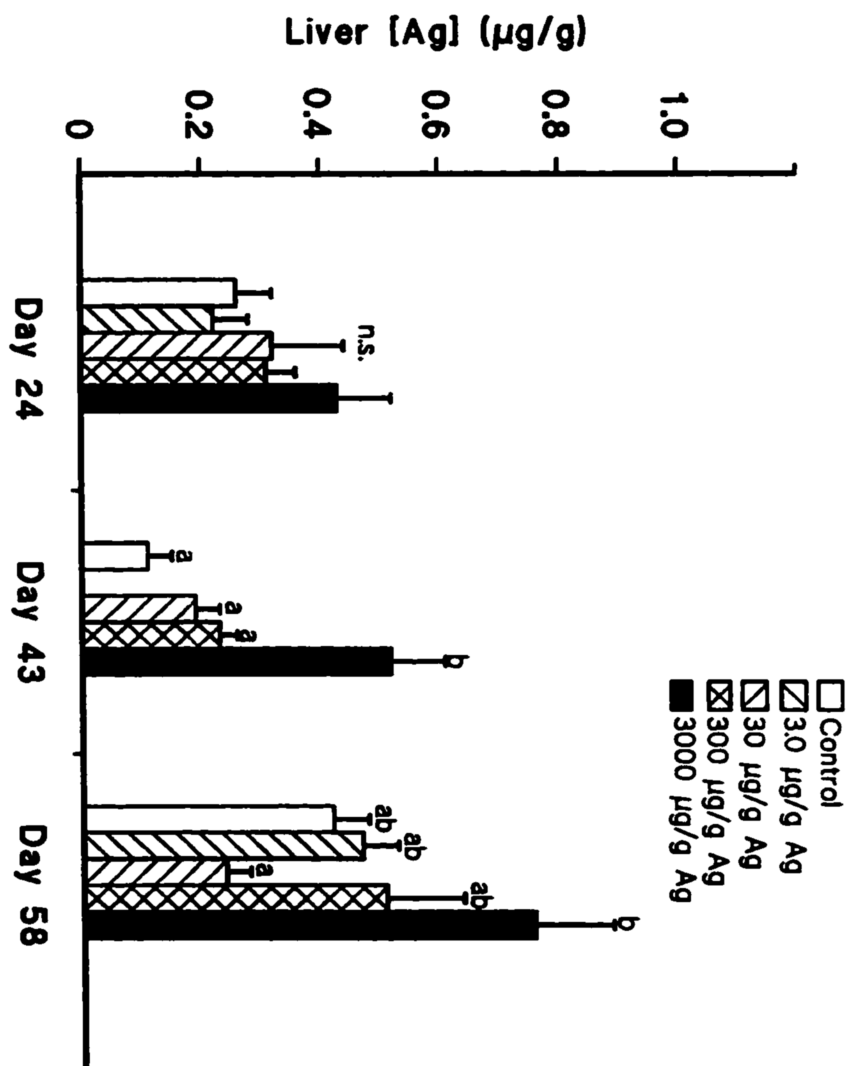
	Silver concentration (µg/g wet weight)				
	Control	*3*	*30*	*300*	*3,000*
Day 24	0.26 ± 0.10	0.12 ± 0.02	0.13 ± 0.03	0.15 ± 0.02	1.24 ± 0.65
Day 43	0.24 ± 0.04	0.17 ± 0.03	0.19 ± 0.04	0.30 ± 0.14	0.38 ± 0.14
Day 58	0.11 ± 0.01	0.10 ± 0.02	0.61 ± 0.43	0.19 ± 0.06	1.80 ± 1.38

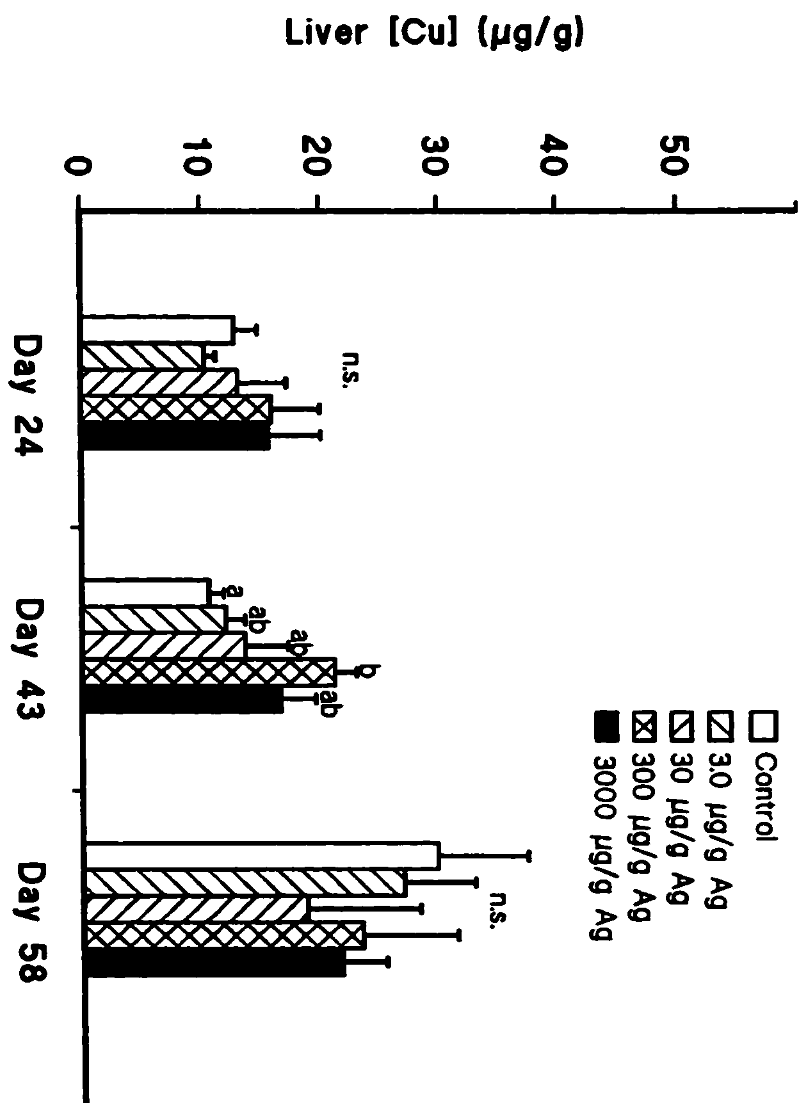
[a]No significant differences among treatments ($p < 0.05$).

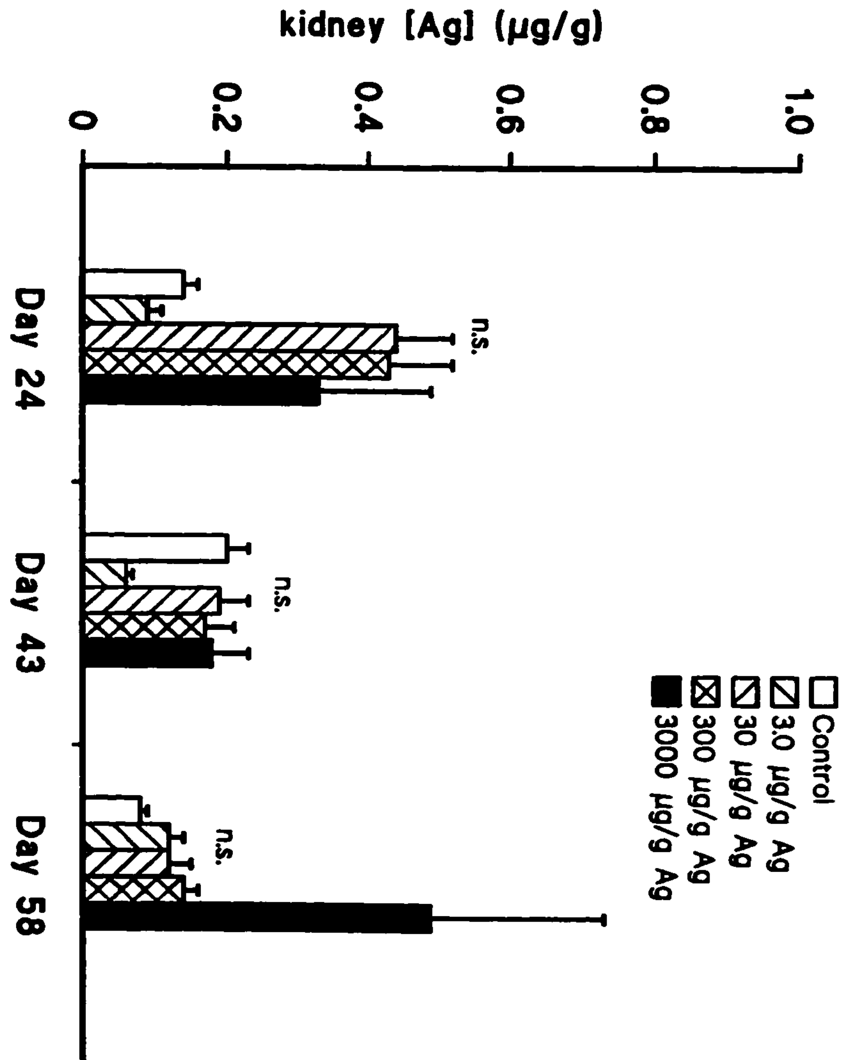
Table 3. Mean gill silver concentrations (± SE) ($n = 8$) of juvenile trout on days 24, 43 and 58 after consuming diets with silver concentrations ranging from 0 to 3,000 µg/g (as Ag_2S).[a]

	Silver concentration (µg/g wet wt.)				
	control	*3*	*30*	*300*	*3,000*
Day 24	0.054 ± 0.005 A	0.431 ± 0.252 A	0.101± 0.059 A	0.673 ± 0.374 AB	2.470 ± 1.200 B
Day 43	0.060 ± 0.013	0.053 ± 0.007	0.126 ± 0.030	0.139 ± 0.050	1.043 ± 0.922

[a]Significant differences ($p < 0.05$) among treatment means exists wherever common letters are not shared. No significant differences were observed between treatments on day 43.


Liver [Ag] (µg/g)
0
0.2
0.4
0.6
0.8
1.0
Day 24
Day 43
Day 58
n.s.
a
b
ab
Control
3.0 µg/g Ag
30 µg/g Ag
300 µg/g Ag
3000 µg/g Ag


Liver [Cu] (µg/g)
0
10
20
30
40
50
n.s.
a
ab
ab
b
ab
n.s.
Day 24
Day 43
Day 58
Control
3.0 µg/g Ag
30 µg/g Ag
300 µg/g Ag
3000 µg/g Ag


kidney [Ag] (µg/g)
0
0.2
0.4
0.6
0.8
1.0
Day 24
Day 43
Day 58
n.s.
n.s.
n.s.
Control
3.0 µg/g Ag
30 µg/g Ag
300 µg/g Ag
3000 µg/g Ag

CHAPTER 9

THE PHYSIOLOGICAL EFFECTS OF A BIOLOGICALLY INCORPORATED SILVER DIET IN RAINBOW TROUT (*Oncorhynchus mykiss*)

ABSTRACT

Silver was biologically incorporated into a diet by exposing rainbow trout for 7 days to 100 mg/L of waterborne silver as silver thiosulfate. These fish were processed into a fine powder (trout meal) and pelleted to form a nutritionally balanced feed which was then fed to juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were fed either a diet containing 3.1 µg/g biologically incorporated silver (an environmentally relevant concentration), or one of three control diets containing approximately 0.05 µg/g Ag, for 128 days. All dietary treatments were fed to satiation once daily. Dietary silver did not significantly affect mortality, growth, food consumption, or food conversion efficiency. Furthermore, ion regulation (plasma Na^+ levels and Na^+ influx rates), hematological parameters (hematocrit, plasma protein, hemoglobin levels), plasma glucose, metabolism (oxygen consumption, ammonia and urea excretion rates) and intestinal Na/K-ATPase and amylase activities were all unaffected. Based on the physiological parameters investigated here, this dietary silver exposure appeared to be physiologically benign to rainbow trout. However, silver concentrations in the livers of the silver-fed fish were significantly elevated at day 16, and reached a steady-state level of ~20 µg/g by day 36.

