SILVER ACCUMULATION, PHYSIOLOGY AND TOXICITY IN TROUT

SILVER ACCUMULATION, PHYSIOLOGY AND TOXICITY IN THE FRESHWATER RAINBOW TROUT: IMPLICATIONS FOR THE SILVER BIOTIC LIGAND MODEL

By

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Abstract

The underlying problems associated with the development of the biotic ligand model (BLM) for prediction of silver toxicity, and its associated physiological and toxicological endpoints, were investigated at a mechanistic, physiological level in juvenile rainbow trout.

An equilibrium level of silver accumulation at the gills is achieved over time during flow-through exposures. The pattern of peak and decline in accumulation during static exposures is due to a decrease in the bioavailability of Ag^+ because of complexation by organic carbon produced by the fish, a consequence of the use of a static exposure system. The decrease in bioavailability leads to a decline in apical silver uptake and together with constant basolateral silver export, a peak and decline in gill silver accumulation.

Inhibition of carbonic anhydrase (CA) by silver is responsible for the early decline in active Na^+ uptake at the gills during flow-through silver exposure, while Na^+K^+-ATPase inhibition is associated with the later decline in uptake. CA activity is inhibited early during silver exposure when Na^+ and Cl^- uptake are decreasing but Na^+K^+-ATPase activity is not inhibited until later. The implication of the data that the rate limiting step in the movement of Na^+ and Cl^- across the gill epithelium is the movement across the apical membrane, as well as the identical time course and degree of Cl^- and

Na^+ uptake inhibition provide additional evidence for a role of CA inhibition in the early decline in Na^+ uptake.

A relationship exists between short-term gill silver accumulation and inhibition of Na^+ uptake. There is also a relationship between silver accumulation on the gills after 3 h, as well as after 24 h, and 96 h mortality.

Together, the results of this thesis indicate that short-term gill silver accumulation is an appropriate endpoint for the prediction of acute silver toxicity in freshwater fish, lending support to the current toxicological version of the silver BLM. These results also indicate that the predictive capabilities of the current physiological BLM may be improved by using Na^+ uptake inhibition rather than Na^+K^+-ATPase inhibition as an endpoint to predict acute silver toxicity.

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Thesis Organization and Format

This thesis is organized into five chapters. Chapter 1 provides a general introduction and statements on the objectives of the thesis. Chapters 2 through 4 are manuscripts that report experimental work and will be submitted for publication in peer-reviewed journals. Chapter 5 provides a summary of results and conclusions of Chapters 2 through 4.

Chapter 1: **General Introduction and Objectives of Thesis**

Chapter 2: **The Time Course of Silver Accumulation in Rainbow Trout During Static Exposure to Silver Nitrate: Physiological Regulation or an Artifact of the Exposure Conditions?**

Authors: Tammie P. Morgan, Martin Grosell, Richard C. Playle and Chris M. Wood

Comments: This study was conducted by TPM under the supervision of CMW and MG. RCP provided advice on geochemical modeling and help with TOC measurements. This paper will be submitted to the journal *Aquatic Toxicology*

Chapter 3: **A Time Course Analysis of Silver Accumulation and Na^+ and**

Cl^- Uptake Inhibition Under Flow-Through Conditions in Freshwater Rainbow Trout

Authors: Tammie P. Morgan, Martin Grosell, Kathleen M. Gilmour, Richard C. Playle and Chris M. Wood

Comments: This study was conducted by TPM under the supervision of CMW and MG. RCP provided advice on geochemical modeling and help with TOC measurements. KMG provided assistance with and facilities for carbonic anhydrase measurements. This paper, with slight modifications, will be submitted to the journal *American Journal of Physiology*

Chapter 4: **A Relationship Between Gill Silver Accumulation and Acute Silver Toxicity in the Freshwater Rainbow Trout: Support for the Silver Biotic Ligand Model**

Authors: Tammie P. Morgan and Chris M. Wood

Comments: This study was conducted by TPM under the supervision of CMW. This paper will be submitted to the journal *Environmental Science and Technology*

Chapter 5: **Summary of Results and Conclusions**

Table of Contents

List of Figures

Chapter 1

General Introduction and Objectives of Thesis

General Introduction

Ionoregulation in freshwater teleosts

Freshwater teleosts have plasma osmolalities of about 300 mosmol kg^{-1} while that of the freshwater environment in which they live is typically less than 10 mosmol kg^{-1}. Thus, these fish are hyperosmotic to their environment and as such, face an osmotic influx of water and a diffusional loss of salt. The fish compensates for the influx of water by excreting large amounts of dilute urine. To counterbalance the loss of salt and therefore maintain ion homeostasis, the fish actively absorbs Na^+ and Cl^- across the gill epithelium from the water (Fig. 1-1).

The first step in the transepithelial absorption of Na^+ and Cl^- from the water is the movement across the apical membrane of the gill cell. For Na^+ this is now generally believed to occur via a proton pump (H^+-ATPase)-coupled Na^+ channel (Potts, 1994; Perry and Fryer, 1997; Nelson et al., 1997; Clarke and Potts, 1998; Fenwick et al., 1999; Wilson et al., 2000). The extrusion of protons across the apical membrane to the water by the H^+-ATPase generates an electrochemical gradient that drives Na^+ entry through the Na^+ channel. For Cl^-, movement across the apical membrane occurs via a chloride-bicarbonate (Cl^-/HCO_3^-) exchanger (Perry, 1997). The basolateral Na^+K^+-ATPase powers apical Na^+ and Cl^- uptake by these mechanisms while the intracellular carbonic

anhydrase (CA) provides the H^+ and HCO_3^- required for exchange of Na^+ and Cl^- by catalyzing the hydration of CO_2 to H^+ and HCO_3^- (Fig. 1-1).

The second step in transepithelial absorption involves the extrusion of Na^+ and Cl^- across the basolateral membrane and into the blood. Extrusion of Na^+ occurs via the Na^+K^+-ATPase located on the basolateral membrane. The mechanism by which Cl^- is extruded from the cell is unknown but likely involves a basolateral Cl^--selective channel (Marshall, 1995, Fig. 1-1).

Cl^- uptake appears to occur through the mitochondria-rich cells of the gill epithelium (Perry, 1997). Na^+ uptake is believed to be performed by the pavement cells (Perry and Fryer, 1997), although this view has been challenged recently in light of evidence localizing the H^+-ATPase and Na^+ channel to the apical membrane of both the pavement cells and mitochondria-rich cells (Lin et al., 1994; Wilson et al., 2000).

The acute toxicity of silver

Ionic silver (Ag^+) is considered the toxic form of silver and in this form it is an ionoregulatory toxicant to freshwater teleosts (see Hogstrand and Wood, 1998; Wood et al., 1999 for reviews, Fig. 1-1). To exert toxicity, silver enters the fish gill via the proton pump-coupled Na^+ channel (Bury and Wood, 1999) and noncompetitively inhibits Na^+ and Cl^- uptake at the gills through inhibition of the basolateral Na^+K^+-ATPase (Morgan et al., 1997). The inhibitory effect on the Na^+K^+-ATPase arises from the blockade by silver of the binding of Mg^{2+} to the enzyme (Ferguson et al., 1996), a step that is critical for phosphorylation and hence proper functioning of the enzyme. Silver also inhibits gill CA but to a lesser extent than Na^+K^+-ATPase (Morgan et al., 1997) and for this reason, at

present, inhibition of CA is considered relatively unimportant in the acute toxicity of silver.

The consequent decline in plasma Na^+ and Cl^- levels due to Na^+K^+-ATPase inhibition eventually leads to an osmotic imbalance between plasma and tissues, causing a shift of fluid from the extracellular to the intracellular space and a reduction in plasma volume. The reduction in plasma volume increases the plasma protein and red blood cell concentration, leading to an increase in blood viscosity that is intensified by splenic contraction. Together with an increase in heart rate and vasoconstriction as the result of sympathetic nervous system activation, the increased blood viscosity leads to increased blood pressure, circulatory collapse and finally death of the fish (Wood et al., 1996a,b; Hogstrand and Wood, 1998).

Water chemistry markedly influences the acute toxicity of silver. Anionic ligands such as Cl^-, sulfide, thiosulfate and dissolved organic carbon (DOC), as well as the cations Ca^{2+}, Mg^{2+} and Na^+ decrease silver toxicity through complexation and competition with Ag^+ thereby reducing its availability to disrupt ionoregulation at the gill (Janes and Playle, 1995; Wood et al., 1996a,b; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury et al., 1999a; Bury et al., 1999b; Wood et al., 1999; Schwartz and Playle, 2001; Bianchini et al., 2002).

From the gill, silver may accumulate in the blood and from there, in other tissues of the body including the liver and kidneys (Wood et al., 1999). Transport of silver across the basolateral membrane of the gill cell is less well characterized than transport across the apical membrane but appears to occur by an ATP-dependent transport

mechanism (Bury et al., 1999c, Fig. 1-1). Once inside the body, silver does not appear to cause any acute toxicity.

Silver in the environment

Silver is released into the freshwater environment from a variety of sources including mining and industrial operations. Of these industrial operations, photographic manufacture and developing release the greatest amount of silver to the water (for a review of sources of silver in the environment, see Purcell and Peters, 1998). Still, even in highly impacted areas, total silver levels seldom exceed a few nM (Kramer et al., 2002). This is because most of the silver in mining and photographic wastewaters is reclaimed due to environmental concerns and economic demand (Purcell and Peters, 1998). In addition, in the case of photographic wastewater, a large percentage of any silver that is not reclaimed is extracted when it passes through publicly owned treatment works (POTW) before being discharged to the freshwater environment (Shafer et al., 1998).

Most of the silver that is released to the freshwater environment exists complexed to anionic ligands such as Cl^-, sulfide, thiosulfate and DOC, or even to particles, such that the concentration of uncomplexed Ag^+ is very low (Purcell and Peters, 1998). Any Ag^+ present in the wastewaters likely combines rapidly with these same ligands and particles, which are prevalent in freshwater systems. For this reason it is believed that acute toxicity from silver in natural freshwater habitats is rare.

Environmental regulation of silver

To protect fish and other organisms from the potential acute toxicity of silver due to discharge into the freshwater environment, Canada and the United States have generated guidelines for silver which state the maximum allowable concentration of total silver in freshwater environments. The current Canadian Water Quality Guideline (CWQG) for total silver is 0.1 μg/L, a value designed to provide protection against both acute and chronic toxicity (CCME, 1999). The United States Environmental Protection Agency's (US-EPA) Ambient Water Quality Criteria (AWQC) for silver are based on water hardness and at present are designed to provide protection only against acute silver toxicity (US-EPA, 1980). For example, at a water hardness of 100 mg/L (as $CaCO_3$), the current criterion for total silver is 4.1 μg/L.

However, the US-EPA now recommends that silver criteria be expressed as dissolved silver rather than total silver (US-EPA, 1999). The dissolved silver concentration is believed to better represent the toxic fraction of silver than does the total silver concentration, providing criteria that achieve protection of freshwater organisms without over-regulation. Based on the conversion factor of 0.85 and a water hardness of 100 mg/L, the current dissolved criterion for silver is 3.5 μg/L.

These guidelines are based on laboratory toxicity tests conducted with silver nitrate ($AgNO_3$, which dissociates in water to yield high levels of Ag^+) in very clean laboratory waters containing low concentrations of anionic ligands, thereby maximizing toxic response. As such, there has been concern by the regulated community such as the photographic industry that these guidelines may be overprotective in natural freshwater

environments where concentrations of Ag^+ are low due to binding with Cl^-, sulfide, thiosulfate, DOC and particles. Overprotective criteria produce an unnecessary economic burden to industries that discharge silver into the freshwater environment.

Recognizing that water chemistry and, as a result, acute silver toxicity varies between freshwater environments, the US-EPA allows adjustment of AWQC on a site-specific basis to more accurately reflect the potential acute toxicity of silver. While a site-specific adjustment for hardness is inherent in the AWQC for silver, other water chemistry characteristics can only be taken into account through the generation of the water-effect ratio (WER). The WER is the ratio of the acute LC50 value (48 h for daphnids, 96 h for fish) in the site water to the LC50 value in the laboratory water for reference species of interest. The approach is complex, time consuming and costly and for these reasons unadjusted criteria may still be used, potentially providing an unnecessary level of protection or even inadequate protection to freshwater organisms depending on the site-specific water chemistry.

The silver biotic ligand models (BLMs)

The silver BLM was developed to predict the acute toxicity of silver on a water chemistry and thus site-specific basis (Paquin et al., 1999; Paquin et al., 2002). The approach has generated interest from both regulatory agencies and the regulated community and is currently being considered for use by the US-EPA as an alternative to the WER to generate site-specific AWQC.

The model is based on the observation that silver, as Ag^+, binds to sites on the gills of the freshwater fish and disrupts ionoregulation. The degree of binding to these

"toxic sites" (components of the Na^+ uptake pathway) on the biotic ligand (gill) in the short term (3 h – 24 h) is thought to be directly proportional to the eventual acute toxicity of silver (mortality at 96 h in fish). Because anionic ligands such as Cl^- and DOC reduce Ag^+ binding at the gills through complexation with the cationic metal, their influence on silver toxicity is considered within the model. In addition, the influence of cations in the water on silver toxicity is considered because cation competition with Ag^+ will also reduce binding (see Fig. 1-2 for a schematic representation of the silver biotic ligand model).

The degree of Ag^+ binding to the gills is predicted by considering the binding sites on the gills in the same geochemical framework as any other Ag^+-complexing ligand. This allows the generation of conditional equilibrium binding constants (affinity and maximum site density) for Ag^+ binding to the gills. These constants, as well as the conditional equilibrium binding constants for silver binding to various anionic ligands in the water and for cations in the water binding to the gill sites, are inserted into an aquatic chemistry equilibrium modeling program, together with the site-specific measured water chemistry. The output allows estimation of short term silver binding to the gills and if the relationship between this factor and 96 h mortality is known, by extension, acute silver toxicity (Paquin et al., 1999; Di Toro et al., 2001).

Although the silver BLM is a simple, inexpensive alternative to the generation of WERs, it has not been accepted for use as a regulatory tool by the US-EPA. This is because silver accumulation on the gills appears to be constantly changing over time, increasing and reaching a peak in the first few hours of exposure followed by a marked

decline with continued exposure (Bury and Wood, 1999; Wood et al., 1999; Wood et al., 2002). As such, an equilibrium level of accumulation may not be achieved, suggesting that short-term gill silver accumulation may not be an appropriate endpoint for prediction of acute silver toxicity. Furthermore, in several studies, there appeared to be no relationship between gill silver burden at fixed time points and toxic physiological effects (McGeer and Wood, 1998; Bury et al., 1999a). In addition, silver accumulation on the gills has never been experimentally correlated with acute silver toxicity (i.e. 96 h mortality), so the current toxicological version of the BLM (Paquin et al., 1999) has been calibrated directly to mortality data using assumed gill burdens.

The physiologically based silver BLM developed by McGeer et al. (2000) bypasses some of the problems associated with the toxicological silver BLM by avoiding the use of total gill silver accumulation entirely. Instead, it assumes that the toxic sites are the Na^+K^+-ATPase molecules that power Na^+ and Cl^- uptake, and that inhibition of gill Na^+K^+-ATPase activity (a physiological endpoint associated with mortality) can serve as a surrogate for Ag^+ binding to toxic sites on the gill. This model uses a conditional equilibrium binding constant developed for Ag^+ inhibiting Na^+K^+-ATPase on the gill (Wood et al., 1999), as well as for protection by competing cations in the water. As with the BLM of Paquin et al. (1999), these constants are inserted into an aquatic chemistry modeling program together with constants for Ag^+ binding to anions in the water and the site-specific water chemistry to predict acute silver toxicity. However, this approach has not received regulatory approval either.

Objectives of Thesis

Overall, the objective of this thesis was to investigate at a mechanistic, physiological level, the underlying problems associated with the development of the BLM approach for silver. In particular, the objectives of this thesis were:

1. To determine the mechanism behind the pattern of silver accumulation on the gills of rainbow trout during waterborne silver exposure, with a peak and decline in accumulation despite continued silver exposure. Specifically, we tested the hypothesis that the mechanism involves physiological regulation of silver movement across the gill epithelium as the result of Na^+K^+-ATPase inhibition by silver (Chapter 2).
2. To determine, under flow-through conditions, the mechanism by which silver causes the early decline in Na^+ uptake at the gills of rainbow trout noted in Chapter 2. Specifically, we investigated whether carbonic anhydrase inhibition by silver was involved (Chapter 3). An additional objective of the flow-through exposure of Chapter 3 was to further investigate the mechanism behind the pattern of silver accumulation on the gills of rainbow trout demonstrated during static silver exposure. Reduced silver bioavailability, a consequence of the use of a static exposure system, as a possible mechanism for the pattern was studied.
3. To evaluate whether there is a relationship between short-term silver accumulation on the gills and acute toxicity as 96 h mortality, potentially providing support for the BLM approach for silver (Chapter 4). In the study of Chapter 4 possible relationships between gill silver accumulation and

physiological toxicity were also tested through measurements of whole body Na^+ uptake and gill Na^+K^+-ATPase activity.

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Fig. 1-1

A schematic representation of Na^+ and Cl^- as well as Ag^+ uptake at the freshwater fish gill, including the sites of toxic action of silver. Dotted lines represent diffusion. Solid lines associated with carriers labeled ATP represent active transport, while solid lines associated with all other carriers represent electroneutral exchange. ATP, adenosine 5' triphosphate; CA, carbonic anhydrase.

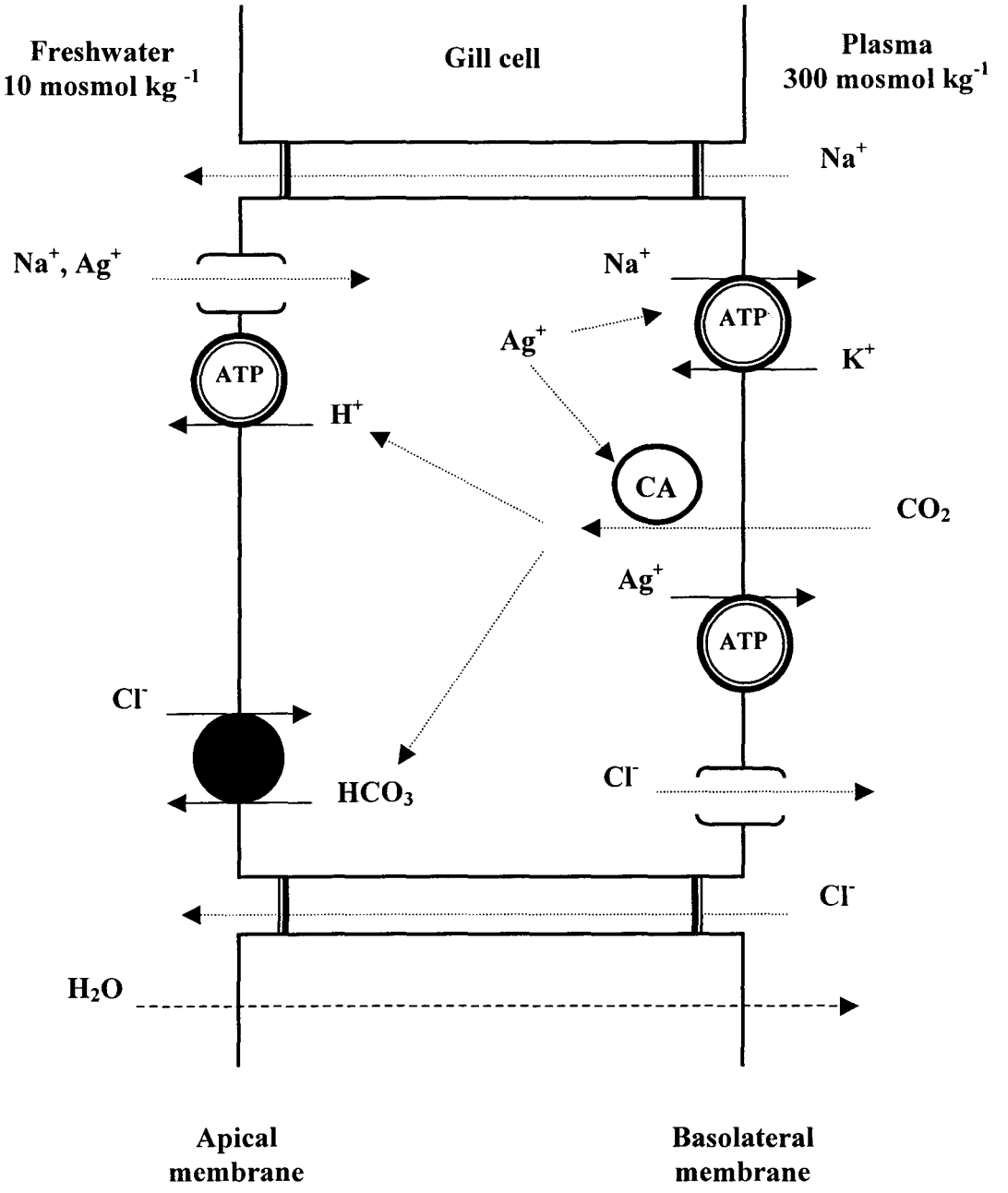
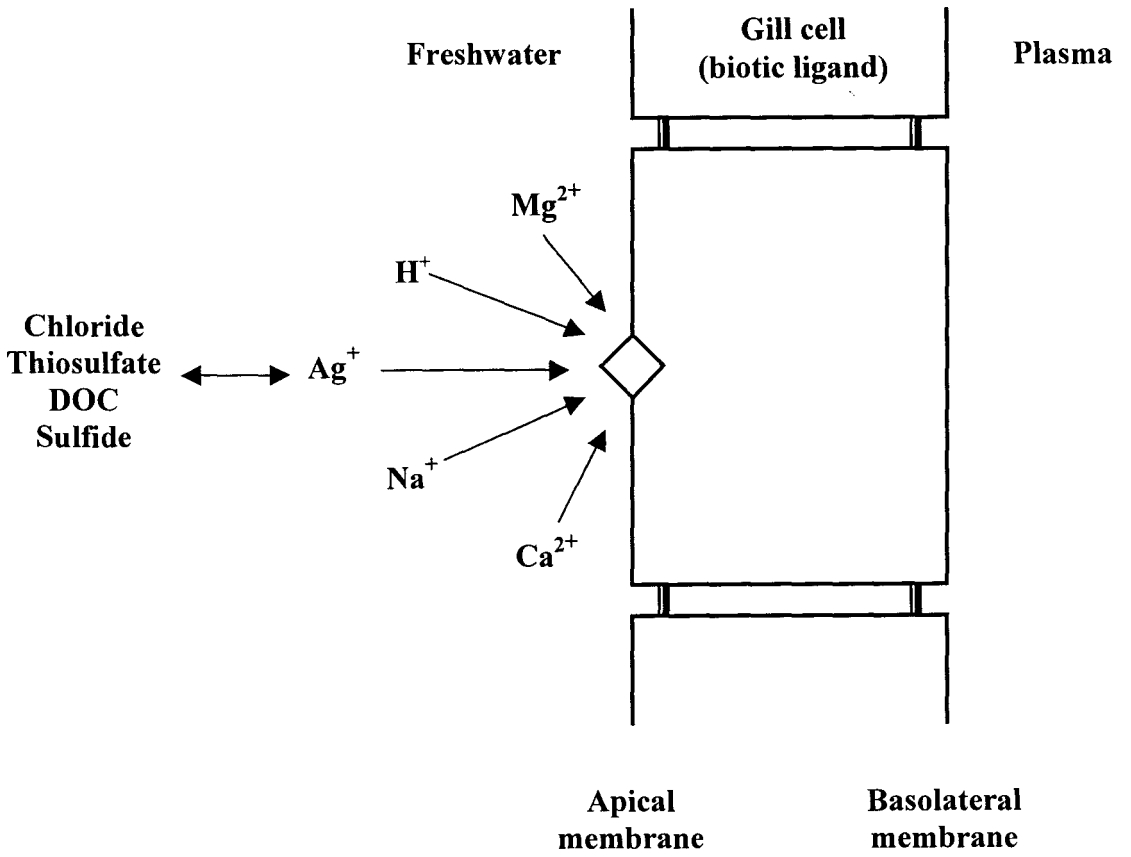

Freshwater
10 mosmol kg -1
Gill cell
Plasma
300 mosmol kg-1
Na+
Na+, Ag+
Na+
ATP
Ag+
ATP
K+
H+
CA
CO2
Ag+
ATP
Cl-
Cl-
HCO3
Cl-
H2O
Apical
membrane
Basolateral
membrane

Fig. 1-2

A schematic diagram of the silver biotic ligand model. The diamond represents a silver binding site on the trout gill. The diagram illustrates the effect of cation competition with Ag^+ and complexation of Ag^+ with anions in the freshwater on Ag^+ binding to binding sites on the trout gill and by extension, acute silver toxicity.


Freshwater
Gill cell
(biotic ligand)
Plasma
Mg2+
H+
Chloride
Thiosulfate
DOC
Sulfide
Ag+
Na+
Ca2+
Apical
membrane
Basolateral
membrane

Chapter 2

The Time Course of Silver Accumulation in Rainbow Trout During Static Exposure to Silver Nitrate: Physiological Regulation or an Artifact of the Exposure Conditions?

Abstract

The pattern of gill silver accumulation in rainbow trout during waterborne silver exposure has been reported to be unusual, reaching a peak in the first few hours of silver exposure followed by a marked decline with continued exposure. The potential causes of the pattern were investigated. Rainbow trout (1-5 g) were exposed in a static system to ^{110m}Ag labeled $AgNO_3$ at a total concentration of 1.92 μg Ag L^{-1} for 24 h in synthetic soft water. Periodically throughout the exposure, gill and body ^{110m}Ag accumulation, gill and body ^{24}Na uptake (from which whole body Na^+ uptake was calculated), gill Na^+K^+-ATPase activity, plus water silver (total and dissolved), Cl^- and total organic carbon (TOC) concentration were measured. Gill silver levels rapidly increased, peaked at 3 h of exposure and then decreased until a plateau was reached at 12 h of exposure. Body (minus gills) silver levels increased steadily over the exposure period until 18 h of exposure. Whole body Na^+ uptake decreased, was maximally inhibited by 3 h of exposure but recovered by 12 h despite continued silver exposure. Gill Na^+K^+-ATPase activity was not inhibited until 5 h of exposure. The water dissolved silver concentration declined by ~70% over the 24 h exposure period and the TOC content of the water

increased over three fold during the first 2 h of exposure. There was a decrease in the calculated contribution of Ag^+ (from 20.9 to 2.5%) and an increase in the calculated contribution of Ag-TOC complexes (from 77 to 97.3%) to the total water silver concentration over the first 2 h of exposure. Apical silver uptake into the gills decreased over the initial 2.5 h of exposure while basolateral silver export out of the gills to the body remained constant throughout the exposure. The results of this study suggest that: 1) physiological regulation of silver movement may explain the pattern of gill silver accumulation observed in rainbow trout, although not by a mechanism coupled to Na^+K^+-ATPase inhibition as originally proposed, 2) alternatively or additionally, a decreased bioavailability of silver, due to the static exposure conditions, may explain the pattern of gill accumulation, 3) the early inhibition of whole body Na^+ uptake observed during silver exposure occurs via a mechanism other than Na^+K^+-ATPase inhibition, and 4) gill silver accumulation may be an appropriate endpoint for biotic ligand modeling.

Introduction

When present as silver nitrate, silver is one of the most acutely toxic metals to freshwater rainbow trout with 96 h LC50 values in the range of 6.5–13 $\mu g\ L^{-1}$ (Davies et al., 1978; Nebeker et al., 1983; Hogstrand et al., 1996). Silver nitrate is highly toxic because it readily dissociates in water to yield ionic silver (Ag^+), the most acutely toxic species of silver (Hogstrand et al., 1996; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury et al., 1999a; Bury et al., 1999b; Grosell et al., 2000). Silver species such as silver complexed by dissolved organic carbon (DOC) or chloride are much less toxic to rainbow trout than Ag^+ (Hogstrand et al., 1996; Galvez and Wood, 1997; McGeer and Wood, 1998; Bury et al., 1999a; Bury et al., 1999b; Karen et al., 1999; Grosell et al., 2000; Rose-Janes and Playle, 2000) because their formation renders the ion less available for binding at key toxic sites on the gill epithelium.

It is generally accepted that the key toxic site of action of Ag^+ is the Na^+K^+-ATPase located on the basolateral membrane of gill cells (Morgan et al., 1997). This enzyme is responsible for extruding Na^+ in exchange for K^+ across the basolateral membrane and into the extracellular fluid (Skou, 1990), thereby providing much of the energy for active Na^+ and Cl^- uptake. In freshwater fish this transport is essential to counteract the diffusive loss of Na^+ and Cl^- to the hypo-osmotic freshwater environment. Waterborne Ag^+ exposure inhibits this enzyme causing an inhibition of Na^+ and Cl^- uptake via the gills (Wood et al., 1996; Morgan et al., 1997; McGeer and Wood, 1998; Webb and Wood, 1998; Bury et al., 1999a; Grosell et al., 2000). The resultant

ionoregulatory disturbance eventually leads to circulatory collapse and death of the fish (Wood et al., 1996; Hogstrand and Wood, 1998).

To exert its toxic action, Ag^+ enters gill cells from the water, at least in part, by the same route as Na^+, via an apical Na^+ channel driven by the electrical gradient established by a H^+-ATPase (Bury and Wood, 1999). In addition to accumulating in the gills, silver readily accumulates in the rest of the body (Hogstrand et al., 1996; Wood et al., 1996; Webb and Wood, 1998). The transport of silver across the basolateral membrane of the gill cell and into the blood (and thereby the rest of the body) is at least in part carrier-mediated and ATP-dependent, suggesting active transport (Bury et al., 1999c).

Two studies recently investigated the temporal patterns of silver accumulation in the gills and body of rainbow trout (Bury and Wood, 1999; Wood et al., 2002). These studies found that the gill silver accumulation constantly changed over time; gill silver levels increased, peaked and then declined despite continued silver exposure. In contrast, body silver accumulation increased steadily to a plateau with time. Relative to other metals this pattern of gill silver accumulation is unusual. Most studies with Cu, Cd and Zn have shown simple linear or hyperbolic accumulation of the metals at the gills over time (Laurén and McDonald, 1986; Giles, 1988; Playle et al., 1993,1993a; Hollis et al., 1997; Grosell et al., 1998; MacRae et al., 1999; Alsop et al., 1999). Albeit unusual, the pattern may be characteristic of silver exposure; it is observed in both rainbow trout and European eel during exposure to either predominantly Ag^+ or predominantly $AgCl_{aq}$ (Wood et al., 2002).

The mechanism behind the pattern is unknown. Wood et al. (2002) hypothesized that the mechanism may involve physiological regulation of silver movement across the gill cell. According to this hypothesis the initial rapid increase in gill silver accumulation is due to entry via the apical H^+-ATPase coupled Na^+ channel. The peak and decline in gill silver accumulation is due to the inhibitory effect that Ag^+ has on the Na^+K^+-ATPase, as well as the continuing active export of silver from the gill across the basolateral membrane and into the body. Inhibition of Na^+K^+-ATPase by Ag^+ could lead to an increase in the intracellular Na^+ concentration over time and a reduction of the membrane potential. In turn, this would inhibit apical Na^+ and Ag^+ uptake because the driving force for entry would be decreased, and/or the apical channel might close.

The objective of this study was to determine if physiological regulation of silver movement across the gill cell as the result of inhibition of Na^+K^+-ATPase by Ag^+ could explain the pattern of gill silver accumulation observed in rainbow trout during waterborne silver exposure. This objective was accomplished by performing a detailed time course analysis of the pattern of gill and body silver accumulation (using radiolabelled ^{110m}Ag), whole body Na^+ uptake (using radiolabelled ^{24}Na), and gill Na^+K^+-ATPase inhibition over the course of 24 h of static silver exposure. A total concentration of silver of 1.92 $\mu g\ L^{-1}$ was used because this value is close to the concentrations used in previous studies which examined silver accumulation in rainbow trout (Bury and Wood, 1999; Wood et al., 2002). Radiolabelled ^{110m}Ag was used to achieve the sensitivity of analysis required for these detailed time course studies, and thereby necessitated the use of a closed exposure system, similar to that used in these earlier investigations.

Materials and Methods

Experimental animals and acclimation

Juvenile rainbow trout (*Oncorhynchus mykiss*; 1-5 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and held for two weeks in a 200 L polyethylene tank supplied with flowing, aerated, dechlorinated Hamilton city tap water (approximate ionic composition in mM: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, DOC ~3 mg C L^{-1}, hardness ~140 mg L^{-1} as $CaCO_3$ and temperature 13.5 ± 0.5 °C). All fish were then acclimated over a two week period to synthetic soft water, created by mixing increasing amounts of reverse osmosis (Culligan Aqua-Cleer Reverse Osmosis System, Toronto, ON, Canada) treated Hamilton city dechlorinated tap water with Hamilton city tap water until the desired water ionic composition was achieved. Fish were maintained in this soft water (approximate ionic composition in mM: 0.05 [Na^+], 0.05 [Cl^-], 0.05 [Ca^{2+}], 0.02 [Mg^{2+}], 0.02 [K^+], pH 7.0, DOC ~0.5 mg C L^{-1}, hardness ~10 mg L^{-1} as $CaCO_3$ and temperature 12 ± 2 °C) for at least an additional two weeks before experimentation. During initial holding and soft water acclimation, fish were fed to satiation once daily with commercial trout pellets (Martin Mills, Tavistock, ON, Canada). Feeding was suspended for 1 day before and during the experiment to minimize silver binding to organic matter in uneaten food and waste products during the exposure period.

Experimental design

Silver, as ^{110m}Ag labeled $AgNO_3$ (70 μCi; specific activity 0.92 μCi μg^{-1} Ag^+; RISOE Nuclear Research Reactor, Roskilde, Denmark), was added to an 80-L

polyethylene tank containing 40 L of aerated, synthetic soft water (composition as above) 24 h before the addition of fish. This was done to ensure saturation of silver binding sites on the tank walls so that silver levels would remain constant during the experiment. The addition of silver yielded a total silver concentration of 1.92 $\mu g\ L^{-1}$ in the exposure water at the start of the experiment.

After the 24 h equilibration period, a total of 152 fish were placed into the exposure tank. Eight fish were sampled at 0.5 h, every hour from 1–8 h and at 12, 18 and 24 h of silver exposure for determination of gill and body silver accumulation (the body was that portion of the rainbow trout remaining after the gills were excised) and gill and body Na^+ uptake (from which whole body Na^+ uptake was calculated; via ^{24}Na). The methods for the ^{24}Na uptake measurements are given separately below. Water samples were also taken at each time point for measurement of water silver (total and dissolved), chloride and TOC concentration. At 1, 3, 5, 8, 12, 18 and 24 h of exposure, an additional eight fish were collected for determination of gill Na^+K^+-ATPase activity. Gill and body silver accumulation, gill and body Na^+ uptake and gill Na^+K^+-ATPase activity were also measured in fish before silver exposure (initial time point on Figures). Sampled fish were rinsed in a concentrated solution containing $AgNO_3$ (7.9 mg L^{-1}) and NaCl (2.9 g L^{-1}) to remove any loosely bound radioisotope (^{110m}Ag and/or ^{24}Na) by displacement, and were euthanized by an overdose of MS-222 (1 g L^{-1}).

Water analyses

Water samples were taken to determine the concentration of total and dissolved silver during the exposure period. Silver was referred to as dissolved if it was able to

pass through a 0.45-micron filter (Acrodisc polyethersulfone syringe filters, Pall Gelman Laboratory, Ann Arbor, MI, USA). Two 5 ml non-filtered (for determination of total silver) and two 5 ml filtered water samples were taken from the exposure tank at each of the time points listed above and counted for ^{110m}Ag radioactivity (MINAXI Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada). In addition, six 5 ml water samples were taken from the exposure tank at the start and end of the experiment. Three of these samples were immediately acidified with 1.0% (v/v) trace metal grade HNO_3 (Fisher Scientific, Canada) and analyzed for total silver by graphite furnace atomic absorption spectrophotometry (Varian AA-1275 with GTA-9 atomizer, Varian Ltd., Mississauga, ON, Canada). The remaining three samples were counted for ^{110m}Ag radioactivity. From these two measurements the specific activity (SA) of silver in the water was calculated (cpm/μg silver). The SA of silver changed by less than 20% between the start and end of the experiment. The total and dissolved silver concentration of the exposure water at each of the time points was determined by dividing the ^{110m}Ag radioactivity (cpm) of the water by the SA of silver.

Concentrations of Cl^- in the water were analyzed using the colorimetric mercuric thiocyanate method (Zall et al., 1956). Total organic carbon (TOC) concentrations were measured on a Shimadzu 5050A total organic carbon analyzer (Mandel Scientific Co. Ltd., Guelph, ON, Canada). TOC is the sum of particulate organic carbon (POC) and dissolved organic carbon (DOC).

Tissue silver accumulation

To determine the silver concentration of the gills and body, the tissues were first counted for ^{110m}Ag radioactivity (MINAXI Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada; see below for details of radioactivity counting). The counts due to ^{110m}Ag radioactivity were then converted to absolute silver concentrations based on the known specific activity of silver in the water during the exposure period (see above).

Na^+ uptake measurements

Na^+ uptake measurements were conducted in a 600 ml Pyrex glass beaker. Before the uptake measurements in silver-exposed fish, two of the beakers were submerged in the exposure water in the exposure tank for 24 h before experimentation. One beaker was removed from the tank, used for the first uptake measurement, and at the completion of the measurement, rinsed with water and replaced in the exposure tank. The additional beaker was then used for the second measurement and so on. This was done to ensure saturation of silver-binding sites on the glass beaker so that the concentration of silver in the water would remain constant during the uptake measurement. For Na^+ uptake measurements conducted in fish before silver exposure, the beaker was not equilibrated in the silver exposure water.

At each sample time fish were netted from the exposure water and added to one of the 600 ml beakers which contained 300 ml of water to which the fish had been exposed. ^{24}Na (6.7 μCi; mean SA 0.009 μCi μg^{-1} Na^+; McMaster University Nuclear Reactor, Hamilton, ON, Canada) was then added to the beaker. The uptake measurement lasted a total of 30 min during which time the water was continuously aerated. Five minutes after the start of the measurement and again at the end, 5 ml water samples were taken in

duplicate for measurement of water ^{24}Na radioactivity by gamma-counting (MINAXI Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada; see below) and total $[Na^+]$ by flame atomic absorption spectrophotometry (Varian AA-1275, Varian Ltd., Mississauga, ON, Canada). In addition, at the end of the measurement, gills were excised and then the gills and the body (minus the gills) were counted separately for ^{24}Na activity on the gamma counter (see below). The gill or body uptake of Na^+ ($\mu mol \cdot g^{-1} \cdot h^{-1}$) was then calculated as follows:

$$\text{Gill or body } Na^+ \text{ uptake} = CT/(SA \cdot wt \cdot t)$$

where CT is the total counts per minute (cpm) in the gills or body, SA is the measured specific activity of the water, wt is the wet weight of the gills or body (g), and t is the time of exposure (h). The specific activity of the water was calculated as follows:

$$SA = [(cpm_i/[Na]_i) + (cpm_f/[Na]_f)]/2$$

where cpm_i represents the ^{24}Na cpm per ml initially in the water, cpm_f represents the final ^{24}Na cpm per ml in the water, and $[Na]_i$ and $[Na]_f$ represent the initial and final sodium concentrations of the water, respectively. Whole body Na^+ uptake was calculated by adding the gill and body Na^+ uptake and correcting for the whole body weight.

Radioactivity counting

Because gill and body silver accumulation and Na^+ uptake were determined at each sample time, the sampled fish were exposed to two radioisotopes, ^{24}Na and ^{110m}Ag, both of which are gamma emitters. To determine the ^{110m}Ag radioactivity in the gills, body, and water the samples were initially counted for the sum of ^{24}Na and ^{110m}Ag radioactivity. ^{24}Na (15-h half-life) was then allowed to decay for approximately two weeks and the samples were counted again to give the ^{110m}Ag radioactivity (250-d half-life). To determine the ^{24}Na radioactivity in the gills, body and water, the ^{110m}Ag radioactivity was subtracted from the sum of ^{24}Na and ^{110m}Ag radioactivity. The ^{24}Na activity was then corrected for decay to a common reference time. Gamma radioactivity counting was conducted as outlined by Hansen et al. (2002) using an energy window of 1050 - 2000 keV.