The Concentration Specific Accumulation Rate in the livers of fish fed biologically incorporated silver was about 4.6 orders of magnitude greater than when fed dietary silver sulfide (Galvez and Wood, 1999), indicating much greater bioavailability. Despite this increase, hepatic metallothionein concentrations remained unchanged, in contrast to waterborne exposures (Hogstrand *et al.*, 1996; Galvez *et al.*, 1998), indicating that bioaccumulated silver behaves differently depending on whether it is taken up from the diet or from the water. Apart from a significant reduction in hepatic Cu at day 16, liver concentrations of Cu and Zn were not affected by dietary silver. Silver concentrations were also significantly elevated (relative to control fish) in the kidneys of the silver-treated fish on days 88 and 126, and in the gills and plasma at day 126.

INTRODUCTION

Since the early 1990s, a concerted effort has been made to understand the physiological mechanisms associated with acute silver toxicity to aquatic organisms. This research demonstrates that the free Ag^+ ion probably is the primary toxic moiety to freshwater fish (Wood *et al.*, 1996a; Galvez and Wood, 1997; Bury *et al.*, 1999a). During waterborne exposures, Ag^+ acts as a surface-active toxicant, eliciting its primary effect at the gill epithelium (Janes and Playle, 1995; Morgan *et al.*, 1997; Hogstrand and Wood, 1998; Wood *et al.*, 1999). Nevertheless, various forms of silver, although seemingly non-toxic in an acute sense, are able to accumulate readily in freshwater fish (Hogstrand *et al.*, 1996; Wood *et al.*, 1996b; Hogstrand and Wood, 1998). The most explicit examples are seen in freshwater fish exposed to Ag thiosulfate complexes. On the one hand, waterborne Ag thiosulfate was found to be at least 4-5 orders of magnitude less toxic than $AgNO_3$ during routine acute toxicity tests. On the other hand, when presented as Ag thiosulfate, Ag accumulated readily in the gills and internal tissues such as the liver. In fact, rainbow trout exposed to ~100 mg/L Ag (as $Ag(S_2O_3)_n$) accumulated ~900 μg/g of Ag in the liver, resulting in a 300 % increase in hepatic metallothionein concentration (Hogstrand *et al.*, 1996).

This is environmentally relevant because up to 99 % of the photographic facilities in the United States discharge their effluents into municipal sewers as soluble silver thiosulfate complexes, accounting for up to 44 % of the total anthropogenic discharge to the aquatic environment (Lytle, 1984; Purcell and Peters, 1998). However, concentrations of silver in the water column near U.S. urban centers are extremely low, ranging from 0.01-0.1 $\mu g \cdot L^{-1}$ (Shafer *et al.*, 1998). At these low concentrations of silver,

acute toxicity to aquatic organisms is not expected, but silver might enter the food chain. The majority of the silver accumulates in sediment either as silver sulfide or bound to particulate or colloidal materials (Purcell and Peters, 1998). Silver bound within sediments can be potentially taken up by benthic invertebrates and then be passed to higher trophic levels (Ratte, 1999). The concentrations of silver in tissues of aquatic plants and animals collected near industrialized sites are typically elevated, implying that environmental silver is bioavailable to aquatic organisms (reviewed in Eisler, 1996). Silver concentrations in bivalves living in contaminated environments may exceed 100 µg/g dry weight (~ 20 µg/g wet weight) (Luoma and Phillips, 1988), whereas silver burdens in freshwater fish have been reported up to 1.9 µg/g wet weight (U.S. Public Health Service, 1990).

A recent interdisciplinary workshop recommended that criteria to protect aquatic ecosystems from metal pollution must in future incorporate a food chain component to account for all routes of exposure to aquatic organisms (Bergman and Dorward-King, 1997). However at present, only a few studies have addressed the trophic transfer of silver to freshwater fish under laboratory conditions (Terhaar *et al.*, 1977; Garnier and Baudin, 1990; Galvez and Wood, 1999). Using mathematical simulations on a simplified 'water-carp-trout' food chain, Garnier and Baudin (1990) concluded that the trophic route would represent the primary source of metal uptake into the organism after only 13 days of continuous exposure to both waterborne and dietary silver in the form of ^{110m}Ag labelled AgCN at 4 µg/L in the original water to which both species were exposed. In contrast, Galvez and Wood (1999) found that food spiked with silver sulfide to levels up to 3,000 µg/g did not result in significant silver accumulation by rainbow trout and

produced no deleterious physiological effects over 58 days of exposure. Only the latter study has attempted to address the physiological effects (other than metal bioavailability) of food chain transfer of silver.

The primary objective of this study was to assess the growth, metabolism and physiology of juvenile rainbow trout fed a diet containing biologically incorporated silver over 126 days. Silver was incorporated into the feed by initially exposing trout (destined to become food) to silver thiosulfate via the water, and then processing these "prey" trout into a commercial grade feed. This is in contrast to the majority of dietary studies on metals performed to date, which have instead relied on direct addition of metal salts to yield contaminated diets. Harrison and Curtis (1992) point out that naturally contaminated foods tend to have higher absorption efficiencies than superficially contaminated diets. This statement is supported by the profound effects seen in rainbow trout fry fed with metal-laden invertebrates collected from the field (Woodward *et al.*, 1994). Additional dietary treatments in the present study were implemented to monitor any effects due to the thiosulfate ion and quality of our synthetic diets. The quality and palatability of our homemade diets (using synthetic trout meals) were tested against a control diet made with commercial herring meal. Each of the additional control dietary treatments was pair-fed at the same ration as consumed by the fish fed the silver-contaminated diet. Pair fed controls tested whether potential physiological effects were due to the dietary silver exposure, or simply a result of differences in feed intake between treatments (Lanno, 1989).

MATERIALS AND METHODS

Fish husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*) (n = ~1100) weighing 5-10 g were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and transported to McMaster University, Hamilton, ON. Upon arrival to the laboratory, fish were placed in a 400-L tank, mixed and then randomly divided amongst five well-aerated 400-L holding tanks. Fish were held for 3 weeks before use in the experiment. Each tank was supplied with 1.5 $L{\cdot}min^{-1}$ of Hamilton dechlorinated tap water. The chemical composition of the water was as follows (in mM): Na^{+} = 0.6; Cl^{-} = 0.7; Ca^{2+} = 1.0; titratable alkalinity to pH 4.0 of 1.0 mM; and pH 7.8-8.0. Water temperature was maintained at ambient conditions, which ranged from 5-11 °C over the course of the study. Fish were fed commercial trout pellets (Martin's Feed, Elmira, ON) until the start of the study, at which time they were switched to one of the prepared diets (See Preparation of "homemade" trout meals section). Fish were fed once daily to satiation during both the acclimation and experimental phases of the study.

In order to distinguish between treatments in tests in which animals were exposed together, each group of fish was uniquely marked with Alcian Blue dye using a Panjet injector (Wright Health Group; Dundee, Scotland). During the marking procedure, fish were lightly anaesthetized with 0.1 gL^{-1} MS-222 buffered with 0.2 gL^{-1} sodium bicarbonate, and allowed to recover in fresh water before being placed back in the holding tanks. All fish were marked two weeks before the start of the study.

Preparation of "homemade" trout meals

Adult rainbow trout (n =120) (Humber Springs Trout Hatchery; average weight ~ 300 g) were randomly selected and transferred in equal numbers (40 fish per tank) to three well-aerated 400-L tanks served with dechlorinated Hamilton tap water. Fish were allowed to acclimate to these holding tanks for two days. At the start of the waterborne exposures, water flow was turned off to each tank and one of three water chemistries was assigned to each. These water chemistries include:

(1) Silver diet. Trout were exposed to waterborne silver at 100 µg/L (as silver thiosulphate; 0.37 $mmol \cdot L^{-1}$) for 7 days. The Ag thiosulphate solution was made by reacting on a molar basis, 1 part AgCl (0.37 $mmol \cdot L^{-1}$) (Johnson Matthey Ltd, Brampton, ON) to 4 parts $Na_2S_2O_3$ (1.48 $mmol \cdot L^{-1}$) (BDH).

(2) Control diet. Trout were exposed to Hamilton dechlorinated tap water for 7 days.

(3) Thiosulfate diet. Trout were exposed to waterborne thiosulfate (1.48 $mmol \cdot L^{-1}$ $Na_2S_2O_3$) (the same concentration of thiosulphate used during the Ag thiosulphate exposure).

Every 24 hours, 50 % of the water from each tank was removed and replaced with 200-L of fresh Hamilton dechlorinated tap water, and the appropriate amount of silver thiosulfate or sodium thiosulfate stock was added to tanks 1 and 3. Waterborne exposures lasted 7 days. Following the seventh day, fish were sacrificed by a quick cephalic blow and the whole fish carcass was portioned into small pieces (~2 cm x 2 cm). Fish chunks were placed in a plastic bag and kept refrigerated until further processed at

the University of Guelph the following day. In total, 10 kg of fish per diet were required to produce sufficient quantities of feed.

Formulation and steam-pelleting of diets

All experimental diets were formulated in the Department of Animal Science and Nutrition at the University of Guelph, using the ingredients listed in Table 1. Each diet was identical, except for differences in the trout meal and herring meal portions. Three of the experimental diets consisted of 45 % fish meal (25 % "home-made" trout meal plus 20 % commercial herring meal (Wedgeport Enterprises Ltd, Yarmouth, NS, Canada) as filler. The remaining 55 % of the mixture consisted of corn gluten meal (Martin Feed Mills), wheat middlings (Martin Feed Mills), vitamin premix (Vit-9408), mineral premix (Min-9504) and fish oil (Martin Feed Mills) (Table 1). The vitamin and mineral premixes were donated by Dr. C.Y. Cho from the Department of Animal Sciences and Nutrition at the University of Guelph. In brief, fish chunks were dried in an oven at ~48 °C for 48 hours. After drying, carcasses were mixed with an equal proportion (on a weight basis) of wheat middlings and ground up into a fine powder using a household blender. The remaining ingredients of the diets were added to the fine powder mixture and mixed for 30 minutes. Diet formulations were then steam-pelleted to produce a commercial-grade pellet. All diets were sieved using a 2 mm sieve to remove any fine particulate material. An additional diet was prepared using only commercial herring meal (herring diet) as the 45 % fish meal component. This feed was incorporated into the study as a benchmark against which to evaluate the palatability of the diets made with trout meal.