Gill Na^+K^+-ATPase activity

Gills were obtained by dissection at the above mentioned sample times and also before silver exposure, immediately frozen in liquid nitrogen, and stored at -80°C for later analysis of Na^+K^+-ATPase activity. The activity of gill Na^+K^+-ATPase was measured according to the method of McCormick (1993). Briefly, gills were homogenized in SEID buffer (0.5 g of sodium deoxycholate in 100 mL of SEI; SEI = 150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) at between 0 and 4 ° C. Using a microplate spectrophotometer (SPECTRAmax 340PC, Molecular Devices Corp., Sunnyvale, California, USA) the linear rate of NADH disappearance was measured in all gill homogenates in the presence and absence of ouabain at 340 nm for 10 minutes at room temperature. Na^+K^+-ATPase activity (μmol ADP mg^{-1} protein h^{-1}) was calculated

as the difference in ATP hydrolysis in the absence and presence of ouabain and was standardized to protein content. The protein concentration in homogenates was measured using the Bradford assay (kit no. B6916, Sigma, Canada) with bovine serum albumin as a standard (Sigma, Canada).

Statistical analyses

Data have been expressed as means ± SEM (n) where n represents the number of different fish contributing to the mean except for the water silver, chloride and TOC concentrations where only the means of duplicate determinations are reported. All comparisons were made using a one-way ANOVA followed by a least significant difference (LSD) test (SPSS 10 for Windows). Linear regression analysis was done using SPSS 10 for Windows. A significance level of $p<0.05$ was used throughout.

Results

Water silver concentrations

The mean measured total and dissolved water silver concentrations over the 24 h exposure period were 1.92 ±0.08 μg L^{-1} and 0.71 ±0.11 μg L^{-1} respectively.

Silver accumulation

When rainbow trout were exposed to silver, gill silver initially increased rapidly and then peaked after 3 h of exposure at ~200 ng g^{-1}. A gradual decline in gill silver with continued silver exposure followed until a plateau was reached at ~125 ng g^{-1} after 12 h of exposure (Fig. 2-1A).

Body (minus the gill) silver increased steadily over the first 18 h of silver exposure to a plateau of ~12 ng g^{-1} at 24 h of exposure (Fig. 2-1B).

Whole body silver levels increased rapidly over the first 3 h of exposure after which levels increased slowly until a plateau was reached of ~15 ng g^{-1} at 24 h of exposure (Fig. 2-1C).

Initially, the gills were the largest contributor (~82%) to the whole body silver burden. However, over the course of the exposure the contribution of the gills decreased steadily to ~30% by 24 h of exposure (Fig. 2-2). In contrast, the contribution of the body to the whole body silver burden increased steadily from only 18% at 0.5 h to almost 70% at 24 h of exposure.

Na^+ uptake

Silver exposure rapidly inhibited whole body Na^+ uptake (Fig. 2-3A). By 3 h of silver exposure, Na^+ uptake was maximally inhibited by ~ 55% (from 834 nmol g^{-1} h^{-1} before silver exposure to 374 nmol g^{-1} h^{-1}). Inhibition was followed by eventual recovery of Na^+ uptake at 12-24 h of silver exposure. The time course of inhibition of body (minus the gills) Na^+ uptake paralleled the time course of inhibition of whole body Na^+ uptake (Fig. 2-3A). Interestingly, Na^+ uptake appeared to vary inversely with gill silver accumulation such that maximal inhibition of Na^+ uptake (3 h of exposure) was associated with the greatest gill silver accumulation (3 h of exposure; Fig. 2-1A). Furthermore, the eventual recovery of Na^+ uptake with continued silver exposure corresponded with the decline in gill silver accumulation (compare Fig. 2-1A and Fig. 2-3A).

Gill Na^+K^+-ATPase activity

Silver exposure significantly inhibited gill Na^+K^+-ATPase activity in rainbow trout by ~40%, but the effect did not occur until 5 h (from 4.2 μmol ADP mg^{-1} protein h^{-1} before silver exposure to 2.6 μmol ADP mg^{-1} protein h^{-1} at 5 h of silver exposure; Fig. 2-3B). The 5 h delay in enzyme inhibition was in contrast to the rapid inhibition of whole body Na^+ uptake (significant at 2 h and maximal at 3 h; Fig. 2-3A) and rapid accumulation of gill silver (significant at 0.5 h and peaking at 3 h; Fig. 2-1A). Na^+K^+-ATPase activity remained inhibited over the remaining 19 h of exposure except at 12 h.

Water chemistry changes

Static exposure of rainbow trout to silver was associated with changes in the chemical constituents of the exposure water over time. In terms of water silver, the total silver concentration remained relatively constant over the 24-h exposure period, declining by only 20% (from 1.92 to 1.56 μg L^{-1}; Fig. 2-4A), likely due to uptake of silver by the fish and adhesion of silver to surfaces. However, the dissolved silver concentration did not remain constant (Fig. 2-4A). Over the first 7 h of exposure the concentration of dissolved silver decreased by approximately 65% (from 1.32 to 0.46 μg L^{-1}) but remained relatively constant over the remaining 17 h of exposure. In total, over the 24-h exposure period the dissolved silver concentration decreased by approximately 70% (from 1.32 to 0.39 μg L^{-1}).

Over the exposure there was a small decrease in the water chloride concentration (Fig. 2-4B). At 1 h of silver exposure the water chloride concentration was 122 μM but by 24 h of exposure the concentration had declined by 7% to 113 μM. In contrast, there was an increase in the TOC concentration of the exposure water from an initial value of

0.6 mg C L^{-1} to 1.9 mg C L^{-1} at 2 h of silver exposure, an increase of over three fold (Fig. 2-4C). TOC levels remained elevated relative to the initial value and constant over the following 22 h of exposure.

Discussion

The mechanism behind the pattern of gill silver accumulation

The unusual and complex pattern of gill silver accumulation reported in this study, with somewhat differing time courses, has been demonstrated before in two separate studies (Bury and Wood, 1999; Wood et al., 2002), suggesting that the pattern is characteristic of silver exposure.

The present data allowed an analysis of the rates of apical silver uptake into the gills and basolateral silver export from the gills. This was accomplished by first calculating silver accumulation at each time point based on the equation of the line of best fit for the whole body (apical uptake; Fig. 2-1C) and body (basolateral export; Fig. 2-1B; adjusted to whole body weight) silver accumulation data and then dividing the change in silver accumulation over each time interval by the length of the time interval. The rates were therefore expressed as rates per unit whole body weight, and were plotted at the midpoint of the time interval. Initially, apical silver uptake was much higher than basolateral silver export (10 ng g^{-1} h^{-1} versus 0.5 ng g^{-1} h^{-1}; Fig. 2-5). However, apical uptake decreased rapidly over the first 2.5 h of exposure and by 3.5 h of exposure and for the remainder of the exposure apical uptake was similar to and slightly lower than basolateral silver export at ~0.5 ng g^{-1} h^{-1}. The peak in gill silver accumulation (Fig. 2-1A) therefore could be attributed to the initially much higher uptake rate than export rate,

a phenomenon that rapidly declined (Fig. 2-5). Basolateral silver export was low but constant over the exposure period (Fig. 2-5). The steady increase in body silver accumulation over time (Fig. 2-1B) reflected the constant basolateral export of silver (Fig. 2-5).

Apical Na^+ uptake and basolateral Na^+ export were also determined. (Apical Na^+ uptake is the sum of gill and body Na^+ uptake because all of the radiolabelled Na^+ present in the fish must have crossed the apical membrane in the 0.5 h measurement period; basolateral Na^+ export is the body (minus the gill) Na^+ uptake because all of the radiolabelled Na^+ present in the body must have crossed the basolateral membrane in the 0.5 h measurement period.) Both apical Na^+ uptake and basolateral Na^+ export decreased and then increased to a plateau with continued silver exposure (Fig. 2-3A). The identical time course of apical Na^+ uptake inhibition and basolateral Na^+ export inhibition suggests that the rate limiting step in the movement of Na^+ across the whole gill epithelium is the movement of Na^+ across the apical membrane.

Physiological regulation of silver uptake?

The temporal patterns of whole body Na^+ uptake and gill Na^+K^+-ATPase activity inhibition observed in this study suggest that physiological regulation of silver movement across the gill epithelium by the mechanism originally proposed (i.e. inhibition of Na^+K^+-ATPase activity) cannot explain the pattern of gill silver accumulation. Upon silver exposure Na^+ uptake was affected rapidly such that by 3 h of exposure, uptake was significantly and maximally inhibited by ~55% (Fig. 2-3A). Morgan et al. (1997) saw a similar large and rapid inhibition of uptake (50% inhibition by 3 h of exposure) during

exposure of larger rainbow trout to 2 μg Ag l^{-1} but did not measure Na^+K^+-ATPase activity at this time. However, in the present study gill Na^+K^+-ATPase activity was not inhibited until 5 h of silver exposure (Fig. 2-3B), two hours after maximal inhibition of Na^+ uptake. If physiological regulation of silver movement through inhibition of Na^+K^+-ATPase activity could explain the pattern of accumulation as hypothesized, we would have expected to see inhibition of Na^+K^+-ATPase activity preceding or simultaneous with the inhibition of Na^+ uptake as the intracellular Na^+ concentration increased and reduced apical Na^+ entry.

However, if it is the movement of Na^+ across the apical membrane rather than the basolateral membrane (i.e. via Na^+K^+-ATPase) that is the rate limiting step in the movement of Na^+ across the gill epithelium (Fig. 2-3A), physiological regulation of silver movement may still explain the pattern of gill silver accumulation by a different mechanism(s). Gill carbonic anhydrase (CA) and the gill epithelial Na^+ channel are associated with Na^+ movement across the apical membrane and CA inhibition and/or blockage of the epithelial Na^+ channel by silver itself could also alter silver movement across the gill epithelium.

Carbonic anhydrase catalyzes the hydration of CO_2 to produce H^+ and HCO_3^- which are exchanged against Na^+ and Cl^- at the apical membrane (Henry, 1996). Ag^+ has been shown to inhibit gill CA, both *in vitro* (Christensen and Tucker, 1976) and *in vivo* (Morgan et al., 1997), although the degree of inhibition is much less than that of Na^+K^+-ATPase. Inhibition of CA by Ag^+ (discussed further below) could lead to a decrease in the intracellular supply of H^+ which would in turn inhibit apical Na^+ and Ag^+ uptake

because the H^+-ATPase requires protons to fuel apical uptake of Na^+. Together with constant silver export across the basolateral membrane, this would lead to a peak and decline in gill silver accumulation.

Uptake of Na^+ from the water and into the gills of rainbow trout is believed to occur via an epithelial Na^+ channel (Perry and Fryer, 1997). Analysis of the structure of the epithelial Na^+ channel in other species has revealed several cysteine-rich domains within the extracellular loop of the channel subunits (Kellenberger and Schild, 2002). Ag^+ has a very high affinity for sulfhydryl groups such as those found on cysteine (Cooper and Jolly, 1970), so that the resulting blockage of epithelial Na^+ channels would result in an inhibition of Na^+, as well as Ag^+ uptake because Ag^+ enters gill cells via the same pathway as Na^+ (Bury and Wood, 1999). With continued silver export across the basolateral membrane, this would lead to a peak and decline in gill silver accumulation. The metal ions Cd^{2+}, Zn^{2+} and Hg^{2+} have been demonstrated to block both skeletal and cardiac Na^+ channels (distinct from the epithelial Na^+ channel) by binding to sulfhydryl groups of cysteinyl residues, albeit at much higher metal concentrations (Ravindran et al., 1991; Ravindran and Moczydlowski, 1991; Schild et al., 1991; Doyle et al., 1993; Schild and Moczydlowski, 1994; Hisatome et al., 2000). Moschen et al. (2001) reported that Hg^{2+} did not affect the epithelial Na^+ channel although this does not preclude an effect of Ag^+ on channel function.

Changes in silver uptake due to changes in silver bioavailability?

It is well known that silver toxicity in rainbow trout is influenced by chemical constituents in the freshwater environment such as Cl^- and dissolved organic carbon

(DOC, Hogstrand et al., 1996; Galvez and Wood, 1997; Bury et al., 1999b; Karen et al., 1999). Complexation of silver by DOC or Cl^- decreases the bioavailability of the free silver ion, Ag^+, thus reducing the degree of inhibition of Na^+ uptake and gill Na^+K^+-ATPase activity. The presence of POC in the water also decreases Ag^+ bioavailability (POC strongly binds Ag^+) and has been associated with decreased silver toxicity (increased LC50 values) in rainbow trout and fathead minnows (Cooper and Jolly, 1970; Nebeker et al., 1983; Erickson et al., 1998). While binding of Ag^+ by POC and DOC decreases gill silver accumulation, binding of Ag^+ by Cl^- does not necessarily prevent accumulation (Janes and Playle, 1995; Hogstrand et al., 1996; McGeer and Wood, 1998; Bury et al., 1999a; Bury et al., 1999b; Grosell et al., 2000; Rose-Janes and Playle, 2000; Wood et al., 2002). Thus, it would appear to be the reduction of free Ag^+ in solution, rather than the prevention of its uptake, that is critical in preventing silver toxicity.

Because the present experiment was performed using a static silver exposure system (similar to that used by Bury and Wood, 1999; Wood et al., 2002), it is possible that the chemistry of the exposure water changed over the course of the experiment. Surprisingly, rather than increasing, the water Cl^- concentration decreased very slightly over the exposure period (Fig. 2-4B). However, there was also a large decrease in the dissolved silver concentration over the course of the exposure (~70% decline over 24 h; Fig. 2-4A). Visible fish waste did accumulate in the bottom of the exposure tank over time (probably reflecting mucus production by fish). Although the silver content of this waste was not monitored, it is assumed that this waste buildup rendered some of the added silver as non-dissolved and non-bioavailable due to silver-POC formation. As

such, it is not surprising that the TOC content of the water increased over time, increasing more than three fold over the first hour of exposure (Fig. 2-4C). An increase in the DOC content of the exposure water probably contributed to the increase in TOC content and therefore resulted in a decrease in Ag^+ bioavailability due to silver-DOC complex formation. We therefore propose that alternatively to physiological regulation, changes in Ag^+ bioavailibility over the exposure period through complexation of Ag^+ by POC and DOC may explain the pattern of gill silver accumulation observed in rainbow trout. TOC was not monitored in earlier studies (Bury and Wood, 1999; Wood et al., 2002) which showed similar patterns of gill silver accumulation over time.

Altered bioavailability of silver could explain the pattern of gill silver accumulation in the following way: Initially during silver exposure there would be rapid accumulation of Ag^+ by the trout gills because it would be available for uptake. However, as time progressed and the fish contributed organic matter to the exposure water, Ag^+ uptake would decrease as it became less available through complexation. Complexation, in combination with the constant removal of silver basolaterally via its carrier mediated, ATP dependent mechanism (Bury et al., 1999c), would produce a peak and decline in gill silver accumulation.

We calculated the speciation of silver in the exposure water using the aquatic chemistry program MINEQL+ (version 4.0, Schecher and McAvoy, 1992) using measured total silver concentrations and TOC values and appropriate binding constants from Janes and Playle (1995). Over the first hour of silver exposure the calculated contribution of Ag^+ to the total silver concentration decreased from 20.9% to only 2.5%,

while the contribution of Ag-TOC complexes increased from 77.0% to 97.3%. Together with the changes in apical silver uptake and basolateral silver export demonstrated over the exposure (Fig. 2-5), these results suggest that altered Ag^+ bioavailability could explain the pattern of gill silver accumulation noted in these experiments.

Further evidence for a silver bioavailability effect comes from the observation that during silver exposure whole body Na^+ uptake initially fell, but with continued silver exposure uptake later increased to pre-silver exposure values (Fig. 2-3A). As the bioavailability of Ag^+ decreased with increased TOC concentration, the degree of inhibition of Na^+ uptake by Ag^+ would be reduced, allowing recovery of whole body Na^+ uptake despite continued silver exposure. If physiological regulation alone (i.e. inhibition of gill CA or blockage of the apical Na^+ channel) were responsible for the time course of inhibition, we would expect the inhibition of Na^+ uptake that occurs initially but not the recovery of Na^+ uptake that occurs with continued silver exposure.

Because altered silver bioavailability appears to be a consequence of static silver exposure, the pattern of gill accumulation may be an artifact of the exposure conditions. A similar reduction in bioavailability may have played a role in the studies of Bury and Wood (1999) and Wood et al. (2002) who used similar static exposure systems. Two recent studies with copper have demonstrated a pattern of gill accumulation for copper similar to that of silver (Grosell et al., 1997; Grosell and Wood, 2002); again the pattern may be an artifact of the exposure conditions as these studies were also performed using static exposure systems. In the future, the use of flow-through exposure conditions that

minimize or prevent changes in silver bioavailability may be helpful in resolving the uncertainty.

A role for gill carbonic anhydrase and/or apical Na^+ channels in the acute toxicity of silver?

For the past six years it has generally been accepted that the inhibition in whole body Na^+ uptake observed in rainbow trout during silver exposure is the result of an inhibition of basolateral Na^+K^+-ATPase, because this enzyme is greatly inhibited during silver exposure (Morgan et al., 1997) and is known to power Na^+ uptake in freshwater fish (Richards and Fromm, 1970). The results of the present study indicate that this may not be a complete explanation. Specifically, Na^+ uptake was very clearly inhibited before Na^+K^+-ATPase activity (2 h versus 5 h; Fig. 2-3A and Fig. 2-3B) suggesting that, at least early during silver exposure, the inhibition of Na^+ uptake may be due to an additional mechanism.

Carbonic anhydrase has been localized to the intracellular compartment of gill epithelial cells (Rahim et al., 1988) while Na^+K^+-ATPase is located on the basolateral membrane. As such, inhibition of CA and hence, inhibition of Na^+ uptake would perhaps be expected to occur before Na^+K^+-ATPase inhibition because Ag^+ must traverse the entire cell before reaching the Na^+K^+-ATPase, but only a short distance before reaching CA. Likewise, blockage of the apical Na^+ channel by silver (see above) would result in an inhibition of Na^+ uptake, although initially Na^+K^+-ATPase activity would remain unchanged. In addition, simple competition between Na^+ and silver for uptake by the channel could explain the inhibition of Na^+ uptake in the absence of Na^+K^+-ATPase

inhibition. Silver has a very high affinity for the uptake pathway relative to Na^+ (Janes and Playle, 1995; Bury and Wood, 1999; McGeer et al., 2000), and as such its presence in the water could reduce apical Na^+ uptake as it competed with Na^+ for uptake by the channel.

A relationship between gill silver accumulation and physiological toxicity

The temporal patterns of gill silver accumulation and whole body Na^+ uptake observed during silver exposure suggest an inverse relationship exists between the two parameters (Fig. 2-1A and Fig. 2-3A). Indeed, regression analysis revealed a significant negative relationship between the two (r^2=0.64; p<0.01; Fig. 2-6A). This result was somewhat surprising because McGeer and Wood (1998) could find no correlation between gill silver concentrations and disruption of Na^+ balance. The reason for the difference between the two studies may be time. McGeer and Wood (1998) examined the relationship between gill silver accumulation and Na^+ balance at 48 h, whereas we looked at the relationship at progressive times up to 24 h. Longer term silver exposures (such as those used by McGeer and Wood, 1998) may obscure a direct relationship between gill silver accumulation and toxic effect because accumulation of silver may occur at "non-toxic" sites (Paquin et al., 1999). In addition, defense mechanisms, such as detoxification, may be activated during longer exposure times. Our result is in agreement with that of Janes and Playle (1995) who demonstrated a significant correlation between gill silver accumulation and net sodium losses from the fish using short, 2-h exposure periods.