Experimental protocol

Feeding regime, growth and food consumption

At the start of the study, each treatment was switched to one of the experimental diets. In total, there were five experimental treatments. Two groups were fed either the control or Ag diets to satiation daily, referred to as the control satiation and Ag satiation treatments, respectively. Satiation was determined using the protocol of Wilson and Wood (1992) and Galvez *et al.* (1998). In brief, small amounts of food (~2 g) were lightly sprinkled on the water surface of each tank every minute. Food was distributed to tanks at one-minute intervals. This process was continued until food was found floating at the surface after the one minute period. Feeding was stopped for one minute. If the administered food had been subsequently consumed, feeding was continued as normal. Satiation was reached once food remained uneaten on the water surface for more than 2 minutes. The amount of food consumed was recorded for both the control and silver satiation treatments daily. Three additional treatments were either fed the control, thiosulphate or herring diet. These groups served as pair-fed controls, fed the same ration on a per body weight basis as the dietary silver treatment. The ration of food consumed at satiation was expressed on a $\%\cdot day^{-1}$ basis, calculated by taking the average amount of food consumed per fish, divided by the mean fish weight and multiplied by 100. Mean fish weights were recorded approximately every two weeks by bulk-weighing all of the fish from each tank. Fish from each treatment were removed from their tanks (one tank at a time) and placed in a bucket containing water and a sieve insert. The total biomass of each tank was calculated from the difference between the mass of the bucket, water and sieve, with and without the fish. Mean fish weight in grams was calculated by

dividing the number of fish in each treatment into the total biomass. Specific growth rates (SGR) were determined by least-squares linear regression (SPSS Version 8.0) through the natural logarithm (ln) of mean fish weight versus time data and expressed on a $\%\cdot day^{-1}$ basis. Food-conversion efficiency (FCE) (in %) for each treatment is the ratio of mean SGR ($\%\cdot day^{-1}$) and daily food consumed ($\%\cdot day^{-1}$), multiplied by 100.

Five-mL water samples were taken weekly from each tank for analysis of total silver.

Tissue sampling

On days 0, 16, 36, 88 and 126, ten fish per tank were randomly selected and immediately sacrificed by a quick cephalic blow. Blood was taken by caudal puncture using a 1-mL Hamilton syringe pre-rinsed with ammonium heparin (50 $i.u \cdot ml^{-1}$). Blood was centrifuged at 10,000 g for two minutes and plasma was collected and analyzed immediately for total protein. The remaining plasma was frozen in liquid nitrogen and stored at –70 °C, until analyzed for plasma Na^+, Ag and glucose. In addition, entire gill baskets, kidneys, livers, and intestines were excised from fish for silver analysis. Although all treatments were sampled, only the data from control satiation and silver satiation treatments were analyzed for silver and presented here. Gills were rinsed in 18 MOhm double-deionized water to remove any fine particulate matter. The intestinal tract was flushed with Cortland saline and scraped with a fine spatula to remove any undigested feed or feces. All tissues were blotted dry, frozen in liquid nitrogen, and stored at -70 C until processed. Liver digests were also measured for total Cu, Zn and metallothionein concentrations to assess the impact of silver accumulation on metal

metabolism, and intestines collected on day 88 were also analyzed for Na/K-ATPase and amylase activities.

Routine metabolism

Routine "in tank" oxygen consumption, ammonia and urea excretions were measured on days 0, 16, 36, 55, 88 and 126. Each sampling period consisted of four cycles performed at 6-hour intervals over 24 hours. Sampling was begun exactly 1.5 hours after fish feeding. At the beginning of each cycle, the surface of the water of each tank was sealed with a transparent, heavy plastic sheet, and water flow and aeration turned off. A small pump was used to transfer water from the bottom of each tank and reintroduce it to the top of the tank at a rate of 10 L/min via a small connector on the plastic lid. Care was taken to ensure that no atmospheric air was introduced into the system during measurement of routine metabolism. Change in water P_{O2} was measured over a half-hour period by taking 10-mL water samples from each tank at 10-minute intervals. Water was injected into a Cameron E101 oxygen electrode, maintained at the temperature of the experimental tanks, and connected to a Cameron OM-200 O_2 meter. Water P_{O2} levels were not allowed to decrease below 100 torr. In-tank oxygen consumption rate (M_{O2}) was calculated according to the following equation.

$$M_{O2} = \frac{\Delta P_{O2} \cdot \alpha O_2 \cdot vol}{mass \cdot time} \qquad \text{Eqn. 1}$$

where ΔP_{O2} is the average change in P_{O2} (torr) over the 30-minute time period, αO_2 is the solubility constant for O_2 in water at the experimental temperature, vol is the volume of

water in the experimental tanks, and mass is the biomass of the entire treatment group in grams.

Ammonia-N and urea-N excretion rates were measured at the same time as oxygen consumption, except that each cycle was extended to 1.5 hours to allow greater analytical accuracy. Water flow was turned off for the entire cycle, but aeration was reinstated after the 30 minute P_{O2} measurement period. Water samples (2 x 5 mL) were collected from each tank at the beginning and end of the 1.5 hour period, and immediately frozen for later analysis of total ammonia-N and urea-N. After the terminal water samples were taken for N-waste excretion, water flow to each tank was resumed. Half the water volume of each tank was removed, and then the tank was flushed with fresh dechlorinated tap water to help return water ammonia and urea levels to normal, prior to the start of the subsequent cycle. The N-waste excretion rate for each cycle was determined from the difference in N concentration of the water samples taken at the start and end of each cycle. Mean M_{O2} and mean N-waste excretion rates were calculated for each treatment from the four replicate values per sample period. All M_{O2} and N-waste excretion rates were normalized to a weight of one-kilogram using the weight exponent of 0.824 determined by Cho (1990) for rainbow trout. The nitrogen quotient (NQ) was calculated from the ratio of moles of N produced (ammonia-N and urea-N excretion) to moles of oxygen consumed.

Na^+ influx and hematological indices

Unidirectional Na^+ influx rates were measured on days 0, 36 and 126. Tests were performed in a single, plastic bucket containing 40-L of aerated, dechlorinated Hamilton

tap water. Two hours before the start of each flux period, 8 fish per treatment were randomly selected and allowed to acclimate to the flux chamber. At time 0, 40 μCi of ^{22}Na (Mandel Scientific, Guelph, ON) were added to the water and allowed to mix for 5 minutes. Two- 5 mL water samples were taken at 5 minutes, and again at 2, 4 and 6 hours, for analysis of total Na^+ concentration and ^{22}Na activity. After four hours, 5 fish were removed from the flux tank and placed in a 15-L bucket of "clean" dechlorinated water to remove any superficially bound ^{22}Na from the fish. After a few minutes, fish were netted one at a time from the "clean" water and quickly sacrificed with buffered 1.0 g/L MS-222. Blood was immediately sampled by caudal puncture using a Hamilton syringe rinsed with ammonium-heparin. Small amounts of blood were placed in ammonium-rinsed capillary tubes, spun and analyzed for blood hematocrit and plasma protein. Blood hemoglobin concentrations were measured using the remaining blood. Once the blood work was completed, fish were blotted dry and weighed, and individual fish were placed in scintillation vials for δ-counting (Canberra-Packard A5000 Minaxi)). After all five fish initially removed from the flux tank had been sampled, an additional 5 fish were transferred into the "clean" water. All forty fish were sampled using the same protocol. The exact time at which each fish was removed from the radioisotopic solution was recorded and substituted into equation 2 below for calculation of Na^+ influx rate (absolute time ranged from 4 h to 6.5 h). The unidirectional Na^+ flux in $nmol \cdot g^{-1} \cdot h^{-1}$ was calculated as:

$$J_{in} = \frac{\text{Fish CPM}}{\text{MSA} \cdot \text{W} \cdot \text{t}} \qquad \text{Eqn. 2}$$

where MSA is the mean specific activity in CPM/nmol Na of the exposure water; W is the weight of fish in grams, and t is time in hours.

Analytical methods

Five-mL water samples were acidified to 0.5 % HNO_3 (trace-metal grade acid) and analyzed for total silver by atomic absorption spectroscopy (AAS) on a Varian AA 1275 unit fitted with a graphite furnace atomizer (Varian GTA-95). Total plasma protein was analyzed immediately after dissection by placing plasma on an optical refractometer and measuring refractive index (American Optical, Buffalo, NY). Plasma Na was analyzed by flame AAS (Varian AA 1275), whereas plasma Ag was measured by graphite furnace AAS (Varian GTA-95). Plasma was diluted 1000-fold or 50-fold with 0.5 % HNO_3 (trace metal grade acid; Fisher Scientific) prior to analysis of total Na and Ag, respectively. Plasma glucose was measured according to the hexokinase method using a commercial reagent kit (Sigma kit no. 16-20, St.Louis, MO). Whole-blood hemoglobin concentrations were measured using the cyanmethemoglobin method (Sigma kit 525-18). In short, 20 µL of whole blood was immediately placed in a vial containing 5 mL of Drabkin's reagent (Sigma) and measured against known standards at 540 nM (LKB, Ultraspec Plus).

Tissues were individually homogenized in 1-3 mL of 50 mM Tris-HCl buffer (Sigma, St. Louis, MI), pH 8.0 at 2 °C using an ice-cold glass-Teflon homogenizer (Thomas Scientific). One-hundred-µL aliquots of tissue homogenate were digested with 1 mL of concentrated trace-metal grade HNO_3 in acid-washed test tubes, and the digests

were slowly brought to 120 °C and evaporated to dryness. Each tube was subsequently reconstituted with ~5 mL of 0.5% HNO_3 and analyzed for total Ag by graphite furnace AAS. The remaining ice-cold homogenate was immediately centrifuged at 16,000g for 20 minutes at 2 °C. The resulting supernatant was collected in centrifuge tubes, frozen in liquid nitrogen, and stored at –70 °C until analysis of tissue metallothionein concentration. The MT assay used a double antibody radioimmunoassay as described by Hogstrand and Haux (1990). This included rabbit antiserum raised against perch (*Perca fluviatilis*) as the first antibody, ^{125}I-labelled rainbow trout MT tracer, and goat anti-rabbit IgG as the second antibody.

Intestinal homogenates for day 88 fish were assayed for Na/K-ATPase and amylase activities. Na/K-ATPase was measured using the phosphate release method described by McGeer and Wood (1998), except that intestine was used rather than gill filaments. Amylase was measured using the method of Bernfeld (1955).

Ammonia-N concentrations in water were determined by the colorimetric salicylate-hypochlorite method of Verdouw *et al.* (1978). Water samples (5 mL) taken for urea-N analysis were completely lyophilized and reconstituted to 1 mL with distilled water, to increase analytical sensitivity. Urea-N analysis was performed using a modification of the colorimetric diacetyl monoxime method described by Lauff and Wood (1996).

Statistics

Specific growth rates of the various dietary exposure groups were statistically compared to the simultaneous control (satiation) group using an unpaired, two-tailed *t*-test. The alpha level was modified using a Bonferroni correction to allow for multiple comparisons. Plasma Na^+, Na^+ influx, blood hematocrit, plasma protein, blood hemoglobin, and plasma glucose in the exposure groups were tested for statistical significance against the control satiation treatment using a one-way analysis of variance, followed by Student-Newman-Keuls test for multiple comparisons. For all metal metabolism data, the dietary silver treatment was statistically compared to the control (satiation) group using an unpaired *t*-test. A p value of 0.05 was considered statistically significant throughout. Because O_2 consumption and N-waste excretions were measured on all of the fish "in-tank" as a single mass but with (4 time replicates), no statistical comparisons could be performed.

RESULTS

The total silver concentrations of water samples taken throughout the course of the study were always below the analytical detection limit of 0.25 μg/L Ag. As a result, any effects seen in the present study were very probably due to dietborne silver rather than waterborne silver.

Food consumption and growth.