The negative relationship between gill silver accumulation and whole body Na^+ uptake demonstrated in the present study is particularly important due to the current interest by regulatory agencies such as the U.S. Environmental Protection Agency in the generation of biotic ligand models (BLMs). BLMs attempt to predict acute metal toxicity in different water qualities by predicting gill metal accumulation (Di Toro et al., 2001). Thus, for the models to be adequately predictive of toxicity, a relationship must exist between gill accumulation and mortality. As of yet the silver version of the BLM (Paquin et al., 1999) has not been accepted for use as a regulatory tool because silver accumulation on the gill has not been satisfactorily correlated with silver toxicity in terms of mortality. In the present study we have demonstrated a relationship between gill silver accumulation and whole body Na^+ uptake. This result suggests that there is also a relationship between gill silver accumulation and mortality because inhibition of whole body Na^+ uptake is the principal cause of eventual mortality of silver-exposed fish (Hogstrand and Wood, 1998). This observation lends support to the BLM approach for predicting silver toxicity. Further support for the approach would come from experiments establishing a direct relationship between short-term silver accumulation and eventual fish mortality. For example, MacRae et al. (1999) demonstrated a relationship between copper concentration on the gill of the fish after 24 h of exposure and mortality of juvenile rainbow trout after 120 h of copper exposure, a finding which underpins the recently approved BLM for copper (Santore et al., 2001).

The temporal patterns of gill silver accumulation and Na^+K^+-ATPase activity do not suggest a relationship between the two (Fig. 2-1A and Fig. 2-3B), an observation that

is supported by regression analysis (r^2=0.08; p>0.05; Fig. 2-6B). This corroborates the findings of other investigators (McGeer and Wood, 1998; Bury et al., 1999a). Because total gill silver accumulation could not be related to the actual toxic mechanism of silver (inhibition of Na^+K^+-ATPase), McGeer et al. (2000) built a physiologically based BLM which calculates acute silver toxicity by predicting inhibition of gill Na^+K^+-ATPase rather than by predicting gill silver accumulation. When this model was developed it was thought that the inhibition of Na^+ uptake observed with silver exposure was solely caused by inhibition of Na^+K^+-ATPase, justifying the use of Na^+K^+-ATPase inhibition as an endpoint. However, as shown in our study, Na^+ uptake inhibition can occur in the absence of Na^+K^+-ATPase inhibition. This suggests that using Na^+ uptake inhibition as an endpoint rather than Na^+K^+-ATPase inhibition may give the physiologically based BLM more predictive power.

Conclusions

Four important conclusions can be drawn from this study. First, physiological regulation of silver movement by the mechanism proposed (i.e. inhibition of Na^+K^+-ATPase activity) cannot explain the complex pattern of gill silver accumulation demonstrated in rainbow trout. However, it remains possible that physiological regulation may still occur as the result of inhibition of carbonic anhydrase or blockage of the apical Na^+ channel by silver. Second, alternatively or additionally to physiological regulation, the mechanism behind the pattern may involve changes in the bioavailability of Ag^+ due to complexation by organic matter, a phenomenon associated with the use of a static exposure system. Third, the inhibition in Na^+ uptake that occurs early during silver

exposure is not due to an inhibition of Na^+K^+-ATPase activity as previously thought. The inhibition may involve the inhibition of carbonic anhydrase activity, blockage of the apical Na^+ channel by Ag^+ or competition between Na^+ and Ag^+ for uptake at the apical Na^+ channel. Fourth, the biotic ligand model approach for predicting acute silver toxicity based on short-term gill silver accumulation is supported. We demonstrated a relationship between gill silver accumulation and the degree of inhibition of whole body Na^+ uptake, the principal physiological effect of silver exposure which eventually leads to mortality in rainbow trout.

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Fig. 2-1

Time course of silver accumulation in the gills (A), body (B) and whole body (C) of rainbow trout acutely exposed for 24 h to 1.92 μg L^{-1} total silver as $AgNO_3$ in synthetic soft water. The body was that portion of the rainbow trout remaining after the gills were excised. Values are means ±SEM (n = 8 at each time point except the initial time point where n = 16). Asterisks indicate significant differences from initial values (before silver exposure; ANOVA followed by least significant difference (LSD) test; $p<0.05$).

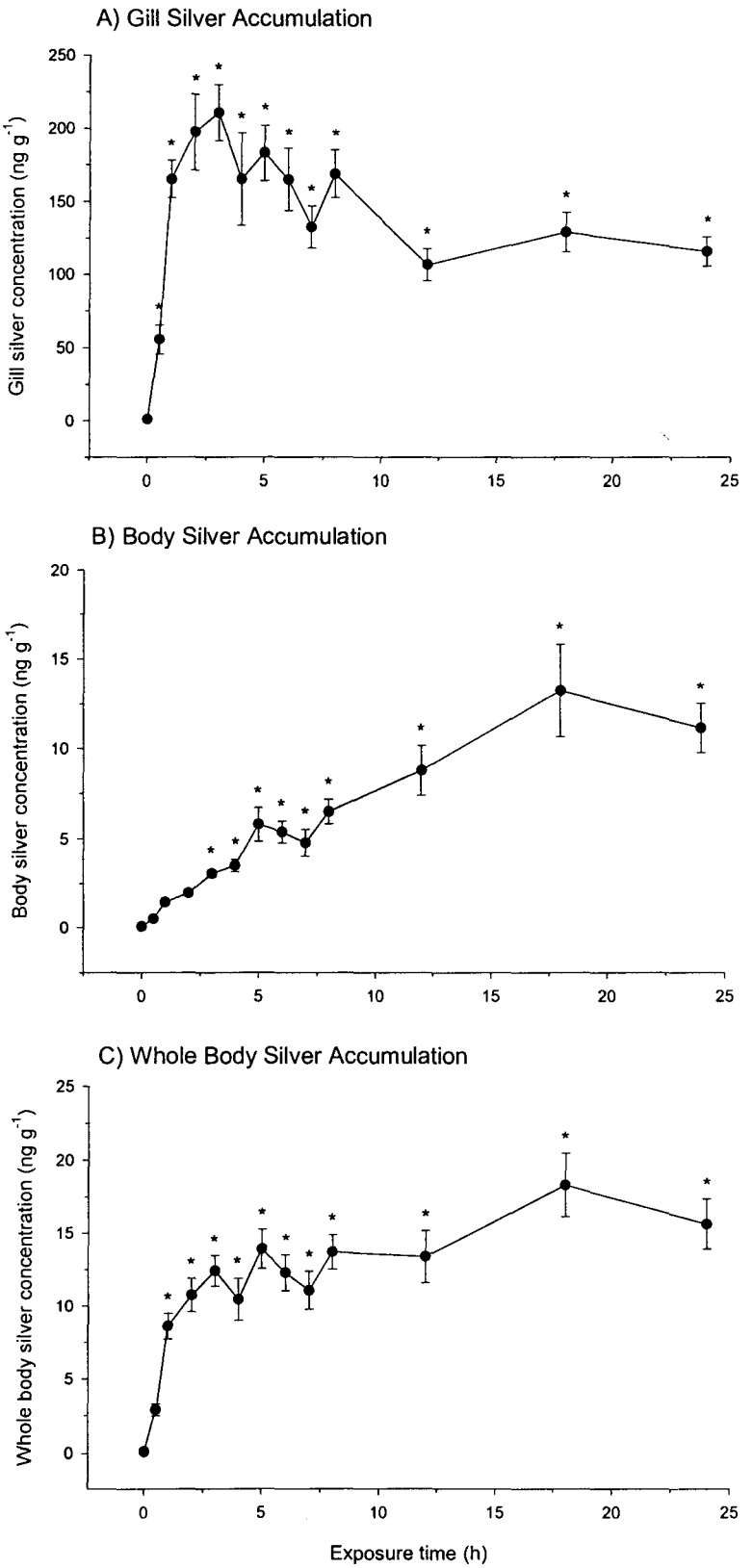

A) Gill Silver Accumulation
Gill silver concentration (ng g-1)
250
200
150
100
50
0
0
5
10
15
20
25
B) Body Silver Accumulation
Body silver concentration (ng g-1)
20
15
10
5
0
0
5
10
15
20
25
C) Whole Body Silver Accumulation
Whole body silver concentration (ng g-1)
25
20
15
10
5
0
0
5
10
15
20
25
Exposure time (h)

Fig. 2-2

Gill (closed circles) and body (open circles) silver burden as a percentage of the whole body silver burden in rainbow trout acutely exposed for up to 24 h to 1.92 μg L^{-1} total silver as $AgNO_3$ in synthetic soft water. The body was that portion of the rainbow trout remaining after the gills were excised. Values are means ±SEM (n = 8 at each time point).

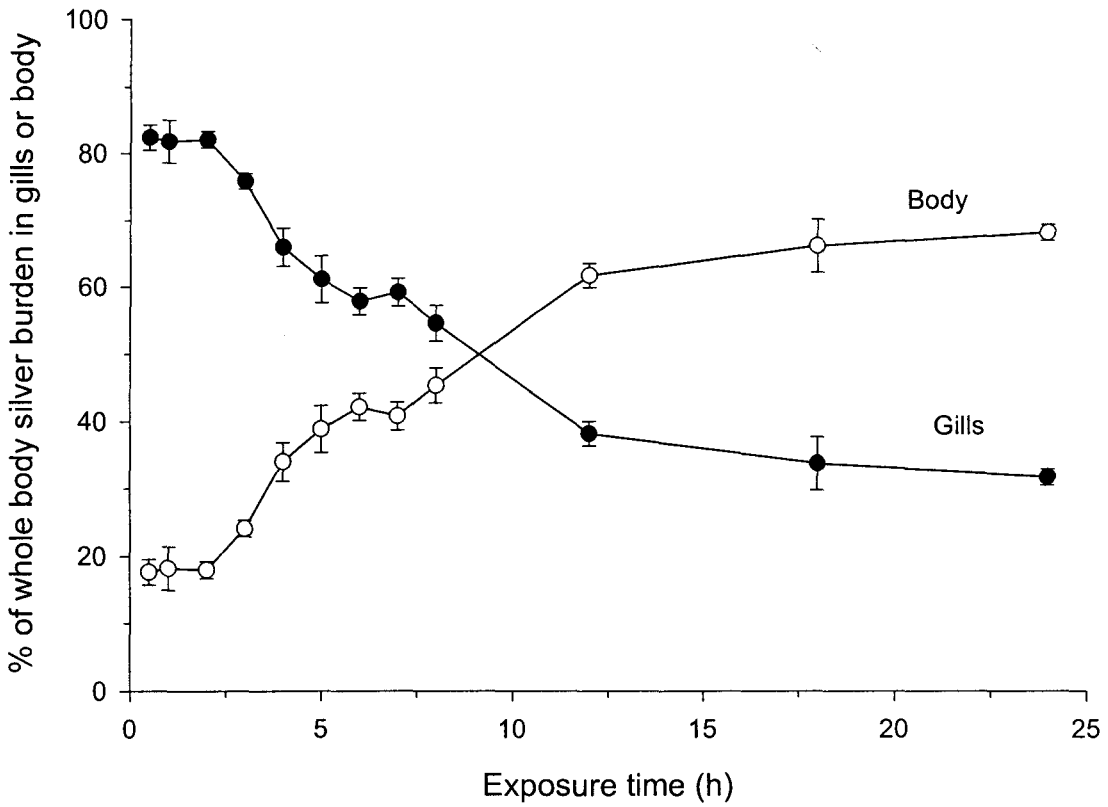

% of whole body silver burden in gills or body
100
80
60
40
20
0
0
5
10
15
20
25
Exposure time (h)
Body
Gills

Fig. 2-3

Time course of inhibition of whole body (closed circles) and body (open circles) Na^+ uptake (A) and gill Na^+K^+-ATPase activity (B) of rainbow trout acutely exposed to 1.92 μg L^{-1} total silver as $AgNO_3$ over 24 h in synthetic soft water. Values are means ±SEM (n = 8 at each time point except the initial time point for (A) and (B) where n = 16 and 14 respectively and the 1 and 3 h time point for (B) where n = 7). Asterisks indicate significant differences from initial values (before silver exposure; ANOVA followed by least significant difference (LSD) test; $p<0.05$).

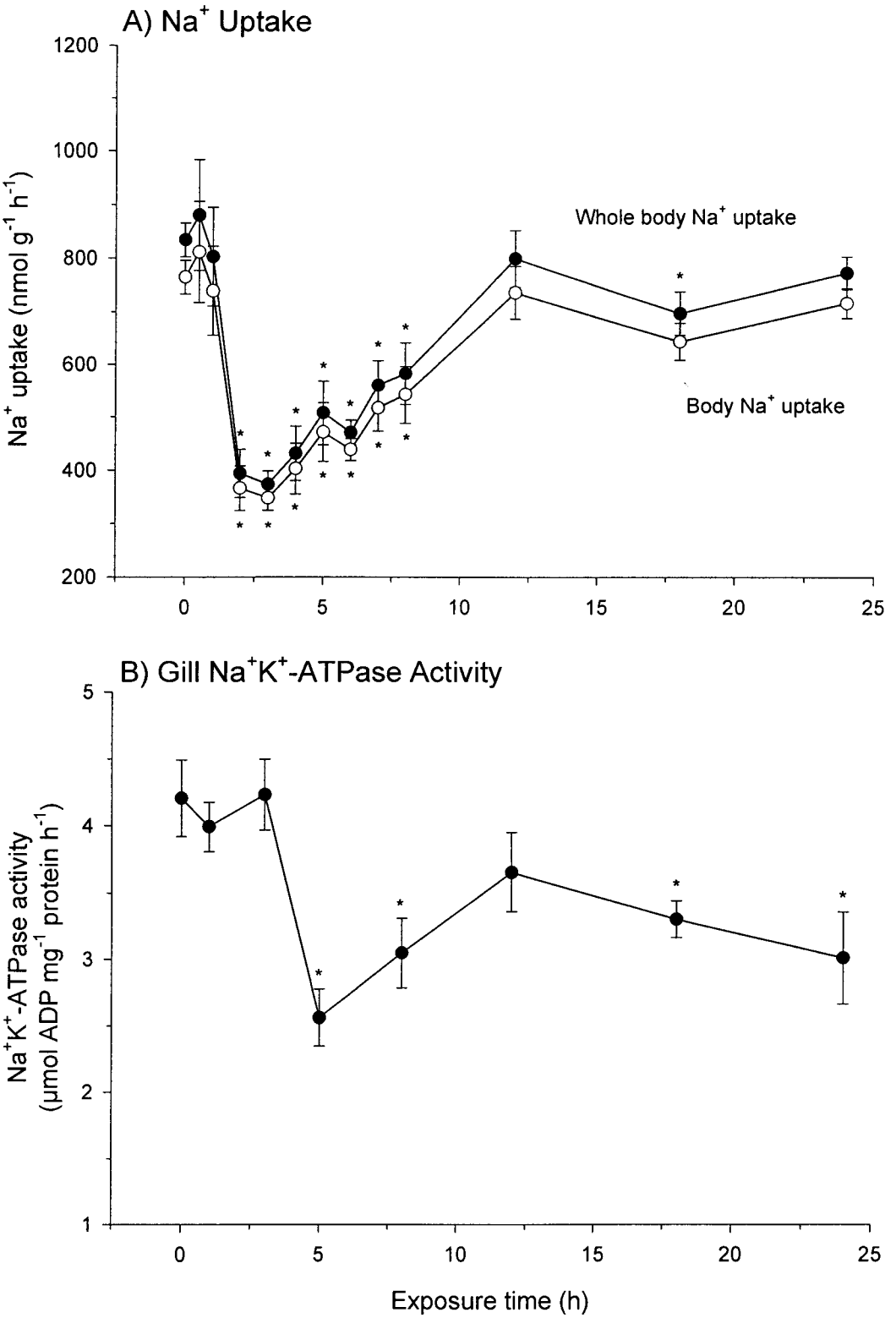

A) Na+ Uptake
Na+ uptake (nmol g-1 h-1)
1200
1000
800
600
400
200
0
5
10
15
20
25
Whole body Na+ uptake
Body Na+ uptake
B) Gill Na+K+-ATPase Activity
Na+K+-ATPase activity (µmol ADP mg-1 protein h-1)
5
4
3
2
1
0
5
10
15
20
25
Exposure time (h)

Fig. 2-4

Changes in the measured water total (closed circles) and dissolved (open circles) silver (A), chloride (B) and TOC (C) concentrations over 24 h of acute exposure of rainbow trout to $AgNO_3$ in synthetic soft water. The dissolved silver concentration is the concentration of silver in the water after passage through a 0.45 μm filter. Values are means ($n = 2$).

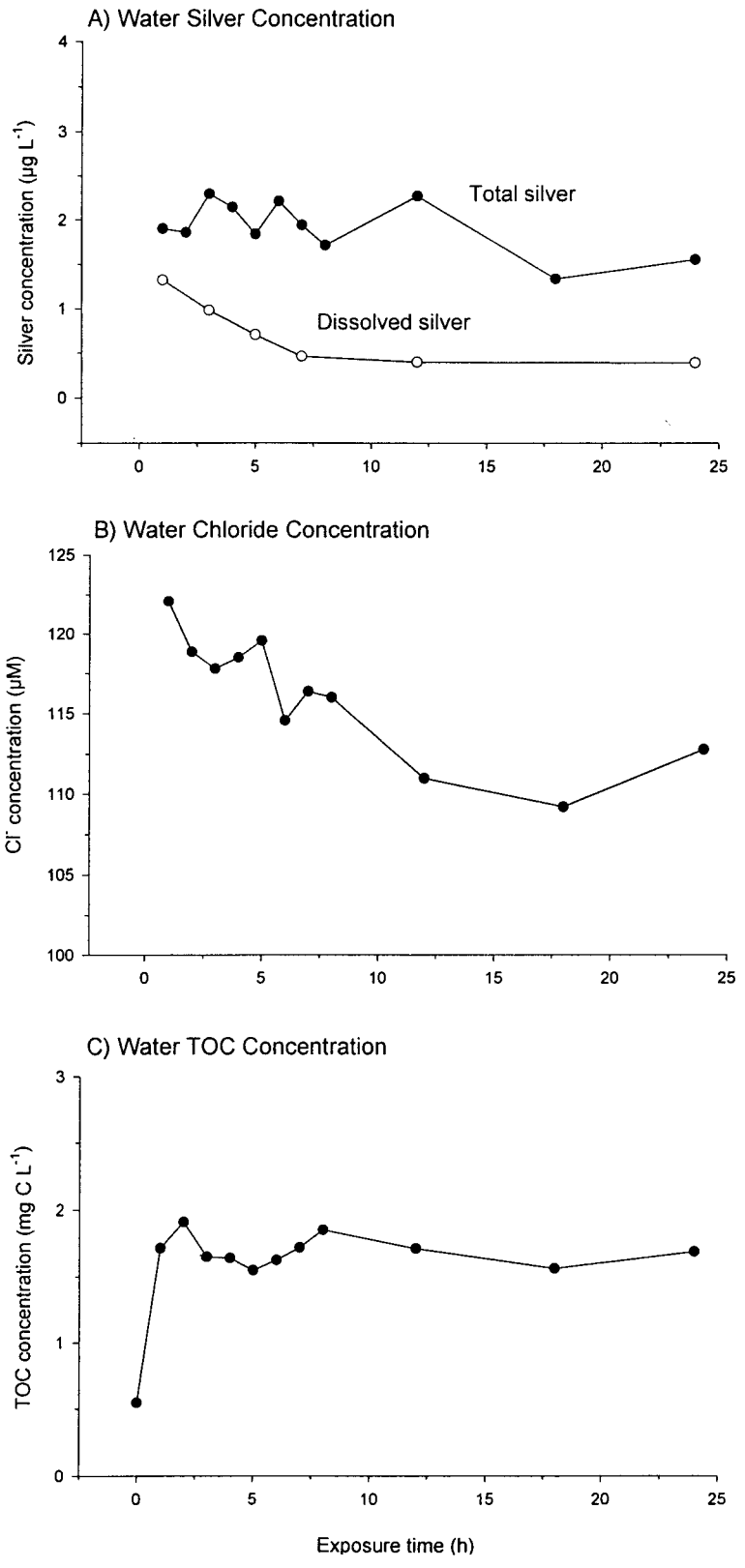

A) Water Silver Concentration
Silver concentration (µg L-1)
Total silver
Dissolved silver
B) Water Chloride Concentration
Cl- concentration (µM)
C) Water TOC Concentration
TOC concentration (mg C L-1)
Exposure time (h)

Fig. 2-5

Apical silver uptake (closed circles) and basolateral silver export (open circles) across the gills of rainbow trout acutely exposed to 1.92 $\mu g\ L^{-1}$ total silver as $AgNO_3$ for 24 h in synthetic soft water. Silver uptake was determined by first calculating silver accumulation at each time point based on the equation of the line of best fit for the whole body and body silver accumulation data (adjusted to whole body weight) and then dividing the change in silver accumulation over each time interval by the length of the time interval.