Food consumption of the dietborne silver treatment did not differ significantly from the control satiation treatment. However, food consumption of these two groups increased approximately 46-65 % between February and May (Table 2). Note that the food consumption rates of the 3 pair-fed treatments were the same as in the silver treatment, and all the food given was consumed. Therefore, appetite did not appear to be suppressed in these groups. Average food consumption rate increased most during the last 30 days of the study when ambient water temperatures increased from 6 to 11 °C. In comparison, specific growth rates were not greatly affected by the 7 °C rise in water temperature; therefore, only a single mean SGR was calculated for each treatment over the entire 126-day period. Specific growth rates did not differ significantly among any of the dietary treatments. Fish in all experimental treatment groups grew well, increasing in mean fish weight approximately 3.5-fold during the study. Furthermore, dietary silver had no apparent effects on food-conversion efficiency, relative to controls, although FCE decreased from 84 % to 52 % in all treatments between the months of February and May. The largest decrease occurred during the last 30 days when water temperature was

increasing. Food-consumption rates, food-conversion efficiencies and growth rates for fish reared on diets formulated with the synthetic trout meals were all within expected ranges for rainbow trout at the reported experimental conditions (Linton *et al.*, 1998). Moreover, there were no significant differences between the four treatments which were fed trout meals versus the group fed the herring meal diet. These results suggest that our home-made diets were nutritionally-balanced formulations that were readily consumed by the trout.

Physiological indices of effect

Dietary silver exposure exerted no significant effect on ion regulation, based on plasma Na^+ concentrations (Fig. 1a) and Na^+ influx (Fig. 1b) measurements during the four month exposure. In addition, no significant differences in hematocrit, plasma protein, and whole blood hemoglobin concentrations were seen among any of the treatments (Figs. 2a-c), although both plasma protein and hemoglobin tended to increase with time in all groups. Plasma glucose showed no consistent response to dietary exposure (Fig. 3), nor were there any effects of dietary silver exposure apparent on oxygen consumption (Fig. 4), or ammonia-N and urea-N excretion rates (Figs. 5a,b). Accordingly, nitrogen quotients (NQ) remained relatively constant (0.15-0.20) treatments (Fig. 5c). Similar to effects on food consumption, steady increases in oxygen consumption and ammonia-N excretion, but not urea-N excretion, were noted in all treatments as water temperature increased. The enzymatic activities of intestinal Na/K-ATPase and intestinal amylase, measured on day 88, were not significantly affected by dietary silver exposure (Table 3).

Metal metabolism

The most pronounced effect of dietary silver exposure was a 12-fold increase in liver Ag burdens after 126 days of exposure (Fig. 6a). Silver concentrations in the liver were significantly higher than controls by day 16, and appeared to saturate at approximately 20 μg/g by day 36. Silver bioaccumulation had no significant effects on hepatic Cu (Fig. 6b) or Zn (Fig. 6c) concentrations, apart from a 38 % reduction in the liver Cu concentration of the dietary silver treatment at day 16. Although dietary silver was readily accumulated in the livers of fish, metallothionein levels were not different between treatments. Metallothionein concentrations ranged from 51.3 and 99.2 μg/g for all treatments, and did not significantly vary with time or liver weight (data not shown). Most importantly, there was no correlation between liver silver concentration and hepatic metallothionein concentrations (Fig. 7).

Concentrations of silver in the intestine were significantly elevated with dietary silver exposure, but only on days 36 and 88 (Fig. 8a). Interestingly, by day 126, Ag concentrations in the control and silver treatment were similar. Silver concentrations were also significantly elevated in the kidney on days 88 and 126 (Fig. 8b), and in the gills and plasma on day 126 only (Figs. 8c,d), in fish fed Ag-laden food. Interestingly, silver concentrations in the kidneys and gills of controls decreased over time. However by day 126, gill and kidney concentrations of the silver treatment were 2-fold higher than the control treatment. Liver silver concentrations did not correlate with liver weight (Fig. 9a), however, kidney and gill silver concentrations appeared to decrease exponentially

with increasing tissue weight (Figs. 9b,c), explaining the reduction in background Ag concentrations over time.

DISCUSSION

Environmental relevance of experimental protocol

Environmental concentrations of waterborne silver are exceedingly low due to silver's tendency of binding with colloidal material or forming complexes with suspended sediments (Shafer *et al.*, 1998). Benthic invertebrates have been shown to assimilate particle-bound silver, allowing the metal to enter the aquatic food chain (Fisher and Wang, 1998). Consequently, the majority of silver accumulated in pelagic organisms is likely derived from food, rather than via the water column (Yamazaki *et al.*, 1996). This is consistent with the bioaccumulation found in feral aquatic organisms for other metals (i.e. Cu, Cd,), despite the fact that the concentrations of these metals were low in water (Dallinger and Kautzky, 1985; Dallinger *et al.*, 1987). Whole body silver burdens as high as 1.9 µg/g have been reported for fish in contaminated sites in the United States (Reviewed in Eisler, 1996). In the present study, a dietary silver burden of 3.1 µg/g (60-fold above background) was used to investigate the effects of food chain transfer of silver at the upper limits of environmental relevance.

This study is unique in that dietary silver was presented in a biologically incorporated form, achieved by exposing rainbow trout (destined to become food) to waterborne silver thiosulfate. Silver thiosulfate was chosen based on its predominance in photographic effluents. Moreover, laboratory studies have indicated that silver thiosulfate is readily accumulated in the liver, kidneys and gills of freshwater fish during aqueous exposures, but does produce acute toxicity (Terhaar *et al.*, 1977; Hogstrand *et al.*, 1996; Wood *et al.*, 1996b). Consequently, it was possible in the present study to expose the "feed" trout to relatively high concentrations of waterborne silver thiosulfate

in order to maximize accumulation. At present, the importance of speciation of dietary metal on bioavailability of the metal to aquatic organisms is not fully appreciated. Hardy *et al.* (1987) observed that a zinc-amino acid chelate was absorbed ~50 % better than $ZnSO_4$ following a single dietary dose in rainbow trout. Furthermore, several studies have observed that natural diets containing elevated concentrations of metals are much more deleterious to freshwater fish than diets artificially laden with metal salts. Metals bound to amino acids may be taken up concurrently across the intestine via amino acid transporters on the mucosal surface of the gut, possibly explaining the improved bioavailability of biologically incorporated diets (Storelli *et al.*, 1989).

In order to ensure that our homemade diets were sufficiently nutritious for juvenile rainbow trout, a variety of supplements were added to the formulations prior to pelletting (Table 1), according to recommendations by Lanno (1989). Woodward *et al.* (1994) noted that the effects of dietary metal exposure were most pronounced when diets were prepared with field-collected invertebrates alone, but that the results may be confounded by nutritional deficiencies in such experimental diets. Considering the length of the present exposure (126 days), we felt that supplementing the experimental diets would help eliminate any such aberrations. In addition, a pair-feeding regime was used in order to account for any potential differences in food consumption rate between treatments. Several studies have demonstrated that food refusal, or regurgitation of food is often elicited in response to elevated concentrations of dietary metals- e.g., Ag (Galvez and Wood, 1999), Cu (Lanno *et al.*, 1985; Handy, 1992) and Cd (Handy, 1992). Consequently, it is often difficult to resolve whether the effects of dietary metal exposure are due to the toxicant or simply a product of reduced food consumption (Lanno, 1989).

However, this was not an issue in the present study because food consumption rates were the same in all treatments.

Physiological effects of dietary silver exposure

Dietary exposure to biologically incorporated silver had no apparent adverse effects on rainbow trout during the 126-day experiment. None of the dietary treatments produced significant mortality, and specific growth rates, food consumption rates and food conversion efficiencies were not significantly different among treatments (Table 2). All the indices of performance were within normal ranges for juvenile rainbow trout reared between 4-11 °C, and fed to satiation daily (Linton *et al.*, 1998). The lack of effect of dietary silver on growth in freshwater fish is consistent with the studies of Terhaar *et al.* (1977), Garnier and Baudin (1990), and Galvez and Wood, (1999). However, only Galvez and Wood (1999) specifically measured food-consumption rates and food-conversion efficiencies: food consumption in trout fed with 3 µg/g silver as silver sulfide was reduced by 21 % from control values. The fact that silver sulfide elicited such a marked reduction in food consumption at only 3 µg/g, whereas the present study produced no effect at 3.1 µg/g, would suggest that the reductions seen by Galvez and Wood (1999) were due to decreased palatability of the diets. Unfortunately, the former study did not include pair-fed controls and therefore it was impossible to make this conclusion definitively.

The physiological mechanism of acute silver toxicity to freshwater fish has been characterized as a severe inhibition of Na^+ uptake mechanisms at the gill epithelium

(Morgan *et al.*, 1997). In contrast, dietborne silver did not impair Na^+ influx or reduce plasma Na^+ concentration (Fig. 1). Furthermore, dietborne silver did not produce any significant effects on intestinal Na/K-ATPase even at concentrations 300-fold higher than previously shown to inhibit gill Na/K-ATPase during waterborne $AgNO_3$ exposure (Morgan *et al.*, 1997; Bury *et al.*, 1999b; McGeer and Wood, 1998).

The ionoregulatory impairment caused by waterborne $AgNO_3$ exposure produces a cascade of physiological perturbations including increased plasma protein, hemoglobin, hematocrit, and plasma glucose concentrations (Wood *et al.*, 1996a; Webb and Wood, 1998). In the present study, the hematological parameters were unaffected, suggesting that hemoconcentration was not likely produced by dietary silver. Plasma glucose concentrations were also not affected by the dietary treatments in any consistent manner (Fig. 2), implying that fish were not acutely stressed as a result of the exposures (Fig. 3).

Routine "in-tank" metabolism was measured to assess subtle effects of dietary silver exposure on metabolic demand, or energy utilization. Oxygen consumption rates were similar among all dietary treatments, suggesting that oxygen uptake at the gills and delivery of oxygen to tissues was not altered. However, oxygen consumption increased in all treatments over time (Fig. 4). Because oxygen consumption rates were size-corrected using the weight exponent developed by Cho (1990), elevations were not likely due to fish growth. Instead, the increase in oxygen consumption over time generally coincided with the 7 °C increase in ambient temperature and the accompanying increase in fish appetite (Table 2) (Brett, 1979).

Waterborne $AgNO_3$ is known to increase ammonia excretion, likely as a result of stress-induced mobilization of cortisol, followed by cortisol-mediated protein catabolism

(Webb and Wood, 1998). In the present study, ammonia and urea excretion rates were monitored under routine "in-tank" conditions to determine the influence of dietary silver on energy utilization and aerobic protein catabolism in fish. Ammonia excretion rates steadily increased in all dietary treatments, likely due to the elevation in water temperature (Fig. 5a). However, dietborne silver did not appear to affect ammonia excretion. In comparison, urea excretion was not greatly affected by changes in water temperature, nor was it influenced by dietborne silver (Fig. 5b). Nitrogen quotient was not affected by dietborne silver, implying that the use of protein as a metabolic fuel (*c.f.* Alsop and Wood, 1997) was not affected (Fig. 5c).

In contrast to the apparent benignity of dietary silver, toxicity to other metals through the diet has routinely been noted. Both dietary Cu (Lanno *et al.*, 1985; Handy, 1993) and Cd (Handy, 1993) have caused mortality in rainbow trout at extremely high doses. More commonly, toxicity is characterized by reduced growth and food-conversion efficiencies in fish. However, these effects are usually attributed to food refusal at elevated concentrations of dietborne metal. To our knowledge, no studies to date have used pair-fed controls in their experimental designs to address the effects of reduced food consumption. Several studies have also assessed the effects of dietary metal on various hematological parameters such as hematocrit, plasma glucose and protein, and blood hemoglobin concentrations. In most cases dietborne metal has produced no significant effects (Lanno *et al.*, 1985), although Murai *et al.* (1981) and Knox *et al.* (1982) observed slight reductions in hematocrit and/or hemoglobin with increasing dietary Cu concentrations. Significant reductions in gill Na/K-ATPase activity, as well as morphological alterations in the gill epithelium after only 14 days of

Cd exposure, have also been noted (Pratap and Wendelaar Bonga, 1993). This effect was attributed to the distribution of dietary cadmium to the gills of fish.

Metal metabolism

Even though physiological disturbances were not produced by dietborne silver, silver accumulated in various tissues of the fish. Significant accumulation of silver was seen in the liver after only 16 days of exposure to a diet containing 3.1 μg/g of biologically incorporated silver (Fig. 6a). Hepatic silver concentrations stabilized at about 14-fold control values after 36 days. In comparison, livers of rainbow trout fed 3000 μg/g dietary Ag as silver sulfide accumulated only four-fold above control concentrations after 43 days (Galvez and Wood, 1999). In the present study, silver accumulated in the liver at a rate of 0.4 μg/g/day during the first 36 days. However, if accumulation is normalized for the silver concentration in the diet (see Hogstrand and Wood, 1998), a Concentration Specific Accumulation Rate of 0.13 ng/g/d/ppb was evident by 16 days. This CSAR is 4.6 orders of magnitude greater than the CSAR measured for dietary silver sulfide exposure, but nonetheless 2,000 to 20,000 times lower than during waterborne $AgNO_3$ exposure. Interestingly, the CSAR presented here (based on food originally contaminated with silver thiosulfate) is similar to the 0.34 - 0.38 ng/g/d/ppb seen in rainbow trout directly exposed to waterborne silver thiosulfate (Hogstrand and Wood, 1998).

In the present study, silver concentrations in the liver increased quickly up until day 36, at which point they appeared to reach a plateau of ~ 15 - 20 μg/g Ag (Fig. 6a). This plateau is almost identical to the maximal Ag concentration reported following

exposure to waterborne $AgNO_3$ for 7 days (Hogstrand *et al.*, 1996). During such waterborne exposures, this bioaccumulated silver is known to significantly increase hepatic MT concentrations (Hogstrand *et al.*, 1996; Wood *et al.*, 1996b; Galvez and Wood, 1998). In contrast, MT was not induced by dietary silver exposure despite the 12-fold increase in silver tissue burden. MT can bind 12 equivalents of Ag^+ per mole of MT at saturation (Li and Otvos, 1996). Accordingly, about 17.3 µg/g of Ag can potentially bind to 80 µg/g MT, the approximate amount of MT in the livers of our fish (Fig. 6d) . Based on these calculations, basal MT concentrations would be theoretically sufficient to bind all of the silver in the liver. It is presently unclear why bioaccumulated silver has a varying ability to induce MT depending on its route of uptake in fish; however, this observation is consistent with other metals (Miller *et al.*, 1993). Lanno *et al.* (1985) and Miller *et al.* (1993) have argued that a functional separation exists between metals accumulated via the diet and water. One possibility is that the difference is rate-related, so that the induction of MT depends on the rate of silver accumulation into the tissue. Therefore, MT induction would only be observed during waterborne silver exposures due to the fast rate of silver accumulation in the liver. Certainly more work needs to be done to clarify this issue.

Several studies have indicated that bioaccumulated silver may interfere with the metabolism of essential metals such as copper and zinc. Coleman and Cearley (1974) noted an inverse relationship between whole-body silver and zinc concentrations in smallmouth bass and bluegills chronically exposed to silver. In comparison, whole-body copper concentrations were unaffected. This contrasts with studies showing that silver can interfere with copper metabolism in the livers of rats (Hirasawa *et al.*, 1994).

Because silver is known to bind to MT *in vivo*, bioaccumulated silver might be able to displace copper and zinc from MT and significantly affect the distribution of these essential metals (Cousins, 1985). In the present study, copper concentrations in the liver were significantly lower in the dietary silver treatment on day 16 only (Fig. 6b), coinciding with the initial increase in hepatic silver burden (Fig. 6a). In comparison, no effect on liver zinc concentrations was noted (Fig. 6c). The transient reduction in liver copper concentration has several explanations. Any copper initially displaced by silver in the cytosol may have been subsequently incorporated into copper-containing enzymes (i.e., ceruloplasmin) and transported out of the liver (Cousins, 1985) and/or excreted into the bile. Alternately, silver may have inhibited copper uptake either across the gut epithelium or into the liver. Regardless, the effect would have had to be transient to explain the subsequent increase in copper concentration in the liver.

Apart from the intestine, silver did accumulate in other tissues until day 88. Accumulation in plasma, gills, and kidney occurred only after the liver silver concentration reached a plateau (Fig 8). Interestingly, concentrations of silver in the kidneys and gills of control fish decreased over time, with tissue Ag concentration decreasing exponentially with increasing tissue weight (Fig. 9). As such, all significant increases in the silver burdens of these two tissues were never higher than initial control values. Silver concentrations in the intestines of the dietary silver treatment were significantly elevated on days 36 and 88 only. Nonetheless, intestinal Na/K-ATPase and amylase activities were not significantly altered on day 88 (Table 3). The lack of effect of dietary silver on amylase activity is particularly interesting because the enzyme is

released into the intestinal lumen to promote digestion of dietary carbohydrates, and should therefore be fully accessible to silver.

In hindsight, the lack of effect of dietary silver on these physiological parameters is not surprising because the acute toxic response to aqueous exposure to silver appears to be mediated by the action of Ag^+ at the gill epithelium. Aqueous concentrations of silver were not elevated at any time during the exposure. Therefore, in order for silver to act at the gills it would first need to be taken up via the gastrointestinal tract and transported via the blood. However, dietary silver was predominantly sequestered within the liver, and never accumulated substantially in any other tissue. In contrast, dietary exposure to Cu (Miller *et al.*, 1993) and Cd (Handy, 1992) resulted in significant accumulations of these metals in tissues such as the kidney and gills. The ability for these metals to accumulate readily in the gills may explain in part why dietary exposure to these metals had toxic effects.

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Table 1. Constituents of control, thiosulfate, silver and herring diets fed to juvenile rainbow trout over a four-month period. All diets are identical except for their proportion of [1]trout meal and [2]herring meal, and the nature of the trout meal. The silver diet① was made from trout exposed to waterborne silver (as silver thiosulfate; 0.37 mmol Ag/L) in dechlorinated Hamilton tap water for one week; the control diet② was made from trout exposed to only Hamilton dechlorinated tap water; and the sodium thiosulfate diet③ was made from trout exposed to 1.48 mmol/L $Na_2S_2O_3$ in dechlorinated Hamilton tap water, the same concentration of thiosulfate used during the Ag thiosulfate exposure. A herring diet④ was formulated using commercial herring meal only. Silver concentrations of diets are given as mean ± SE (n=4).

	Ingredients (as % of total)			
Ingredients	*Control diet ②*	*Thiosulfate diet ③*	*Silver diet ①*	*Herring diet ④*
Trout meal[1]	25	25	25	0
Herring meal[2]	20	20	20	45
Wheat middlings	25	25	25	25
Corn gluten meal	15	15	15	15
Vitamin premix	1	1	1	1
Mineral premix	1	1	1	1
Fish Oil	13	13	13	13
Silver conc. (μg/g)	0.048 ± 0.0001	0.048 ± 0.0001	3.12 ± 0.13	0.058 ± 0.0003

Table 2. Specific growth rates (SGR), food consumption rates, and food conversion efficiencies (FCE) of juvenile rainbow trout fed control, thiosulfate, herring, or silver diets over 126 days. The "control satiation" and "silver satiation" treatments were fed the control and silver diets, respectively, to satiation daily. The three additional treatments were fed either the control, thiosulfate or herring diets pair-fed at the same daily ration as the "silver satiation" treatment. SGR and food consumption are mean ± SEM, whereas FCE are calculated by SGR ÷ food consumption rates. SGRs were calculated by linear regression analysis of the natural logarithm (ln) of mean fish weight over time. Food consumption and FCE are reported on a monthly basis due to changes in their rates over time.

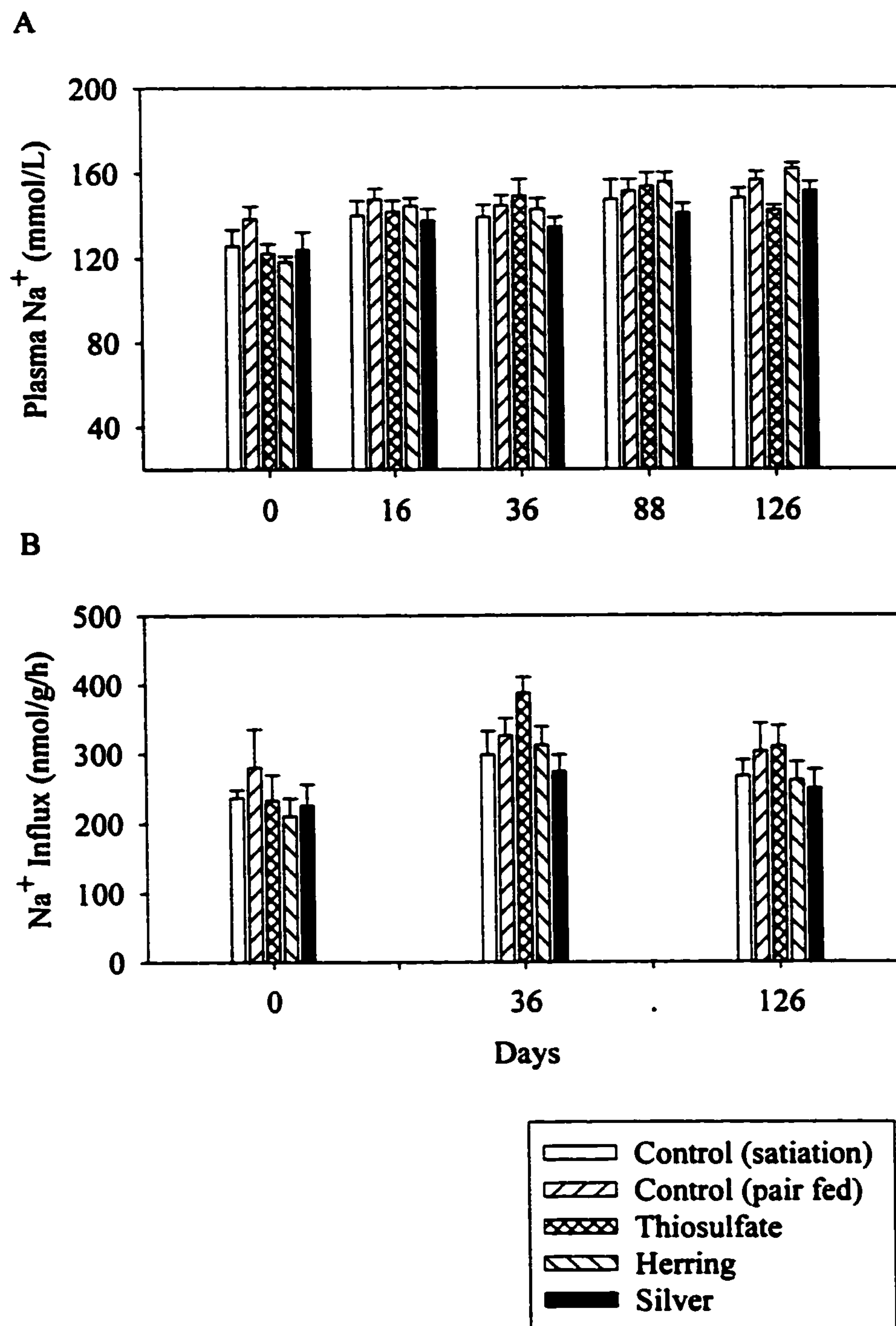
	Diet Ration	Control Satiation	Silver Satiation	Control Pair-fed	Thiosulfate Pair-fed	Herring Pair-fed
SGR (%/day)		1.03 ± 0.04	1.03 ± 0.04	1.01 ± 0.04	1.10 ± 0.04	0.99 ± 0.05