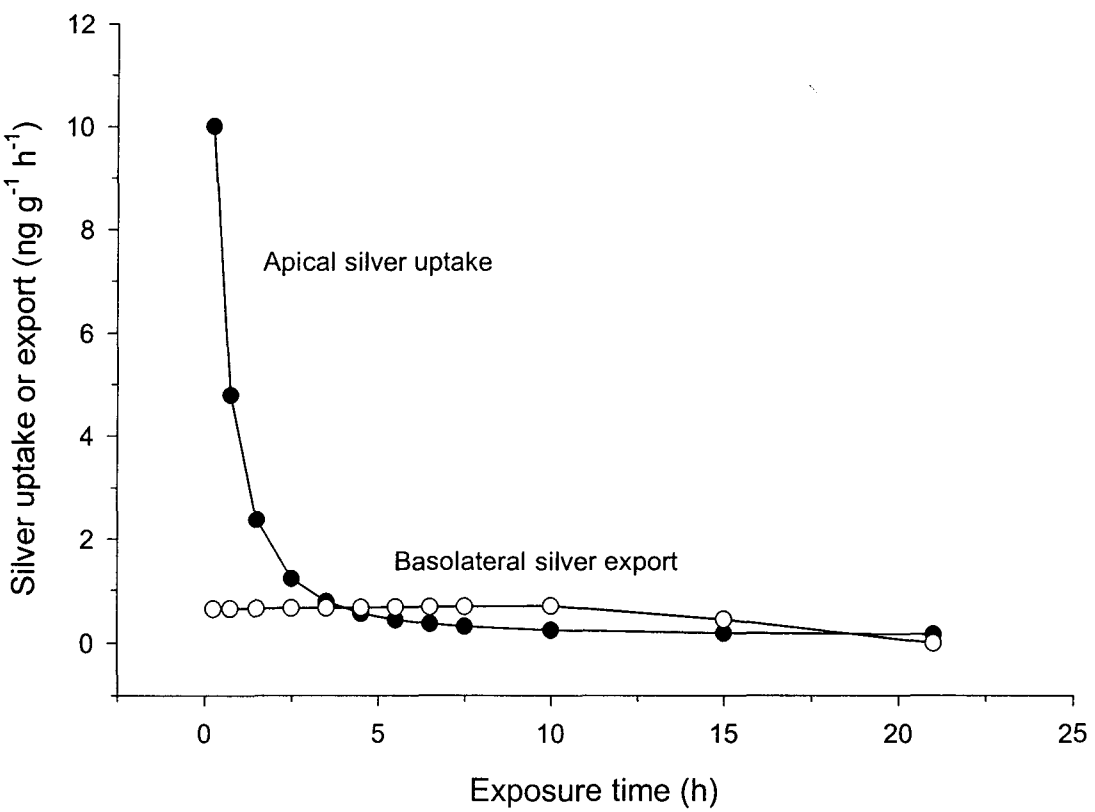
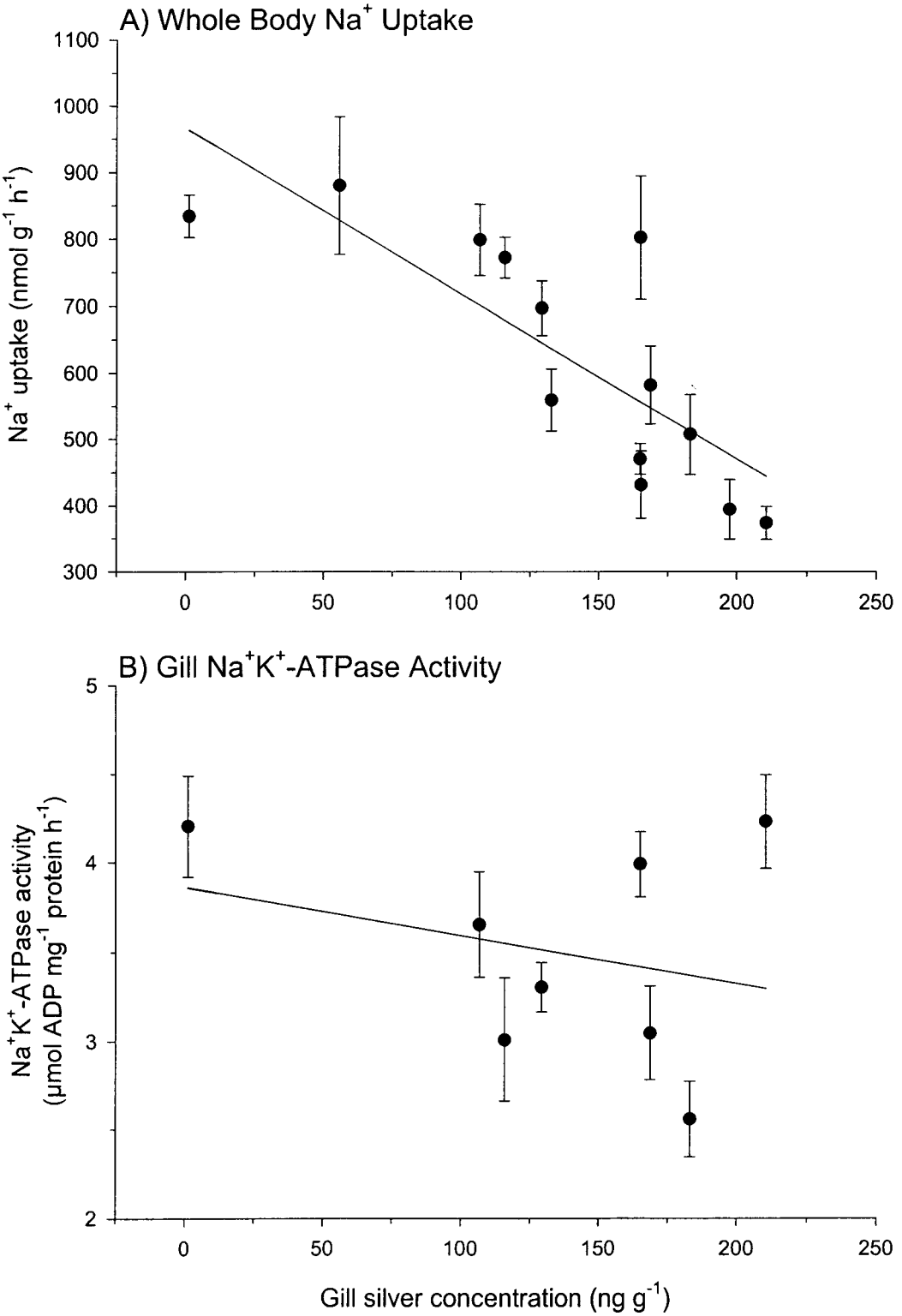

Silver uptake or export (ng g-1 h-1)
12
10
8
6
4
2
0
Apical silver uptake
Basolateral silver export
0
5
10
15
20
25
Exposure time (h)

Fig. 2-6

Plot of whole body Na^+ uptake (A) and gill Na^+K^+-ATPase activity (B) as a function of gill silver concentration. Whole body Na^+ uptake was inversely correlated to the gill silver concentration by the following equation: whole body Na^+ uptake = 966.4 – 2.5 * gill silver concentration, slope significantly different from zero ($p<0.01$), $r^2 = 0.64$. There was no significant correlation between gill Na^+K^+-ATPase activity and gill silver concentration ($r^2 = 0.08$; $p>0.05$).


A) Whole Body Na+ Uptake
Na+ uptake (nmol g-1 h-1)
1100
1000
900
800
700
600
500
400
300
0
50
100
150
200
250
B) Gill Na+K+-ATPase Activity
Na+K+-ATPase activity
(µmol ADP mg-1 protein h-1)
5
4
3
2
0
50
100
150
200
250
Gill silver concentration (ng g-1)

Chapter 3

A Time Course Analysis of Silver Accumulation and Na^+ and Cl^- Uptake Inhibition Under Flow-Through Conditions in Freshwater Rainbow Trout

Abstract

A detailed time course analysis under flow-through conditions examined the mechanism by which silver causes the decline in Na^+ uptake at the gills of the freshwater rainbow trout. During flow-through exposure at a concentration of 4.3 μg/L total silver, Na^+ and Cl^- uptake declined simultaneously and equally initially, with both uptakes reduced by ~500 nmol/g/h by the first hour of silver exposure. There was a further progressive decline in Na^+ uptake until 24 h. Carbonic anhydrase activity was inhibited by 1 h of exposure, whereas Na^+K^+-ATPase activity was not significantly inhibited until 24 h of exposure. These results indicate that carbonic anhydrase inhibition can explain the early decline in Na^+ (and Cl^-) uptake, while the later decline is probably related to Na^+K^+-ATPase blockade. Gill silver accumulation increased steadily to a plateau. Silver bioavailability remained constant and apical silver uptake increased until 10 h, before finally decreasing thereafter. These results suggest that reduced silver bioavailability is the mechanism behind the pattern of peak and decline gill silver accumulation previously reported for static exposures to silver.

Introduction

Waterborne exposure of freshwater rainbow trout to silver results in accumulation of the metal in the gill cells and its appearance in the blood (Wood et al., 1999). Silver, at least in part, enters the cells via the same route as Na^+, presumably by an apical membrane Na^+ channel coupled to an electrogenic proton pump (H^+-ATPase, Bury and Wood, 1999). Transfer of silver across the basolateral membrane and into the blood is at least partly by a carrier-mediated, ATP-dependent mechanism (Bury et al., 1999). Inside the gill cell, silver causes toxicity. In rainbow trout, the free ionic form of silver (Ag^+, as liberated from dissolved $AgNO_3$) is most toxic while various forms of complexed silver, such as $AgCl_{(n)}$ and silver bound to dissolved organic carbon (Ag-DOC), are relatively benign because they decrease the bioavailability of Ag^+ to the fish (Wood et al., 1999). Acute silver toxicity occurs due to silver inhibiting Na^+ uptake at the gill cells, which leads to a net loss of Na^+ by the fish (Morgan et al., 1997). In addition, impaired Cl^- uptake is observed during silver exposure (Morgan et al., 1997). The ensuing ionoregulatory disturbance eventually leads to circulatory collapse and death (Wood et al., 1996; Hogstrand and Wood, 1998). The inhibition of ion uptake is believed to be primarily due to silver binding and inhibiting the gill basolateral membrane Na^+K^+-ATPase (Morgan et al., 1997), the key enzyme powering active Na^+ and Cl^- uptake in freshwater fish (Perry, 1997).

However, a decline in Na^+ uptake occurring before Na^+K^+-ATPase inhibition has recently been demonstrated by us in rainbow trout during silver exposure (Chapter 2). Na^+ uptake was significantly inhibited by 2 h of exposure, although Na^+K^+-ATPase

activity was not inhibited until 5 h of silver exposure, suggesting an alternative reason for the early decline in Na^+ uptake occurring in these fish. In addition to Na^+K^+-ATPase, the gill enzyme carbonic anhydrase (CA) is involved in Na^+ uptake, producing H^+ which is extruded by the H^+-ATPase to drive Na^+ uptake at the apical membrane (Henry and Swenson, 2000). Silver inhibits CA both *in vitro* (Christensen and Tucker, 1976) and *in vivo* (Morgan et al., 1997), so CA inhibition during silver exposure could also alter Na^+ uptake in rainbow trout gills. Because CA has been localized to the intracellular compartment of gill cells, inhibition of the enzyme and hence, a decline in Na^+ uptake might be expected to occur before inhibition of Na^+K^+-ATPase.

The pattern of silver accumulation in rainbow trout gills during static silver exposure is unusual compared to the simple linear or hyperbolic pattern of accumulation of most other metals (Laurén and McDonald, 1986; Giles, 1988; Playle et al., 1993; Hollis et al., 1997; Grosell et al., 1998; Bury and Wood, 1999; Alsop et al., 1999; Wood et al., 2002). During static exposure, silver accumulation increases rapidly, peaks in the first few hours of exposure and then markedly declines despite continued silver exposure (Bury and Wood, 1999; Wood et al., 2002, Chapter 2). It has been suggested that the mechanism behind the pattern of accumulation involves physiological regulation of silver movement across the gill epithelium as a result of Na^+K^+-ATPase inhibition (Wood et al., 2002). A detailed time course experiment did not support this theory, but rather suggested that the mechanism behind the pattern may involve either, (1) a decline in the bioavailability of silver due to complexation by organic matter, a consequence of the use of a static exposure system (Chapter 2) or (2) a blockade event at apical silver entry, such

as CA inhibition or Na^+ channel inhibition. Either mechanism could explain the observed high initial silver uptake rate into the gills followed by the rapid decline in uptake, while basolateral silver export out of the gills to the body remained constant (Chapter 2). Together, these events would lead to a peak and decline in gill silver accumulation.

The primary objective of this study was to determine if CA inhibition by silver was responsible for the early decline in Na^+ uptake that occurs during exposure of rainbow trout to silver. This objective was accomplished by exposing rainbow trout to 4.3 μg/L total silver as $AgNO_3$ for 24 h and performing a detailed time course analysis of the pattern of gill and body silver accumulation, gill and body Na^+ and Cl^- uptake (from which whole body Na^+ and Cl^- uptake were calculated) and gill Na^+K^+-ATPase and CA inhibition. The secondary objective of this study was to determine if reduced silver bioavailability is the mechanism behind the pattern of gill silver accumulation previously observed in rainbow trout during static silver exposures. This objective was accomplished by performing the time course analysis outlined above under flow-through conditions. Under flow-through conditions changes in Ag^+ bioavailability would be minimized as silver is continually renewed by the inflowing water and organic matter produced by the fish is washed away. As such, it was predicted that under these conditions there would be an absence of a peak and decline in gill silver accumulation. In support of this approach, Erickson et al. (1998) found that the organic matter content of the exposure water using flow-through conditions was 0.5 to 1.0 mg C/L lower than that of the exposure water using static conditions. Moreover, the LC50 values for flow-

through exposures were lower than for static exposures (Erickson et al., 1998), suggesting greater Ag^+ bioavailability in flow-through tests.

Materials and Methods

Experimental animals and acclimation

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 0.5 to 3 g, were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). Fish were held in a 200 L polyethylene tank initially supplied with flowing, aerated, dechlorinated Hamilton city tap water (approximate ionic composition in mM: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+], pH 7.8-8.0, DOC ~3 mg C/L, hardness ~140 mg/L as $CaCO_3$ and temperature 14°C). All fish were then acclimated to synthetic soft water over a two week period (generated by reverse osmosis of Hamilton city dechlorinated tap water; Culligan Aqua-Cleer Reverse Osmosis System, Toronto, ON, Canada). During this period the ratio of soft water to tap water that the fish received was increased until the desired ionic composition was achieved. Fish were maintained in this soft water (approximate ionic composition in mM: 0.05 [Na^+], 0.05 [Cl^-], 0.05 [Ca^{2+}], 0.02 [Mg^{2+}] and 0.02 [K^+], pH 7.0, DOC ~0.7 mg C/L, hardness ~10 mg/L as $CaCO_3$ and temperature 16 ± 3 °C) for at least an additional two weeks before experimentation. During soft water acclimation, fish were fed to satiation once daily with commercial trout pellets (Martin Mills, Tavistock, ON, Canada). Feeding was suspended for 1 day before and during the experiment to minimize silver binding to organic matter in uneaten food and waste products during the exposure period.

Experimental Design

After acclimation, 160 fish were transferred to an 80-L polyethylene tank containing 20 L of aerated synthetic soft water (composition as above). The tank was supplied with a continuous flow-through of water at a rate of 130 ml/min. Before the addition of fish to the tank, the walls of the tank were pre-equilibrated with ^{110m}Ag-labeled $AgNO_3$ to ensure saturation of silver binding sites on the tank walls, then the water in the tank was spiked with ^{110m}Ag-labeled $AgNO_3$ (400μCi; specific activity 0.3 μCi/μg Ag; RISOE Nuclear Research Reactor, Roskilde, Denmark) from a light shielded stock bottle to bring the silver immediately up to the exposure concentration. The addition of silver gave a total silver concentration of 3.6 μg/L in the exposure water at the start of the experiment. To maintain the exposure concentration during the experiment, ^{110m}Ag-labeled $AgNO_3$ was added directly to the exposure water from the stock bottle by a peristaltic pump. Vigorous aeration of the exposure water ensured thorough mixing.

Before silver exposure and at 0.5 h, every hour from 1 to 8 h and at 12, 18 and 24 h of silver exposure, eight fish were sampled for gill and body silver accumulation (the body was that portion of the rainbow trout remaining after the gills were excised; via ^{110m}Ag), and gill and body Na^+ and Cl^- uptake (from which whole body Na^+ and Cl^- uptake was calculated; via ^{24}Na and ^{36}Cl; see below for methods of uptake measurements). An additional eight fish were sampled before silver exposure, every hour from 1 to 5 h and at 8, 12 and 24 h of silver exposure for determination of gill Na^+K^+-ATPase and gill carbonic anhydrase activity. All sampled fish (including non-silver exposed controls) were washed in a concentrated solution containing $AgNO_3$ (9.4 mg/L)

and NaCl (2.9 g/L) to remove any loosely bound radioisotopes by displacement and then were killed by an overdose of MS-222 (1 g/L). The gills were obtained by dissection (non-perfused gills were used in this study) and either counted for radioactivity for analysis of silver concentration and whole body ion uptake or frozen immediately in liquid nitrogen and stored at –80°C for later analysis of Na^+K^+-ATPase and carbonic anhydrase activity. The bodies were counted for radioactivity for analysis of silver concentration and whole body ion uptake. At each time point two, 5 ml non-filtered and two, 5 ml filtered (Acrodisc polyethersulfone 0.45 micron syringe filters, Pall Gelman Laboratory, Ann Arbor, MI, USA) water samples were taken and counted for ^{110m}Ag radioactivity for analysis of the total and dissolved silver concentration, respectively. The non-filtered samples were also analyzed for chloride and TOC concentration. Three, 5 ml samples were taken from the silver stock bottle and were acidified with 1% HNO_3 (trace-metal grade; Fisher Scientific, Canada) for determination of ^{110m}Ag radioactivity and total silver concentration to calculate the specific activity (SA) of silver.

Na^+ and Cl^- uptake measurements

For measurement of Na^+ and Cl^- uptake in silver-exposed fish, trout were netted from the exposure water 30 minutes before each sample time (uptake measurements lasted a total of 30 min) and were placed in a 600 ml Pyrex glass beaker containing 300 ml of continuously aerated water to which the fish had been exposed. Before the uptake measurements, two of these beakers were submerged in the exposure water in the exposure tank to ensure saturation of silver-binding sites on the glass beaker so that the concentration of silver in the water would remain constant during the uptake

measurement. For the first uptake measurement, one beaker was removed from the tank, used for the measurement and at the end of the measurement rinsed with water and replaced in the exposure tank. For the second uptake measurement, the second beaker was used. For the third measurement, the first beaker was used and so on. Na^+ and Cl^- uptake measurements were also conducted in fish before silver exposure, but for these measurements the fish were netted from the acclimation tank and were placed in a beaker containing acclimation water. The beaker used for this uptake measurement was not pre-equilibrated in the silver exposure water. ^{24}Na (6.7 μCi; mean SA 0.004 μCi/μg Na^+; McMaster University Nuclear Reactor, Hamilton, ON, Canada) and ^{36}Cl (2.7 μCi; mean SA 0.0013 μCi/μg Cl^-; ICN Radiochemicals, Irvine, CA, USA), were added to the beaker after the addition of fish. Five minutes after the start of the measurement and again at the completion of the measurement, two 5 ml water samples were taken for measurement of water ^{24}Na and ^{36}Cl radioactivity and total [Na^+] and [Cl^-]. At the end of the measurement the fish were sampled as described above and counted for ^{24}Na and ^{36}Cl radioactivity.