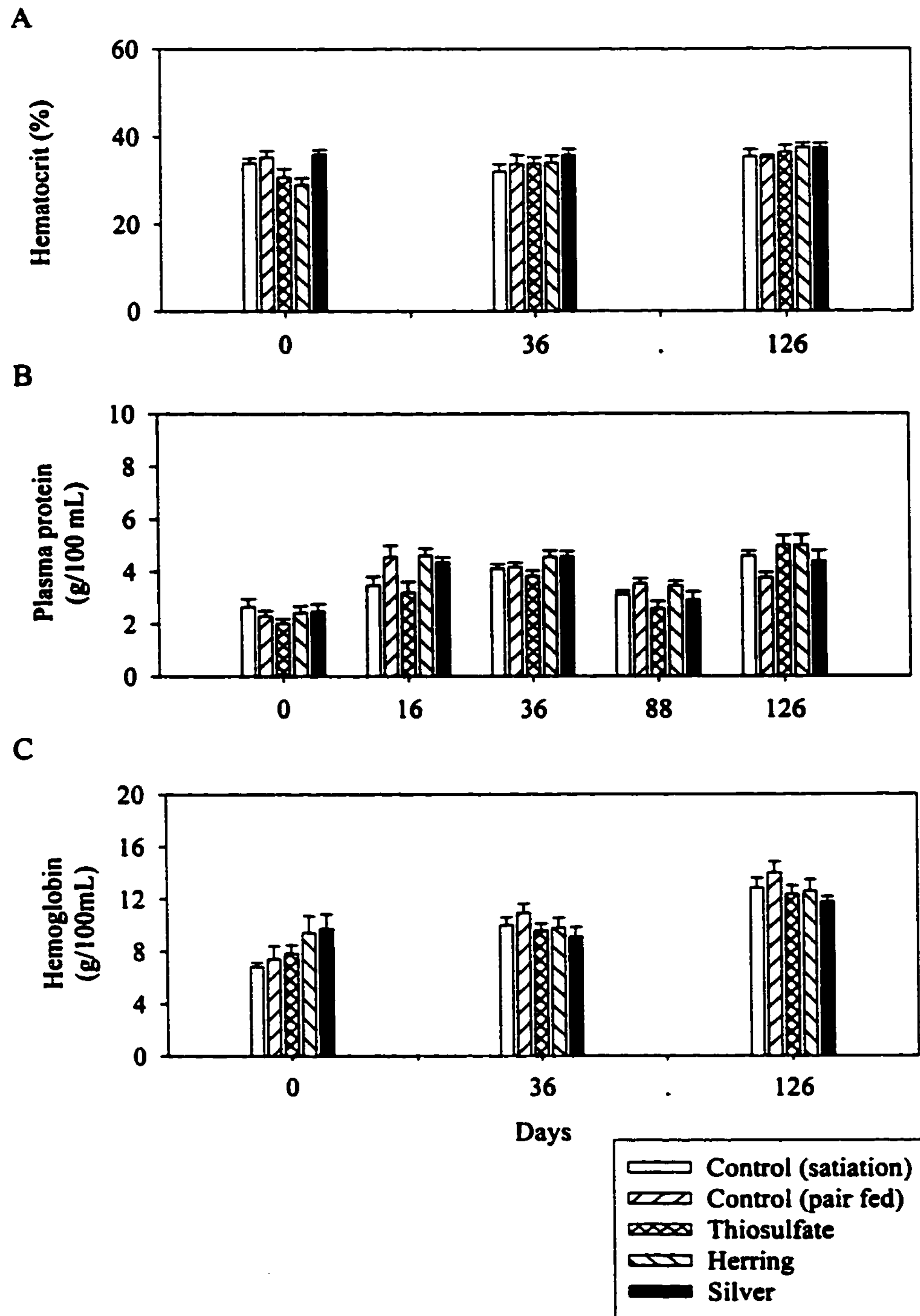
Food consumption	Month	Temp	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
(%/day)	Feb	4.0	1.16 ± 0.10	1.25 ± 0.11	1.25 ± 0.11	1.25 ± 0.11	1.25 ± 0.11
	Mar	4.7	1.14 ± 0.05	1.16 ± 0.06	1.16 ± 0.06	1.16 ± 0.06	1.16 ± 0.06
	Apr	5.6	1.45 ± 0.11	1.46 ± 0.12	1.46 ± 0.12	1.46 ± 0.12	1.46 ± 0.12
	May	11.0	1.69 ± 0.17	2.06 ± 0.19	2.06 ± 0.19	2.06 ± 0.19	2.06 ± 0.19
FCE	Month	Temp	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
(%)	Feb	4.0	89	82	81	88	79
	Mar	4.7	90	89	87	95	85
	Apr	5.6	71	71	69	75	68
	May	11.0	61	50	49	53	48

Table 2. cont.

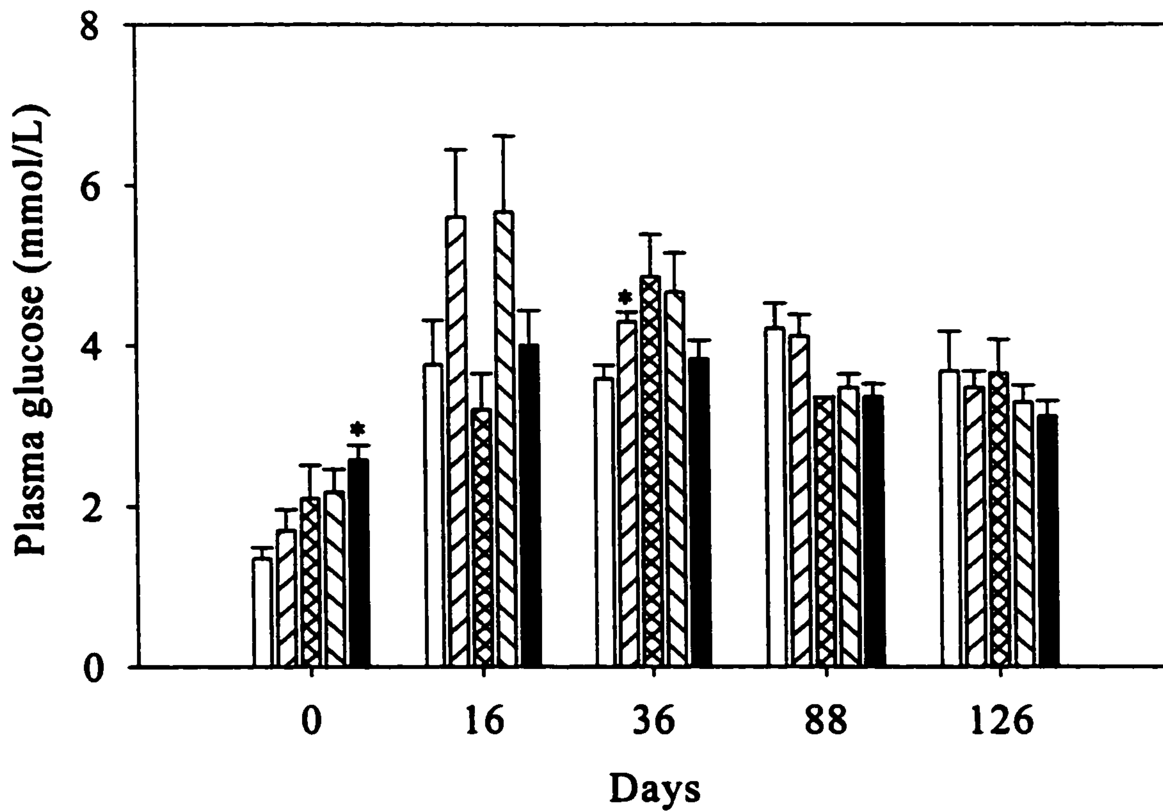
Table 3. Intestinal Na/K-ATPase and amylase activities in juvenile rainbow trout fed to satiation daily for 88 days with either a control (0.05 µg/g Ag) or a silver-contaminated (3.1 µg/g Ag) diet. Additional treatments were fed once daily either the control or thiosulfate diets (each 0.05 µg/g Ag) at the same rate as the silver treatment. No data were available from the herring treatment. Values are mean ± SEM (n = 10). There were no significant differences among treatments.

	Control Satiation	*Silver Satiation*	*Control Pair fed*	*Thiosulfate Pair fed*
Na/K-ATPase (µmol / mg protein / hour)	1.08 ± 0.20	0.89 ± 0.18	0.99 ± 0.21	1.29 ± 0.22
Amylase (µg / mg protein / hour)	97.2 ± 11.2	81.8 ± 6.4	79.9 ± 9.4	111.5 ± 11.7

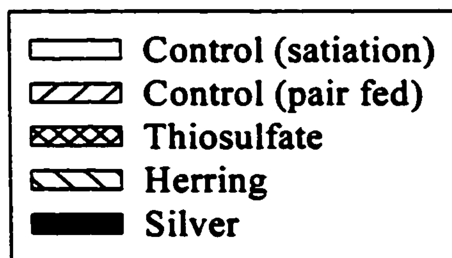

A
200
160
120
80
40
Plasma Na$^+$ (mmol/L)
0
16
36
88
126
B
500
400
300
200
100
0
Na$^+$ Influx (nmol/g/h)
0
36
126
Days
Control (satiation)
Control (pair fed)
Thiosulfate
Herring
Silver



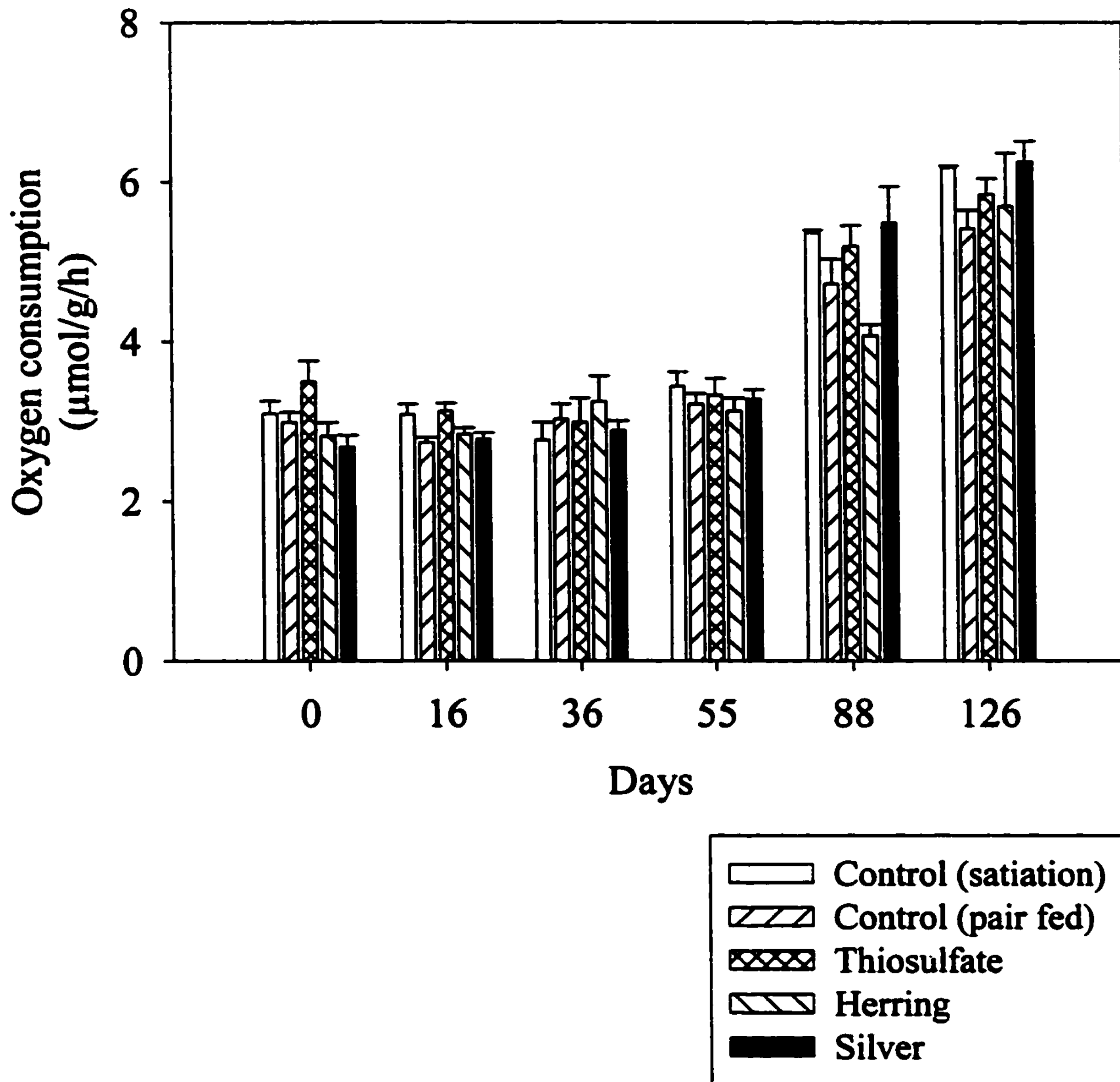
A
Hematocrit (%)
60
40
20
0
0
36
126
B
Plasma protein (g/100 mL)
10
8
6
4
2
0
0
16
36
88
126
C
Hemoglobin (g/100mL)
20
16
12
8
4
0
0
36
126
Days
Control (satiation)
Control (pair fed)
Thiosulfate
Herring
Silver

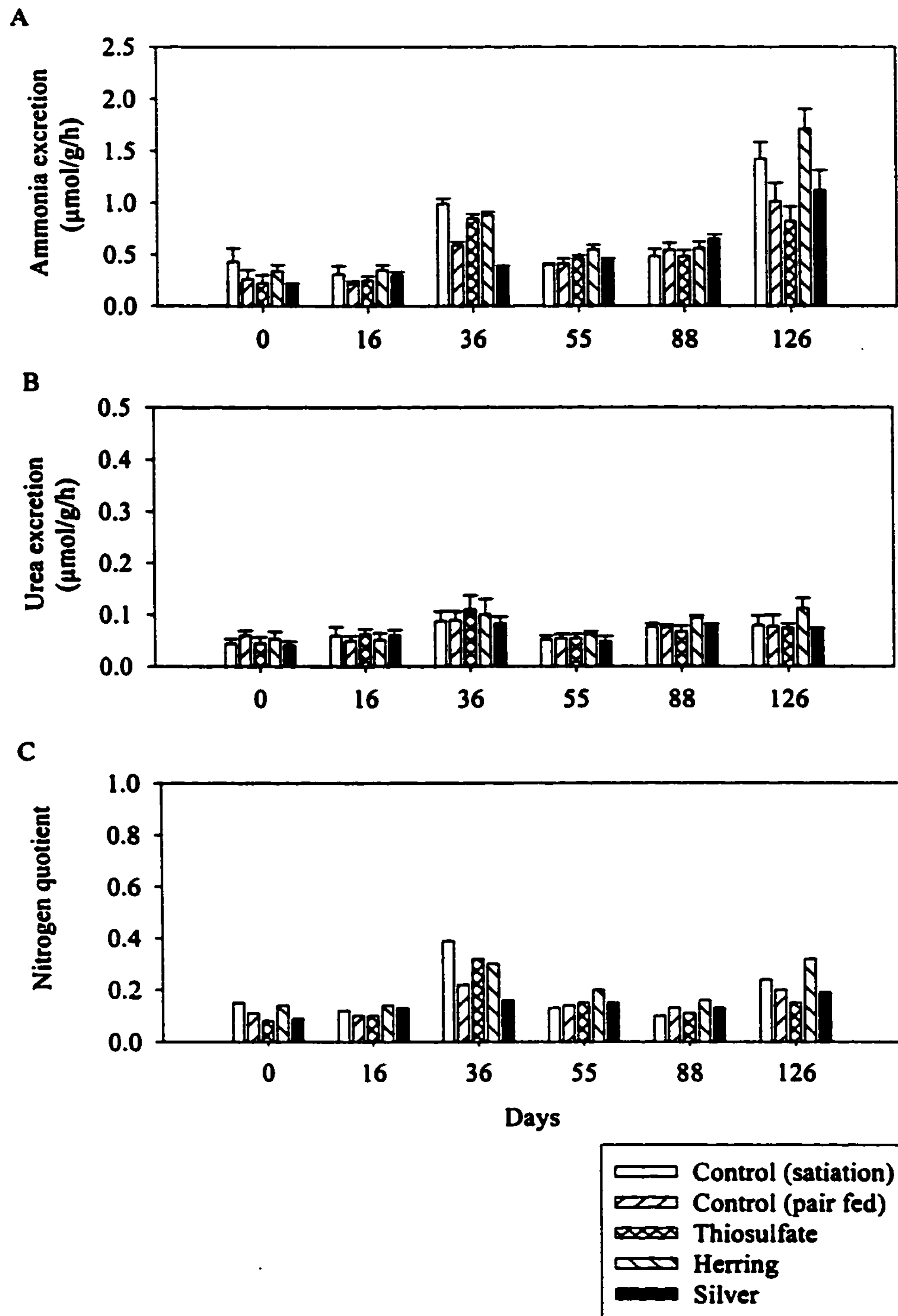


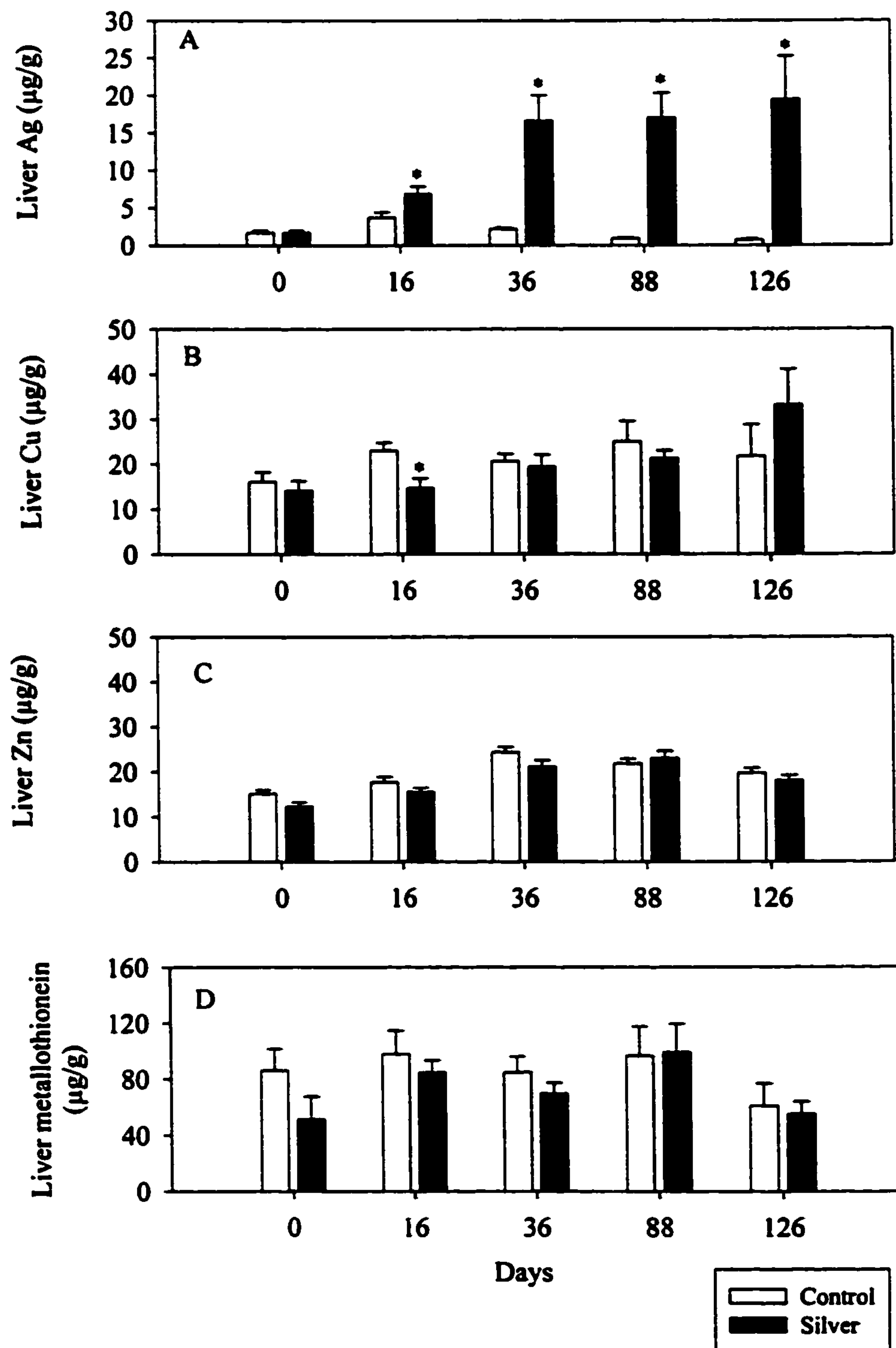
Plasma glucose (mmol/L)
0
2
4
6
8
*
*
0
16
36
88
126
Days