Analytical techniques and calculations

During the experiment fish were exposed to three different isotopes: ^{110m}Ag, ^{24}Na and ^{36}Cl. Isotopes ^{110m}Ag and ^{24}Na are mixed γ- and β- emitters while ^{36}Cl is a pure β-emitter. To determine the ^{110m}Ag radioactivity in the gills, body and water, the samples were initially counted for the sum of ^{110m}Ag and ^{24}Na by gamma counting. ^{24}Na was then allowed to decay (approximately two weeks; 15 h half-life) and the samples were counted again by gamma counting to give the ^{110m}Ag radioactivity (256 d half-life). Gamma

counting was done according to Hansen et al. (2002) using a MINAXI Auto-Gamma 5000 gamma counter (Canberra-Packard, Toronto, ON, Canada) with an energy window of 1050 to 2000 keV. To determine the ^{24}Na radioactivity in the gills, body and water, the ^{110m}Ag radioactivity, determined after decay of ^{24}Na, was subtracted from the initial sum of ^{24}Na and ^{110m}Ag radioactivity. The ^{24}Na activity was then corrected for decay to a common reference time. The samples were counted a third time by beta counting (LKB-Wallac 1217 Rackbeta Liquid Scintillation Counter, Turku, Finland) to give the ^{110m}Ag plus ^{36}Cl radioactivity (300,000 y half-life). ^{36}Cl radioactivity in the gills, body and water was obtained by subtracting the ^{110m}Ag radioactivity (after accounting for differences in efficiency of ^{110m}Ag counting by the two instruments) from the sum of ^{110m}Ag and ^{36}Cl radioactivity.

The concentrations of silver in the gills, body and water were calculated by dividing their ^{110m}Ag radioactivities by the SA of silver. The SA of silver was calculated by dividing the ^{110m}Ag radioactivity of the silver stock solution by the total silver concentration as measured by graphite furnace atomic absorption spectrophotometry (Varian AA-1275 with GTA atomizer, Varian Ltd., Mississauga, ON, Canada).

Water chloride was measured using the colorimetric assay of Zall et al. (1956) while TOC was measured with a Shimadzu TOC-5050A total organic carbon analyzer (Mandel Scientific Co. Ltd., Guelph, ON, Canada). TOC is the sum of particulate organic carbon (POC) and dissolved organic carbon (DOC). Water Na^+ concentration was determined by flame atomic absorption spectrophotometry (Varian AA-1275, Varian Ltd., Mississauga, ON, Canada).

Uptake rates for sodium and chloride were determined by monitoring the appearance of radioisotopes in the fish (gill or body) from the water as described above and were calculated as follows:

Gill or body ion (Na^+ or Cl^-) uptake + $CT/(SA \cdot wt \cdot t)$

where CT is the total counts per min (cpm) in the gills or body, SA is the measured specific activity of the water, wt is the wet weight of the gills or body (g) and t is the time of exposure (h). The specific activity of the water was calculated as follows:

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

where cpm_i represents the cpm per ml initially in the water, cpm_f represents the final cpm per ml in the water and $[ion]_i$ and $[ion]_f$ represent the initial and final ion concentrations of the water, respectively. Whole body uptake of Na^+ and Cl^- was calculated by adding the gill to the body Na^+ or Cl^- uptake and correcting for whole body weight.

Gills obtained for Na^+K^+-ATPase and CA activity determination were homogenized in 500µl of SEID buffer (0.5 g of sodium deoxycholate in 100 ml of SEI; SEI = 150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) using a Teflon-glass homogenizer. All preparations were kept on ice during the homogenization process. The homogenates were subsequently assayed for Na^+K^+-ATPase activity using the microplate method of McCormick (1993) and gill CA activity was assayed using the electrometric

delta pH method as described by Henry (1991). Because non-perfused gills were used in this study, the CA activity measured reflects both gill and red blood cell (RBC) activities. Gill Na^+K^+-ATPase and CA activities were standardized to protein content, which was measured using the Bradford assay (kit no. B6916, Sigma, Canada) with bovine serum albumin as a standard (Sigma, Canada).

Statistical analyses

Data have been expressed as means ±SEM throughout, except for the water silver, chloride and TOC concentrations where only the mean of two samples is reported. All comparisons were made using a one-way ANOVA followed by a least significant difference (LSD) test (SPSS 10 for Windows). Regression analysis was done using SPSS 10 for Windows. A significance level of $p<0.05$ was used throughout.

Results

Water silver concentrations

The mean measured total and dissolved water silver concentrations over the 24 h flow-through exposure period were 4.32 ±0.09 μg/L and 3.02 ±0.10 μg/L respectively.

Silver accumulation

Gill silver accumulation increased steadily over the first 18 h of silver exposure, reaching a plateau of ~2000 ng/g gill wet wt. by 24 h of exposure (Fig. 3-1A). Body silver accumulation increased continually throughout the exposure reaching ~110 ng/g by 24 h of silver exposure (Fig. 3-1B). Whole body silver accumulation increased steadily to a plateau of ~175 ng/g at 24 h of silver exposure (Fig. 3-1C). Note the use of three different scales for silver accumulation in Figure 1.

The contribution of the gills to the whole body silver burden decreased from 62% to 41% over approximately the first 5 h of exposure, while the contribution of the body increased from 38% to 59% (Fig. 3-2), demonstrating movement of silver across the gill epithelium and into the body with time. Thereafter, the proportional distribution of the silver burden was stable.

Na^+ and Cl^- uptake

Initially there was a large and rapid inhibition of whole body Na^+ uptake. Uptake was reduced from 1550.4 ±131.6 nmol/g/h to 997.5 ±66.6 nmol/g/h over the first hour of exposure, a reduction of ~500 nmol/g/h. Uptake remained at this rate for the subsequent 2 h and was followed by another progressive inhibition over the next 21 h to only 136.6 ±26.9 nmol/g/h (Fig. 3-3A). Body Na^+ uptake inhibition showed a similar biphasic trend (Fig. 3-3A).

The time course of whole body Cl^- uptake inhibition was similar to the first phase of the time course of Na^+ uptake inhibition, with uptake rapidly inhibited over the first hour of silver exposure but reaching a plateau by 2 h of exposure. The degree of whole body Cl^- uptake inhibition was also similar, with uptake reduced by ~500 nmol/g/h over the first hour of exposure (from 521.5 ±166.3 nmol/g/h to 40.9 ±10.0 nmol/g/h; Fig. 3-3B). The pattern of body Cl^- uptake inhibition was very similar to the pattern of whole body Cl^- uptake inhibition (Fig. 3-3B).

Gill Na^+K^+-ATPase and CA activity

Gill Na^+K^+-ATPase activity decreased steadily over the exposure period but was not significantly inhibited until 24 h of silver exposure. At this time activity was

inhibited by 41% (from 2.7 ±0.5 before silver exposure to 1.6 ±0.2 μmol ADP/mg protein/h at 24 h of exposure; Fig 3-4A).

Gill CA activity decreased rapidly over the first 2 h of silver exposure and remained inhibited over the following 22 h of exposure apart from an apparent increase in activity between 5 and 8 h of exposure (Fig. 3-4B). Inhibition was first significant by 1 h of silver exposure reaching about 30% (from 1421.1 ±119.1 before silver exposure to 952.6 ±100.2 μmol CO_2/mg protein/min at 1 h of silver exposure). Maximal inhibition of the enzyme occurred at 24 h of exposure with activity inhibited by 42% (from 1421.1 ±119.1 to 817.9 ±145.5 μmol CO_2/mg protein/min; Fig. 3-4B).

Na^+ uptake and enzyme activities versus gill silver accumulation

Na^+ uptake appeared to vary inversely with gill silver accumulation. As gill silver accumulation increased, Na^+ uptake decreased (compare Fig. 3-1A and Fig. 3-3A). Indeed, plotting values of whole body Na^+ uptake as a function of corresponding values of gill silver accumulation revealed a significant negative relationship ($r^2 = 0.97$, $p<0.05$; Fig. 3-5A).

At least over the first 12 h of silver exposure, gill Na^+K^+-ATPase activity also appeared to vary inversely with gill silver accumulation (compare Fig. 3-1A and Fig. 3-4A). Regression analysis revealed a significant negative relationship between these two parameters ($r^2 = 0.85$, $p<0.05$; Fig. 3-5B).

There was no significant negative relationship between gill silver accumulation and CA activity ($r^2 = 0.3$, $p>0.05$; Fig. 3-5C).

Water chemistry changes

The water total silver concentration remained relatively constant over the 24 h exposure period at about 4 μg/L, apart from an increase in the concentration of 47% over the first half-hour of exposure (from 3.6 to 5.3 μg/L; Fig. 3-6A). Other than a small initial decrease of 23% over the first hour of silver exposure (from 3.1 to 2.4 μg/L), the water dissolved silver concentration also remained relatively constant over the exposure period at about 3 μg/L (Fig. 3-6A).

There was a small increase in the water Cl^- concentration of 13% over the first 2 h of silver exposure (from 122.6 to 138.0 μM). The concentration remained elevated until 8 h of exposure but returned to the initial level by 12 h of silver exposure (Fig. 3-6B).

The TOC content of the water increased by 67% over the first 4 h of silver exposure (from 1.2 to 2.0 mg C/L). By 5 h of exposure and for the remaining 19 h of exposure the TOC content was not different from the initial value (Fig. 3-6C).

Discussion

The mechanism by which silver inhibits Na^+ and Cl^- uptake at the gills of rainbow trout

Na^+ uptake declined very clearly before Na^+K^+-ATPase activity during flow-through exposure of rainbow trout to 4.3 μg/L total silver (Fig. 3-3A and Fig. 3-4A). We have demonstrated a similar trend during static exposure of rainbow trout to a lower silver concentration (Chapter 2).

The present study provides three pieces of evidence that the early fall in Na^+ uptake is due to an inhibition of CA activity. First, the time courses of inhibition of whole body Na^+ uptake (apical uptake) and body Na^+ uptake (basolateral export) were

identical (Fig. 3-3A), and the time courses of inhibition of whole body Cl^- uptake (apical uptake) and body Cl^- uptake (basolateral export) were identical (Fig. 3-3B). An identical time course implies that the rate-limiting step in the movement of these ions across the whole gill epithelium during silver exposure is the movement across the apical membrane (rather than the basolateral membrane) and implicates CA inhibition in the early decline in Na^+ uptake because the activity of CA provides H^+ and HCO_3^- for apical uptake of Na^+ and Cl^-, respectively. Second, the time course and degree of Cl^- uptake inhibition were quantitatively similar to the time course and degree of the first phase of Na^+ uptake inhibition (Fig. 3-3A and Fig. 3-3B). Inhibition of CA would be expected to cause a simultaneous and equal inhibition of Na^+ and Cl^- uptake because, as outlined above, the activity of CA provides protons to the H^+-ATPase for apical uptake of Na^+ and HCO_3^- for apical exchange with Cl^- (Henry and Swenson, 2000). Third, CA activity was inhibited by ~30% by 1 h of silver exposure (Fig. 3-4B), at a time when Na^+ uptake and Cl^- uptake were decreasing but Na^+K^+-ATPase activity was not significantly inhibited (Fig. 3-4A).

Morgan et al. (1997) saw a similar degree of inhibition of CA activity during exposure of rainbow trout to 10 µg/L silver (~30%) and a simultaneous and equal inhibition of Na^+ and Cl^- uptake, but only measured CA activity at 48 h. In our study, CA activity was almost maximally inhibited by 1 h of exposure and remained inhibited at 24 h of exposure (Fig. 3-4B). This suggests that the degree of inhibition of CA activity early in the exposure of Morgan et al. (1997) would be similar to the degree of inhibition reported at 48 h and could explain the simultaneous and equal decreases of Na^+ and Cl^- uptake which were similarly noted in their study.

The mechanism by which silver inhibits CA is not known, but the often striking similarities between copper and silver in terms of physiology and toxicology (Hogstrand and Wood, 1998) suggest that silver would inhibit CA by the same or a similar mechanism by which copper inhibits the enzyme. Studies with human carbonic anhydrase II (CA II) have shown that copper inhibits the enzyme by binding to His-64, an amino acid located in the active site of the enzyme (Tu et al., 1981; Tu and Silverman, 1989). Binding of copper to His-64 blocks its role in the release of a proton from the active site, preventing regeneration of the active form of the enzyme. Properties exhibited by the CA of gills of rainbow trout are similar to the those exhibited by human CA II (Henry et al., 1993), suggesting that copper (and potentially silver) would inhibit gill CA by the same mechanism by which it inhibits human CA II.

Although the results of this study implicate CA inhibition in the early decline in Na^+ uptake (phase 1; Fig. 3-3A), the decline in Na^+ uptake that occurred later (phase 2; Fig. 3-3A) is likely due to Na^+K^+-ATPase inhibition. Because Na^+K^+-ATPase is located on the basolateral membrane, it would take some time for silver to reach and inhibit the enzyme. However, Na^+K^+-ATPase activity was not significantly inhibited until 24 h of silver exposure yet silver had clearly reached the basolateral membrane after only a few hours (Fig. 3-7). This suggests that there may be a kinetic limitation affecting the time course of Na^+K^+-ATPase inhibition. Nevertheless, Na^+K^+-ATPase activity was clearly decreasing during phase two of Na^+ uptake inhibition (Fig. 3-3A and Fig. 3-4A). Interestingly, although both Na^+ and Cl^- uptake were reduced to a similar degree (~500 nmol/g/h) over the first hour of silver exposure, the reductions represented a 36% and

92% inhibition in Na^+ and Cl^- uptake, respectively. The immediate, almost complete inhibition in Cl^- uptake suggests that, in contrast to Na^+ uptake, inhibition of Na^+K^+-ATPase does not contribute to the decline in Cl^- uptake observed during silver exposure.

An additional mechanism of gill silver uptake in rainbow trout?

By fitting a line of best fit to the whole body (apical silver uptake; Fig. 3-1C) and body (basolateral silver export; Fig. 3-1B) silver accumulation data, we were able to calculate the rates of apical silver uptake and basolateral silver export from the gills (Fig. 3-7). This was done by first using the equation of the line of best fit to determine silver accumulation at each time point and then dividing the change in silver accumulation over each time interval by the length of the time interval. The calculated rates were expressed as rates per whole body weight and were plotted at the midpoint of the time interval.

Because silver is thought to enter gill cells by the same mechanism as Na^+ (Bury and Wood, 1999), the decline in Na^+ uptake over time caused by CA and Na^+K^+-ATPase inhibition (Fig. 3-3A) was expected to be paralleled by a decline in silver uptake. However, apical silver uptake increased steadily initially and did not begin to decrease until 10 h of silver exposure (Fig. 3-7). The increase in uptake was probably not due to AgCl entry as speciation analysis using MINEQL+ showed that AgCl was less than 3% of the total silver concentration. The increasing silver uptake was surprising, but silver could have continued to enter via the Na^+ channel if silver uptake does not depend on the same driving force for entry as Na^+. Alternatively or additionally, if multiple pathways exist for apical silver uptake at the gill cells (in addition to the Na^+ channel), than silver uptake may not decrease unless more than one pathway is disrupted. Bury and Wood

(1999) demonstrated that approximately one third of silver uptake continued in the presence of phenamil and bafilomycin A_1 (Na^+ channel and V-type ATPase blocker, respectively) or high levels of competing Na^+ in the water, suggesting that in addition to the proton-coupled Na^+ channel, other apical uptake pathways for silver exist. Recent evidence that silver may replace copper in some transport processes in bacteria (Solioz and Odermatt, 1995) suggests that the other apical uptake pathway for silver may involve a copper transporter. The CTR-type copper transporter, although as of yet unidentified in teleost fish gills, is a potential mechanism for apical copper (and silver) uptake in rainbow trout because this transporter has been documented in species from yeast to humans (Camakaris et al., 1999). Recently, Grosell and Wood (2002) identified two pathways of apical copper uptake in freshwater trout gills, a Na^+-sensitive, phenamil-sensitive, bafilomycin-sensitive pathway (likely the Na^+ channel) and a Na^+ insensitive pathway, possibly the CTR-type transporter. By analogy, these pathways could also be responsible for apical silver uptake.

The time course of gill silver accumulation during flow-through silver exposure

The results of Chapter 2 suggested that the pattern of gill silver accumulation observed in rainbow trout during static silver exposure could be due to a decrease in the bioavailability of silver. The results of this study support this idea: during the flow-through exposure of the present study, the bioavailability of silver remained relatively constant and there was no pattern of a peak and decline in gill silver accumulation, rather gill silver levels increased steadily to a plateau with time (Fig. 3-1A). This pattern of accumulation is similar to that which has been reported for other metals such as copper,

cadmium and zinc (Laurén and McDonald, 1986; Giles, 1988; Playle et al., 1993; Hollis et al., 1997; Grosell et al., 1998; Alsop et al., 1999).

Evidence for relatively constant silver bioavailability over the flow-through exposure comes from the observation that there were only initial, temporary changes in the chemistry of the exposure water over time. The dissolved silver concentration decreased, while the Cl^- and TOC concentrations increased over the first few hours of exposure but initial levels were re-established with continued flow-through exposure (Fig. 3-6). This suggests that silver was not being rendered non-dissolved and/or non-bioavailable due to silver complexation by accumulated organic matter or chloride. The relatively small increase in Cl^- was likely due to reduced Cl^- uptake by the fish.

The bioavailability of silver and hence, the pattern of gill silver accumulation is different between the flow-through exposure of this study and the static exposure of Chapter 2 because of the difference in test water renewal between the two exposures. During the static study, the exposure water was not continually replaced over the exposure period, resulting in the accumulation of organic matter and a decrease in the bioavailability of Ag^+. This caused a fall in apical silver uptake and together with constant basolateral export of silver, a rapid peak and following decline in gill silver accumulation. However, during the present flow-through study, the exposure water was continually replaced by the inflowing water, and organic matter produced by the fish was washed away. In addition, silver was continually added to the exposure water by the inflowing water, replacing any Ag^+ lost due to complexation. As such, the bioavailability of Ag^+ remained relatively constant, and apical silver uptake did not quickly decline, but

rather initially increased before finally starting to fall at 10 h (Fig. 3-7), leading to an increase in gill silver accumulation to a plateau over time (Fig. 3-1A).

Toxicological implications

Under static conditions, whole body Na^+ uptake rapidly declined but recovered with continued silver exposure (Chapter 2). This pattern of Na^+ uptake inhibition was unlike that under the flow-through conditions of the present study where whole body Na^+ uptake decreased in two phases, and there was no sign of recovery of Na^+ uptake with continued silver exposure (Fig. 3-3A). As the bioavailability of Ag^+ decreases with organic matter accumulation during static exposure, the degree of inhibition of Na^+ uptake by Ag^+ is reduced, allowing recovery of whole body Na^+ uptake despite continued silver exposure. Because Ag^+ bioavailability presumably was not reduced over time during the present study, the degree of inhibition of Na^+ uptake by Ag^+ was not reduced, but rather persisted with continued silver exposure.

The difference in the time course of Na^+ uptake inhibition between static and flow-through exposures has toxicological implications. Because static exposures show recovery of gill function with continued silver exposure, these exposures may underestimate the toxic effects of silver. In fact, Nebeker et al. (1983) and Erickson et al. (1998) reported differences in silver toxicity between static and flow-through tests. Flow-through LC50 values were lower than static LC50 values for rainbow trout and fathead minnow (i.e. silver was more toxic under flow-through conditions). Those workers attributed the difference in LC50 values between the two tests to the accumulation of organic matter in the static exposure system. This, together with the

differences in gill silver accumulation between the two types of exposures indicates that the exposure conditions must be considered when assessing the risk of silver to freshwater fish in natural waters. Results of flow-through tests probably are more representative of natural freshwater lotic habitats.

Implications for the silver biotic ligand models (BLMs)

In this study, a strong negative relationship existed between gill silver accumulation and whole body Na^+ uptake over 24 h of silver exposure under flow-through conditions (Fig. 3-5A). A negative relationship has been demonstrated before over the same time frame (Chapter 2). In contrast, McGeer and Wood (1998) did not find a correlation between gill silver accumulation and Na^+ balance, but only compared the two at 48 h of exposure. The longer-term silver exposure of McGeer and Wood (1998) may have obscured the relationship between gill silver accumulation and Na^+ uptake because accumulation of silver likely occurred at physiologically inert as well as physiologically active sites (Paquin et al., 1999). During the shorter-term silver exposure of this study, accumulation of silver likely occurred only at physiologically active sites, resulting in the significant, negative relationship between short-term gill silver accumulation and whole body Na^+ uptake. A negative relationship also existed between gill silver accumulation and Na^+K^+-ATPase activity (Fig. 3-5B). In total these observations suggest that there is also a relationship between gill silver accumulation and mortality, because inhibition of Na^+ uptake and Na^+K^+-ATPase activity are causes of eventual mortality in rainbow trout (Wood et al., 1996; Morgan et al., 1997; Wood et al., 1999).