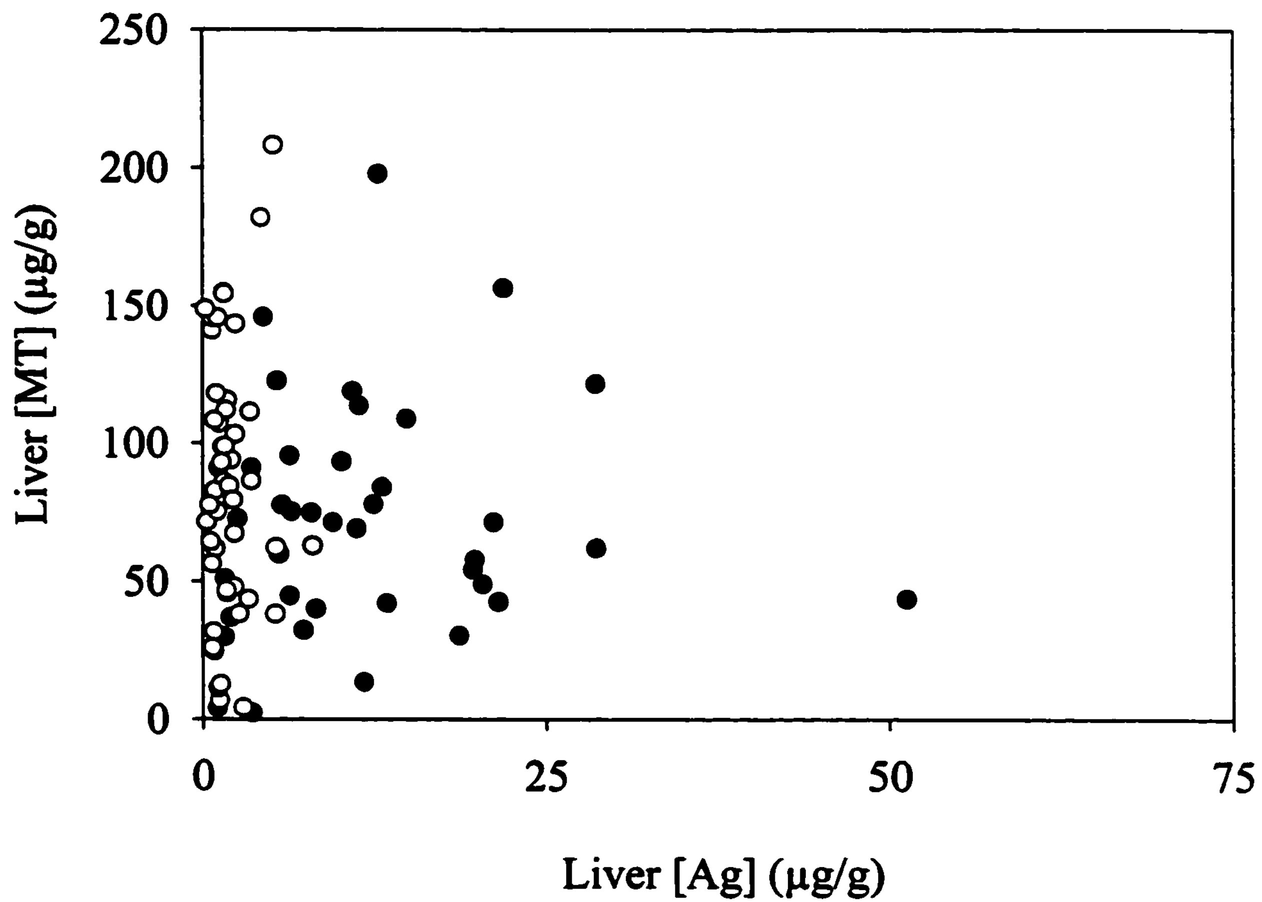
Control (satiation)
Control (pair fed)
Thiosulfate
Herring
Silver

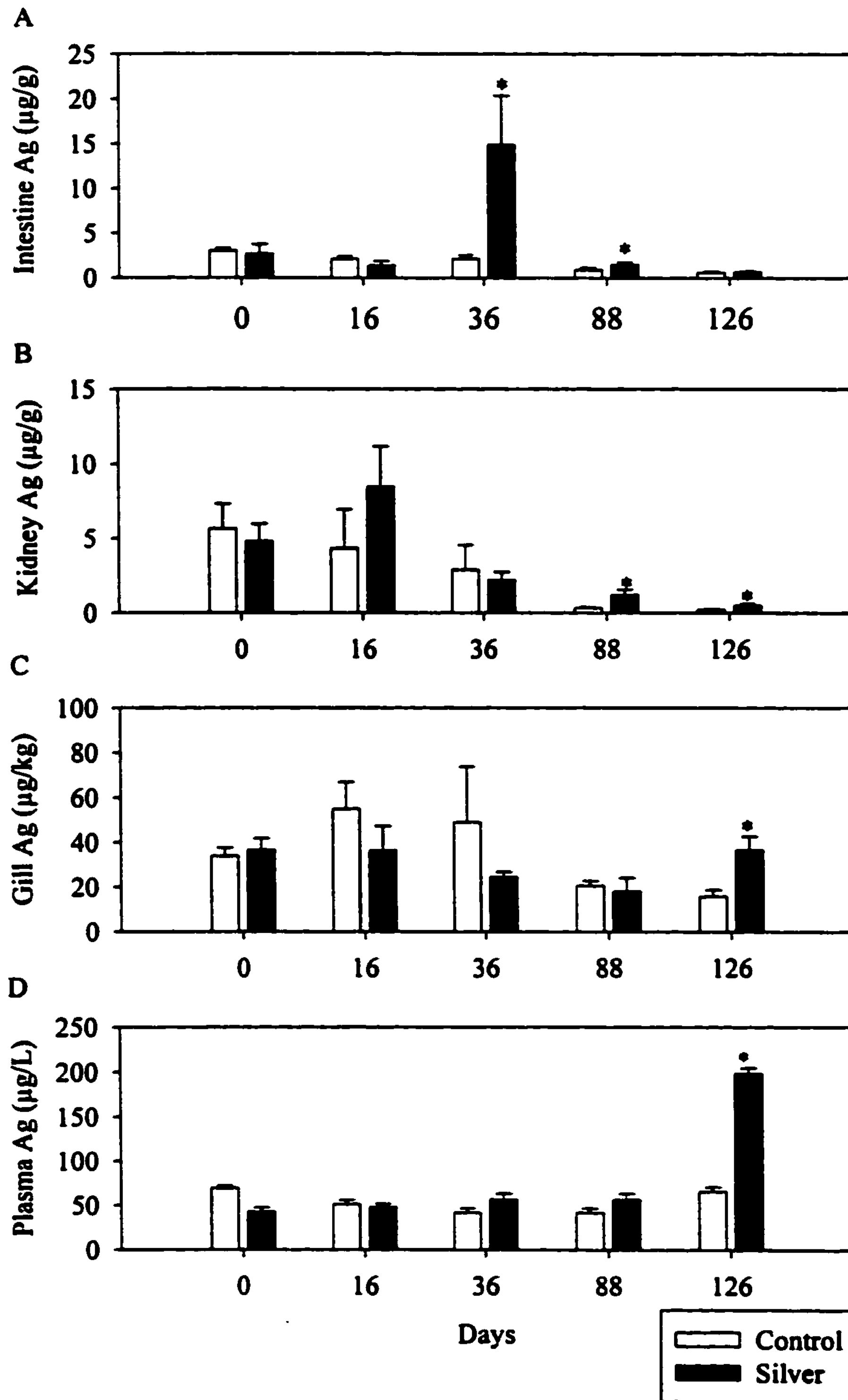

8
6
4
2
0
Oxygen consumption
(µmol/g/h)
0
16
36
55
88
126
Days
Control (satiation)
Control (pair fed)
Thiosulfate
Herring
Silver

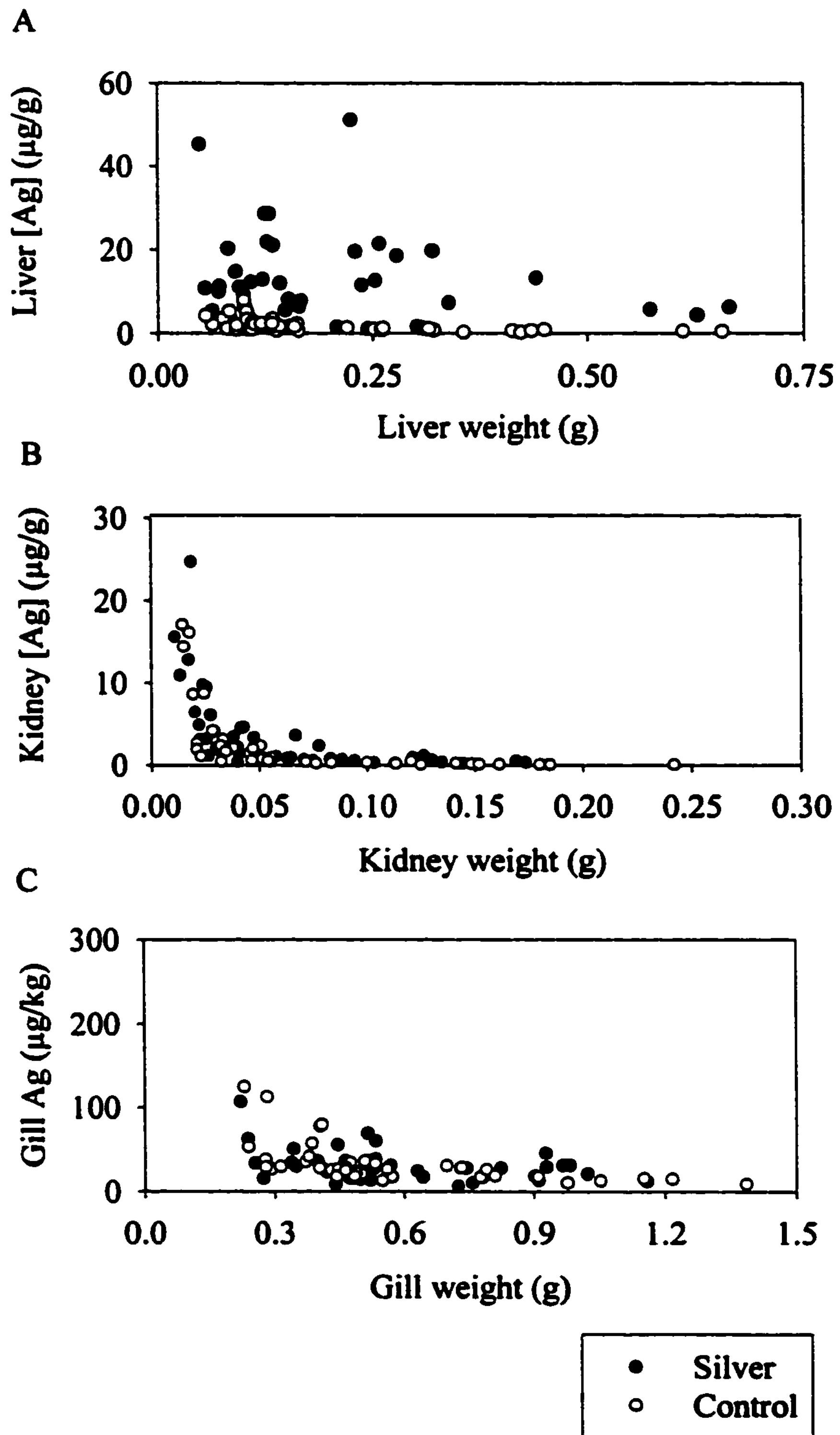

A
Ammonia excretion (µmol/g/h)
2.5
2.0
1.5
1.0
0.5
0.0
0
16
36
55
88
126
B
Urea excretion (µmol/g/h)
0.5
0.4
0.3
0.2
0.1
0.0
0
16
36
55
88
126
C
Nitrogen quotient
1.0
0.8
0.6
0.4
0.2
0.0
0
16
36
55
88
126
Days
Control (satiation)
Control (pair fed)
Thiosulfate
Herring
Silver



A
Liver Ag (µg/g)
30
25
20
15
10
5
0
*
0
16
36
88
126
B
Liver Cu (µg/g)
50
40
30
20
10
0
C
Liver Zn (µg/g)
D
Liver metallothionein (µg/g)
160
120
80
Days
Control
Silver


250
200
150
100
50
0
0
25
50
75
Liver [MT] (µg/g)
Liver [Ag] (µg/g)


A
Intestine Ag (µg/g)
25
20
15
10
5
0
*
*
0
16
36
88
126
B
Kidney Ag (µg/g)
15
10
5
0
*
*
0
16
36
88
126
C
Gill Ag (µg/kg)
100
80
60
40
20
0
*
0
16
36
88
126
D
Plasma Ag (µg/L)
250
200
150
100
50
0
*
0
16
36
88
126
Days
Control
Silver


A
Liver [Ag] (μg/g)
60
40
20
0
0.00
0.25
0.50
0.75
Liver weight (g)
B
Kidney [Ag] (μg/g)
30
20
10
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Kidney weight (g)
C
Gill Ag (μg/kg)
300
200
100
0
0.0
0.3
0.6
0.9
1.2
1.5
Gill weight (g)
Silver
Control

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