These findings, together with the observation that an equilibrium level of silver accumulation at the gills is achieved over time during flow-through exposure (Fig. 3-1A), lend support to the biotic ligand model (BLM) approach for predicting silver toxicity. The silver BLM attempts to predict mortality in different water qualities by predicting gill silver accumulation (Paquin et al., 1999). Because silver accumulation on the gills appeared to constantly change over time and silver accumulation on the gills had not been satisfactorily correlated with mortality, the present silver version of the BLM (Paquin et al., 1999) has not been accepted for use as a regulatory tool. Although the results of the present study suggest a relationship between gill silver accumulation and mortality, definitive evidence would come from experiments establishing a direct relationship between short-term gill silver accumulation and eventual mortality. For example, MacRae et al. (1999) were able to demonstrate a relationship between gill copper accumulation at 24 h and mortality of juvenile rainbow trout after 120 h of copper exposure, a finding which was instrumental in the acceptance of the copper version of the BLM (Santore et al., 2001). Demonstration of such a relationship for silver together with the results presented here would help promote incorporation of the silver BLM into regulatory practice.

A physiologically based BLM also exists for silver (McGeer et al., 2000). The model was developed to provide a mechanistically based method for predicting acute silver toxicity and uses Na^+K^+-ATPase inhibition as an endpoint, the mechanism believed to be wholly responsible for the decline in Na^+ uptake that leads to mortality in rainbow trout. However, as shown in this study, CA inhibition contributes to the decline in Na^+

uptake observed in these fish (Fig. 3-3A and Fig. 3-4B). As such, the model could be improved by using Na^+ uptake inhibition as the endpoint, because Na^+ uptake inhibition includes the role of both CA and Na^+K^+-ATPase inhibition in the acute toxicity of silver.

Perspectives

Silver inhibits Na^+ and Cl^- uptake at the gills of freshwater rainbow trout (Morgan et al., 1997). Inhibition of the gill basolateral membrane Na^+K^+-ATPase has generally been believed to be responsible for the inhibition of Na^+ and Cl^- uptake that occurs in these fish (Wood et al., 1999) and as such, research on the toxicity of silver has focused on this enzyme. The present study provides evidence that gill carbonic anhydrase inhibition is also involved in the inhibition of ion uptake, explaining the early decline in Na^+ and Cl^- uptake that occurs during silver exposure and suggesting that the role of this enzyme in silver toxicity should be considered in future studies. In addition, the present study supports the idea that the mechanism behind the previously reported pattern of gill silver accumulation (Chapter 2) involves reduced silver bioavailability during static silver exposures. Silver bioavailability was not reduced under the flow-through conditions of the present study and therefore there was no pattern of a peak and decline in gill silver accumulation. Rather, gill silver increased steadily to a plateau, a pattern of accumulation similar to that of other metals (Laurén and McDonald, 1986; Giles, 1988; Playle et al., 1993; Hollis et al., 1997; Grosell et al., 1998; Alsop et al., 1999). The differences in the patterns of silver accumulation and of Na^+ uptake inhibition between flow-through and static exposures suggests that care must be taken when interpreting the

results of static tests, because flow-through exposures are probably more representative of natural freshwater lotic environments.

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Fig. 3-1

Time course of silver accumulation in the (A) gills, (B) body and (C) whole body of rainbow trout exposed to 4.3 μg/L total silver as $AgNO_3$ for 24 h in synthetic soft water under flow-through conditions. The body was that portion of the rainbow trout remaining after the gills were excised and the whole body is the sum of the gills and body. Values are means ±SEM (n = 8 at each time point). Asterisks indicate significant differences from the initial value (before silver exposure; ANOVA followed by least significant difference (LSD) test; $p<0.05$).

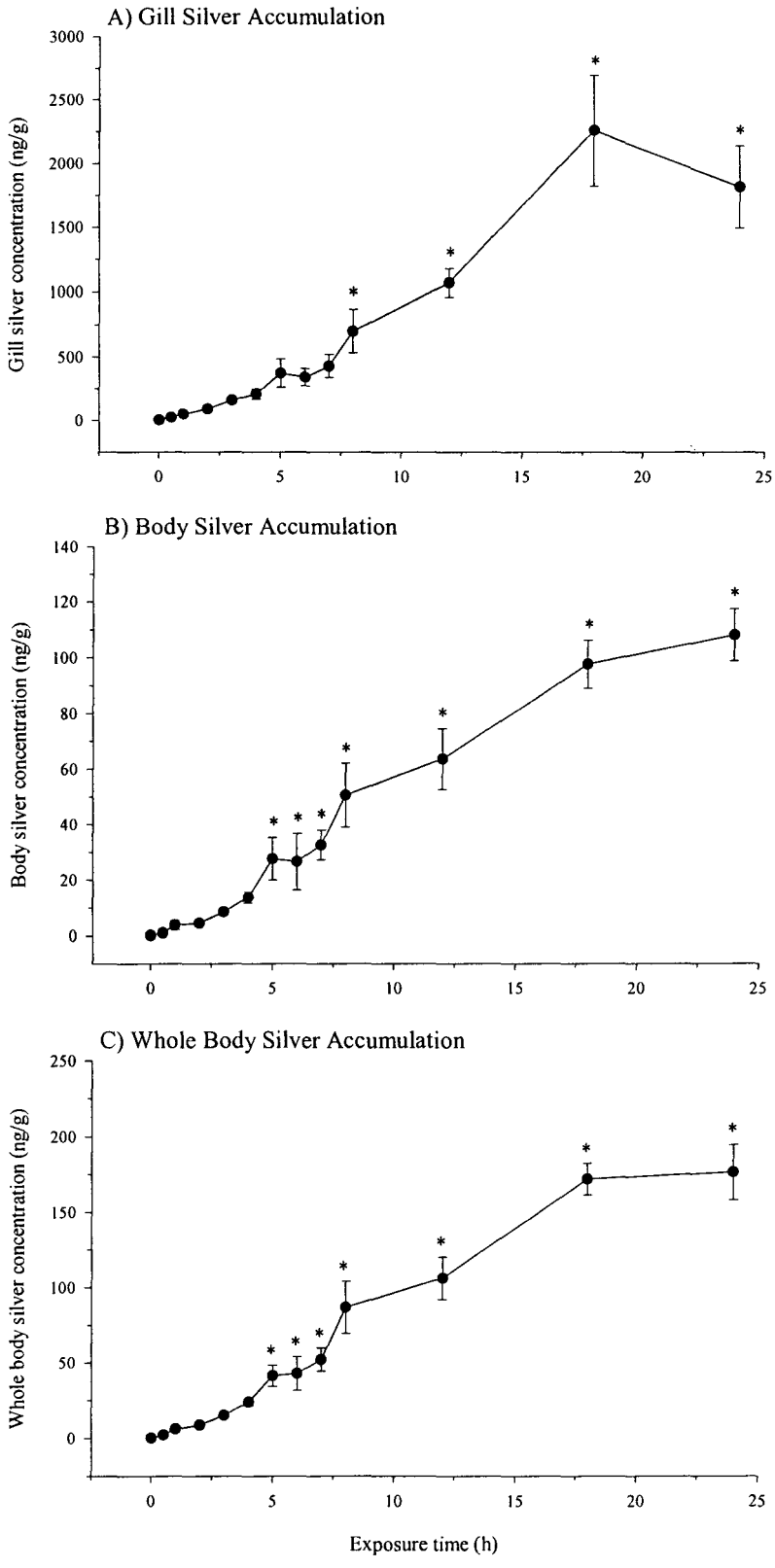

A) Gill Silver Accumulation
Gill silver concentration (ng/g)
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
B) Body Silver Accumulation
Body silver concentration (ng/g)
140
120
100
80
60
40
20
0
C) Whole Body Silver Accumulation
Whole body silver concentration (ng/g)
250
200
150
100
50
0
Exposure time (h)

Fig. 3-2

Gill (closed circles) and body (open circles) silver burden as a percentage of the whole body silver burden during flow-through exposure of rainbow trout to 4.3 μg/L total silver as $AgNO_3$ for 24 h in synthetic soft water. The body was that portion of the rainbow trout remaining after the gills were excised. Values are means ±SEM (n = 8 at each time point).

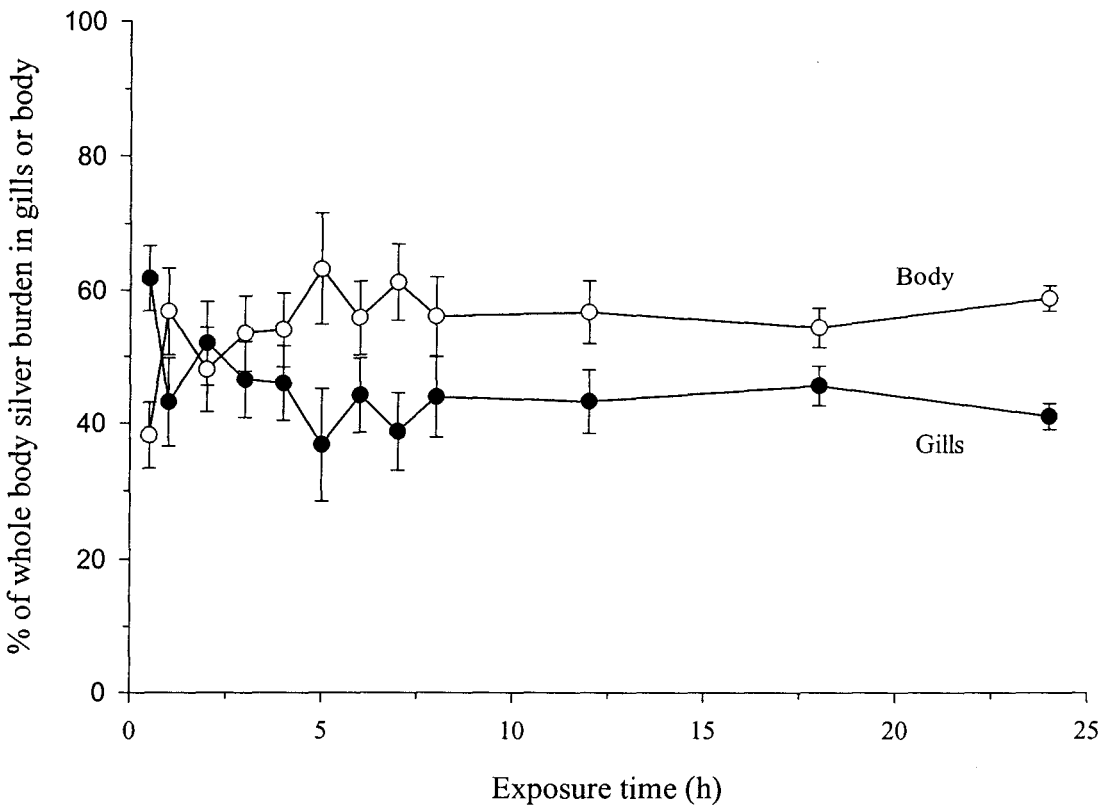

100
80
60
40
20
0
% of whole body silver burden in gills or body
0
5
10
15
20
25
Exposure time (h)
Body
Gills

Fig. 3-3

Time course of inhibition of (A) whole body Na^+ uptake (closed circles) and body Na^+ uptake (open circles) and (B) whole body Cl^- uptake (closed circles) and body Cl^- uptake (open circles) of rainbow trout exposed to 4.3 μg/L total silver as $AgNO_3$ for 24 h in synthetic soft water under flow-through conditions. Values are means ±SEM (n = 8 at each time point). Asterisks indicate significant differences from the initial value (before silver exposure; ANOVA followed by least significant difference (LSD) test; $p<0.05$).

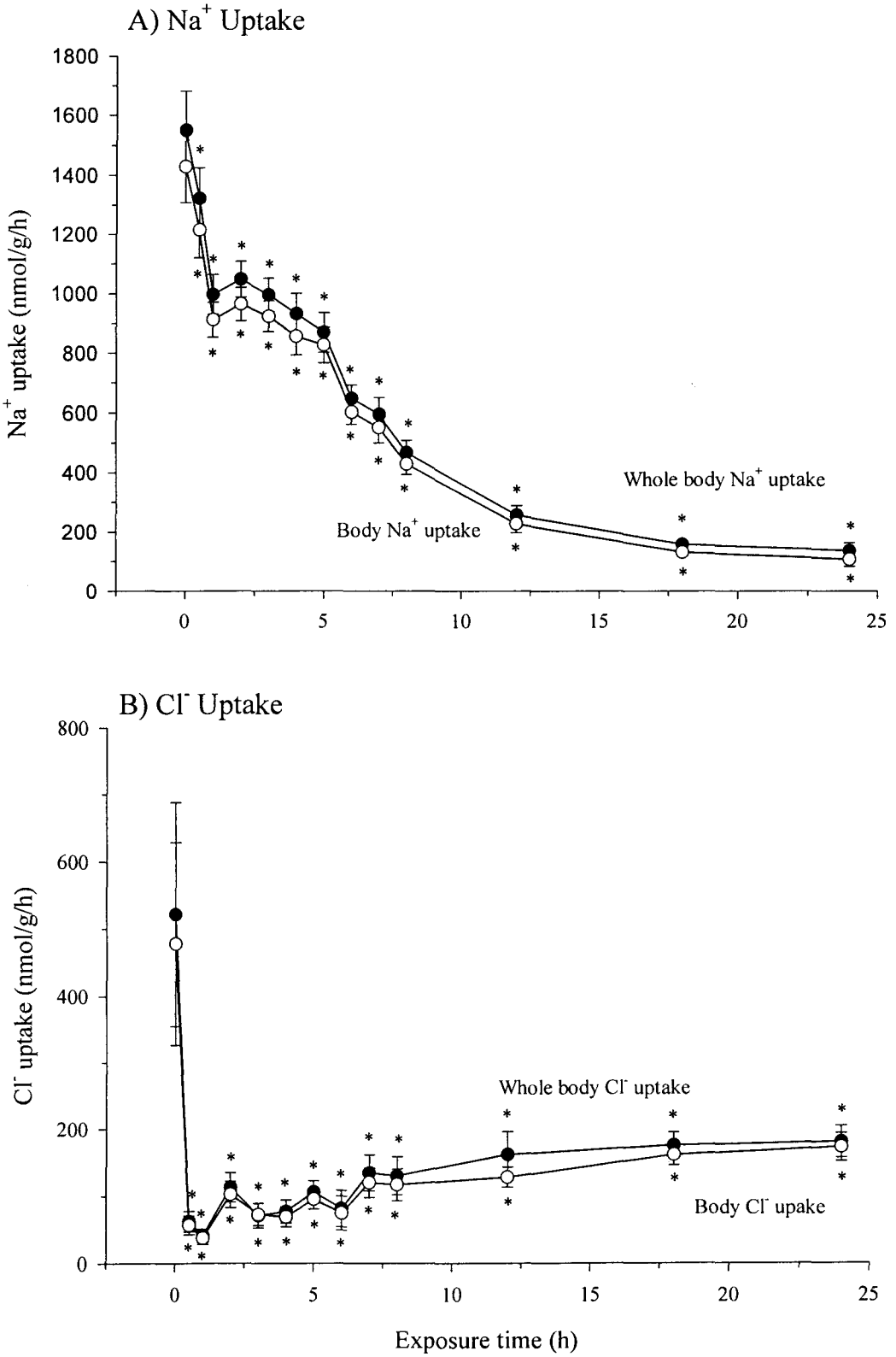

A) Na+ Uptake
Na+ uptake (nmol/g/h)
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
Whole body Na+ uptake
Body Na+ uptake
B) Cl- Uptake
Cl- uptake (nmol/g/h)
800
600
400
200
0
Whole body Cl- uptake
Body Cl- upake
Exposure time (h)

Fig. 3-4

Time course of inhibition of (A) gill Na^+K^+-ATPase activity and (B) gill CA activity of rainbow trout exposed to 4.3 μg/L total silver as $AgNO_3$ for 24 h in synthetic soft water under flow-through conditions. Values are means ±SEM (n = 8 at each time point for (A) except the 5 h time point where n = 7, n = 4 at each time point for (B) except the 1 and 12 h time point where n = 3). Asterisks indicate significant differences from the initial value (before silver exposure; ANOVA followed by least significant difference (LSD) test; $p<0.05$).

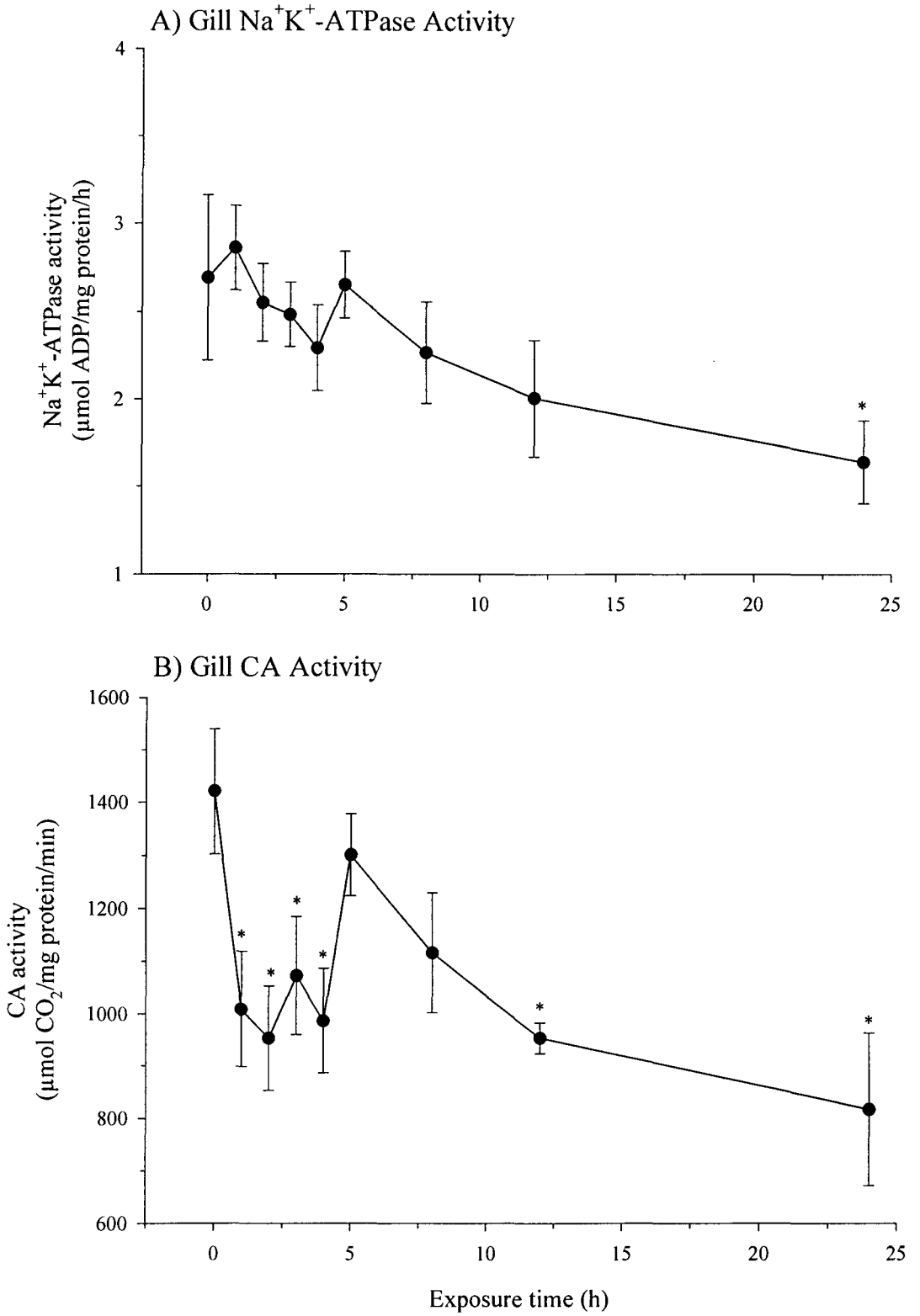
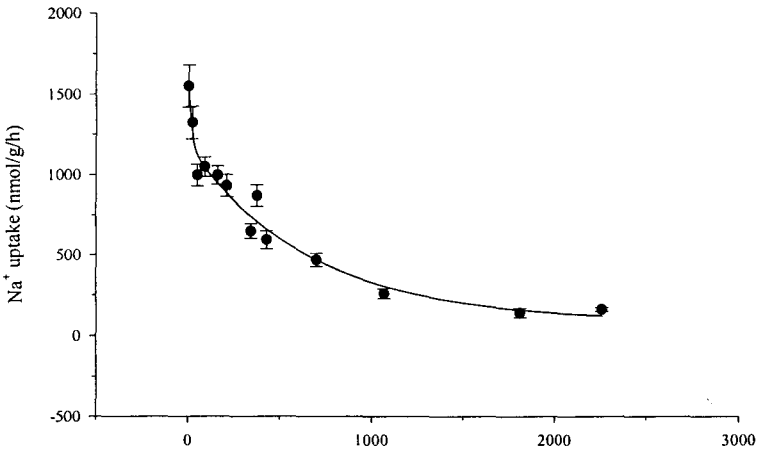

A) Gill Na+K+-ATPase Activity
Na+K+-ATPase activity
(µmol ADP/mg protein/h)
4
3
2
1
0
5
10
15
20
25
*
B) Gill CA Activity
CA activity
(µmol CO2/mg protein/min)
1600
1400
1200
1000
800
600
0
5
10
15
20
25
*
Exposure time (h)

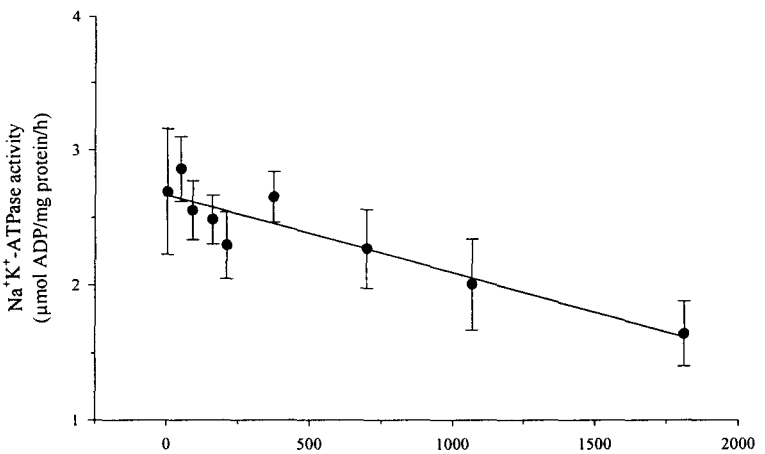
Fig. 3-5

Plot of (A) whole body Na^+ uptake rate, (B) gill Na^+K^+-ATPase activity and (C) gill CA activity as a function of gill silver concentration. Whole body Na^+ uptake rate and gill Na^+K^+-ATPase activity were inversely correlated to the gill silver concentration by the following equations: whole body Na^+ uptake rate = $81.04 + 468.82^{-0.0577*\text{gill silver concentration}} + 1099.92^{-0.0015*\text{gill silver concentration}}$, slope significantly different from zero ($p<0.05$), $r^2 = 0.97$; gill Na^+K^+-ATPase activity = 2.67 - 5.80 * gill silver concentration, slope significantly different from zero ($p<0.05$), $r^2 = 0.85$. There was no significant relationship between gill silver accumulation and CA activity ($r^2 = 0.3$, $p>0.05$).

A) Whole Body Na^+ Uptake



B) Gill Na^+K^+-ATPase Activity



C) Gill CA Activity

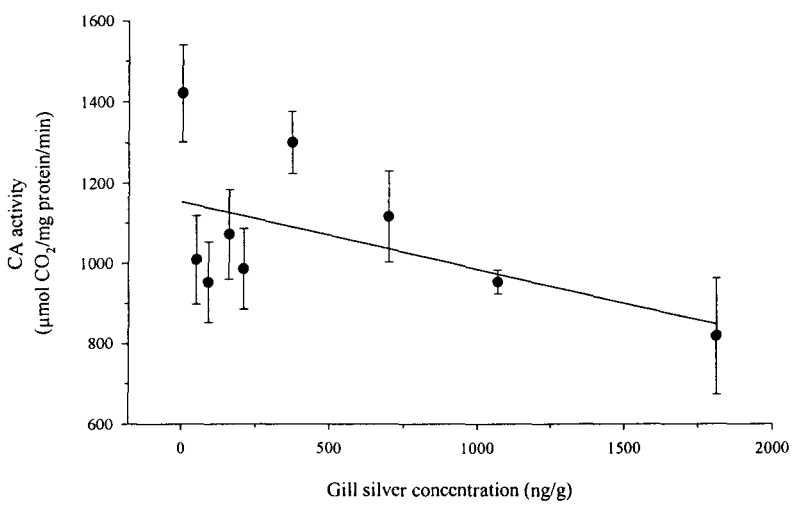


Fig. 3-6

Measured water (A) total (closed circles) and dissolved (open circles) silver, (B) chloride and (C) TOC concentrations over 24 h of flow-through exposure of rainbow trout to $AgNO_3$ in synthetic soft water. The dissolved silver concentration is the concentration of silver in the water after passage through a 0.45 μm filter. Values are means (n = 2 at each time point).

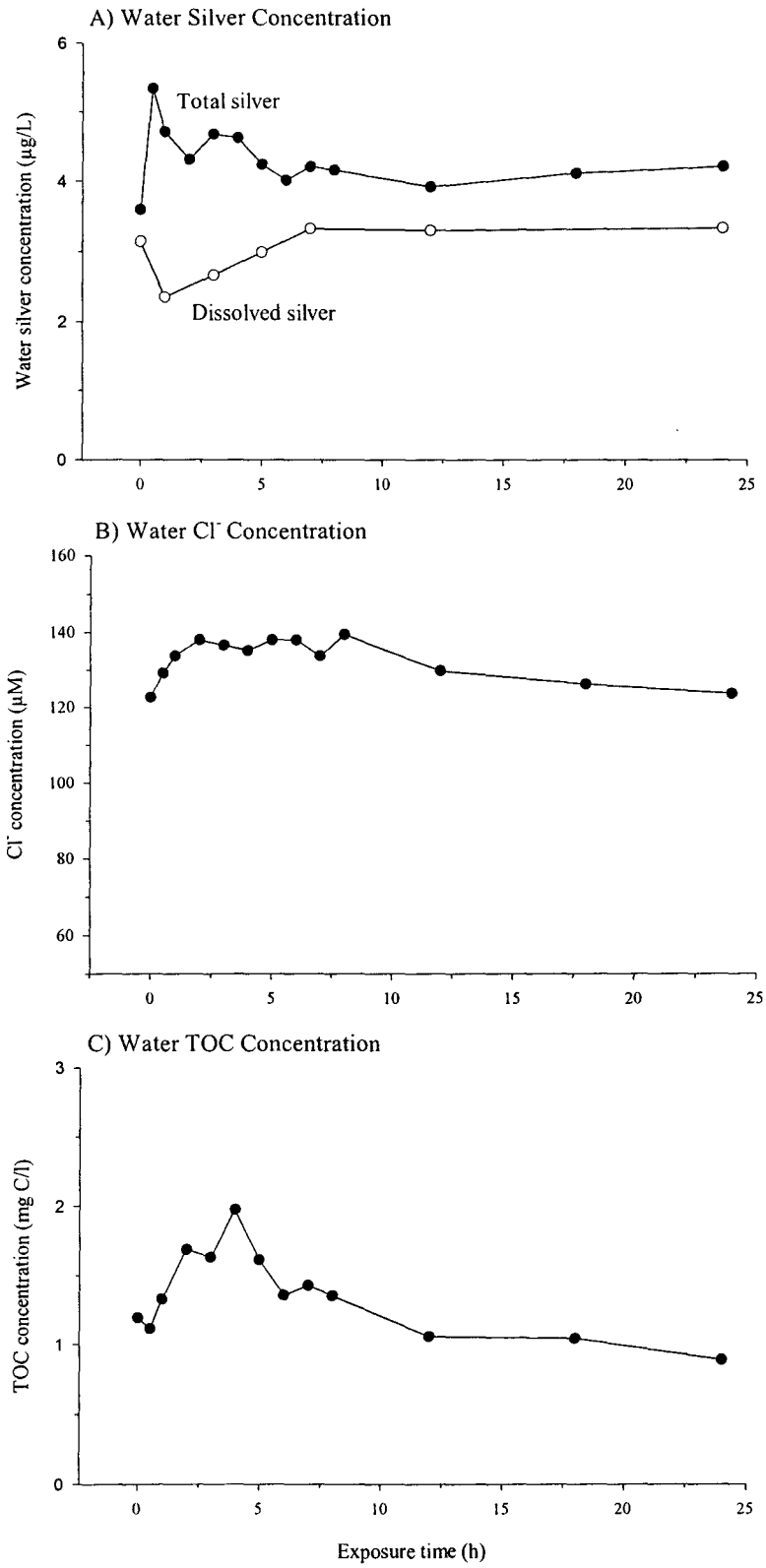
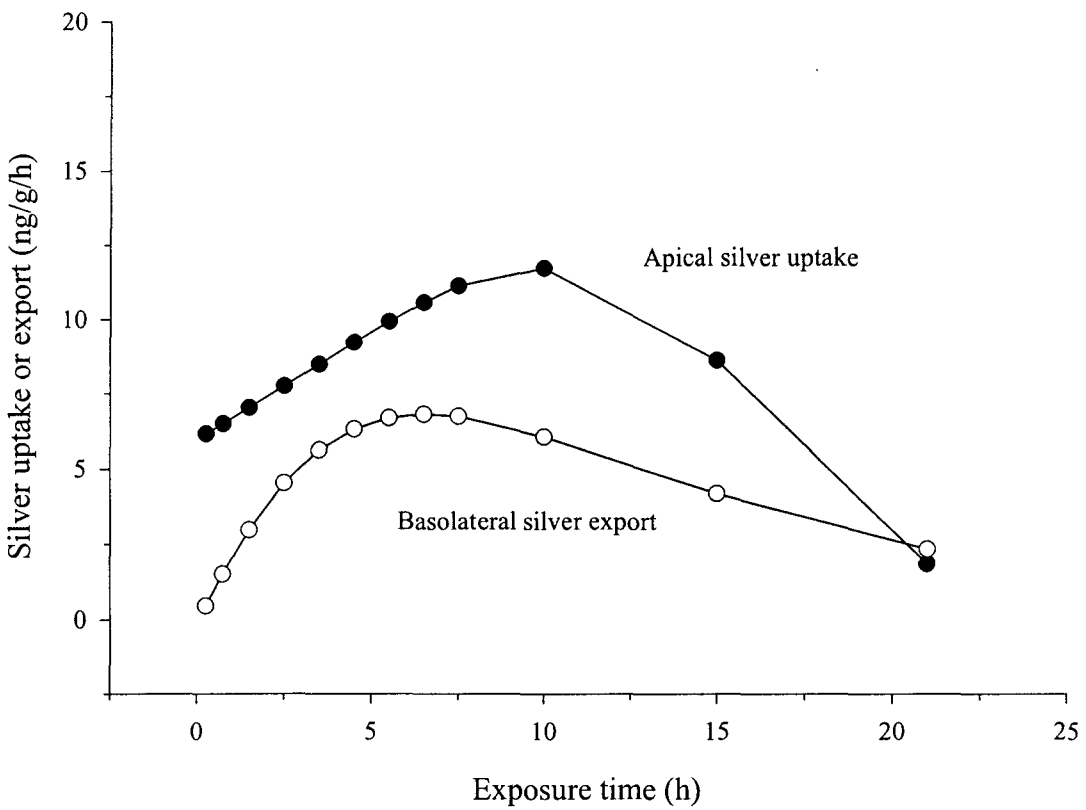

A) Water Silver Concentration
Water silver concentration (µg/L)
Total silver
Dissolved silver
B) Water Cl⁻ Concentration
Cl⁻ concentration (µM)
C) Water TOC Concentration
TOC concentration (mg C/l)
Exposure time (h)

Fig. 3-7

Apical silver uptake (closed circles) and basolateral silver export (open circles) across the gills of rainbow trout over 24 h of flow-through exposure to 4.3 μg/L total silver as $AgNO_3$ in synthetic soft water. Silver uptake was determined by first calculating silver accumulation at each time point using the equation of the line of best fit for the whole body and body silver accumulation data (adjusted to whole body weight) and then dividing the change in silver accumulation over each time interval by the length of the time interval.


Silver uptake or export (ng/g/h)
20
15
10
5
0
Apical silver uptake
Basolateral silver export
0
5
10
15
20
25
Exposure time (h)

Chapter 4

A Relationship Between Gill Silver Accumulation and Acute Silver Toxicity in the Freshwater Rainbow Trout: Support for the Silver Biotic Ligand Model

Abstract

The biotic ligand model (BLM) for silver predicts acute silver toxicity in freshwater fish on the basis of gill silver accumulation. The model has not yet been accepted as a regulatory tool by agencies such as the US-EPA because accumulation on the gills appeared to be unstable over time and has never been experimentally correlated with acute silver toxicity. A recent study indicated that the former may be an artifact of the static exposure conditions. The objective of the present study was to evaluate whether there is a relationship under flow-through conditions between short-term gill silver accumulation and acute silver toxicity in terms of 96 h mortality. Rainbow trout were exposed to a series of increasing silver concentrations (as $AgNO_3$) in flowing synthetic soft water (approximate ionic composition in mM: 0.05 [Na^+], 0.05 [Cl^-], 0.05 [Ca^{2+}], 0.02 [Mg^{2+}] and 0.02 [K^+]; pH 7.0; DOC ~0.7 mg C/L; hardness ~10 mg/L as $CaCO_3$; temperature 10 ±2 °C). At 3 h and 24 h, gill silver accumulation, whole body Na^+ uptake and gill Na^+K^+-ATPase activity were determined. 96 h mortality, as well as, water total and dissolved silver concentration (averaged over the 96 h) were also monitored. The 96 h LC50 values were 13.27 (95% confidence limits: 10.81-16.29) μg total Ag L^{-1} and 3.28 (95% confidence limits: 2.63-4.08) μg dissolved Ag L^{-1}. A

relationship was demonstrated between both 3 h and 24 h gill silver accumulation and 96 h mortality. A relationship was also demonstrated between gill silver accumulation and inhibition of Na^+ uptake after 24 h of exposure and between water silver concentration (total and dissolved) and gill silver accumulation after 24 h of exposure. There was no relationship between gill silver accumulation and inhibition of gill Na^+K^+-ATPase activity. LA50 values (short-term gill accumulation associated with 50% mortality at 96 h) of 129 and 191 ng g^{-1} using the 3 h and 24 h gill silver concentration respectively and a conditional equilibrium binding constant for Ag^+ binding on the gills of 8.0 were calculated from these results. These observations validate the silver BLM and support its use as a regulatory tool to predict acute silver toxicity.

Introduction

When present as the free silver ion (Ag^+), silver is one of the most acutely toxic metals to freshwater rainbow trout with 96 h LC50 values in the range of 6.5-13 $\mu g\ L^{-1}$ (Davies et al., 1978; Nebeker et al., 1983; Hogstrand et al., 1996). Acute toxicity arises because Ag^+ noncompetitively inhibits active Na^+ and Cl^- uptake at the gills by binding and inhibiting the gill enzymes Na^+K^+-ATPase and carbonic anhydrase (CA, Morgan et al., 1997, Chapters 2 and 3). This results in a decline in blood plasma ion levels, leading to circulatory collapse and death of the fish (Wood et al., 1996; Hogstrand and Wood, 1998). Water chemistry influences the toxicity of Ag^+. For example, complexing ligands such as Cl^-, DOC and sulfide and competing cations such as Na^+ and H^+, as well as, the water hardness cations Ca^{2+} and Mg^{2+} decrease toxicity by reducing the bioavailability of Ag^+ to toxic sites (i.e. Na^+K^+-ATPase and CA molecules) at the gills (Wood et al., 1999).

The US Environmental Protection Agency's (US-EPA) current water quality criteria (WQC) for silver corrects for hardness only (US-EPA, 1980), although the ameliorating effect of hardness against silver toxicity is modest compared to other water chemistry parameters (Galvez and Wood, 1997; Bury et al., 1999a; Bury et al., 1999b; Bianchini et al., 2002). As such, the WQC may be over- or under-protective at a particular freshwater site, depending on the water chemistry at that site. Site-specific modifications to WQC that take into account other water chemistry parameters can be made but these modifications involve the generation of water effect ratios (WERs, US-EPA, 1994), a process which is time consuming and costly because it involves many toxicity tests. The biotic ligand model (BLM, Paquin et al., 1999; McGeer et al., 2000;

Paquin et al., 2002) is a computational approach that has been developed to predict the acute toxicity of silver to freshwater fish on a site-specific basis using a comprehensive set of water chemistry parameters. It thereby provides a simple and inexpensive alternative to the generation of WERs. As such, the model has been considered for use by the US-EPA in the establishment and modification of site-specific WQC for silver.

In the model, the gill is considered a negatively charged ligand to which Ag^+ can bind. In theory (Di Toro et al., 2001), the BLM framework uses conditional equilibrium binding constants experimentally determined for the cationic metal and for protective cations (e.g. Na^+, Ca^{2+}, H^+, Mg^{2+}) binding to the gills of reference species such as the rainbow trout, together with the known chemistry of the water at a particular site. These values are inserted into an aquatic chemical equilibrium computer program to estimate metal binding at the gills. By assuming that the amount of metal binding to the gills is proportional to mortality, the model predicts acute toxicity. Because complexation of Ag^+ by ligands and competition with Ag^+ by various cations reduces Ag^+ binding to the gills (Janes and Playle, 1995; Rose-Janes and Playle, 2000; Schwartz and Playle, 2001), their influence on silver toxicity is considered within the context of the model. Specifically, based on experimentally determined calibration data, the BLM predicts the 96 h LC50 of silver (i.e. acute toxicity) in the water quality of interest on the basis of the short term (3-24 h) gill silver accumulation which is associated with 50% mortality (LA50) at 96 h. The dissolved silver concentration at the LA50 is the predicted LC50 at that site (reviewed in Di Toro et al., 2001; Paquin et al., 2002).

In practice, there has been difficulty in relating an experimentally determined gill silver load (i.e. LA50) with 96 h toxicity, for at least two reasons. Firstly, the level of silver accumulation on the gills of trout appeared to fluctuate over time during constant exposure conditions (Bury and Wood, 1999; Wood et al., 2002, Chapter 2). Secondly, in several studies (McGeer and Wood, 1998; Bury et al., 1999a), there appeared to be no relationship between gill silver burden at fixed time points and toxic physiological effects. In combination, these results suggest that there may not be a unique gill silver accumulation associated with a given acute effect. Finally, silver accumulation on the gills has never been experimentally correlated with acute silver toxicity in terms of mortality. As a result, silver versions of the BLM have to date used surrogates for gill silver burden for the purposes of establishing predictive relationships. The toxicological BLM of Paquin et al. (1999) was calibrated directly to mortality data using assumed gill burdens, while the physiological BLM of McGeer et al. (2000) used Na^+K^+-ATPase inhibition (a physiological endpoint associated with mortality) rather than gill silver burden.

These observations suggested that gill silver accumulation may not be an appropriate endpoint for predicting acute silver toxicity, and for this reason, the toxicological silver BLM (Paquin et al., 1999) was not accepted for use as a regulatory tool when presented to the Science Advisory Board of the US-EPA (www.epa.gov/sab/drrp.htm). However, we now know that the unstable pattern of silver accumulation on the gills seen in earlier studies was likely an artifact of changing silver bioavailability in closed system exposures (Chapter 2), and that under flow-through

conditions, gill silver burden increases steadily to a plateau over time (Chapter 3). Furthermore, we have demonstrated a relationship between short-term gill silver accumulation and inhibition of Na^+ uptake (Chapter 2 and Chapter 3), the primary physiological response to silver exposure which eventually leads to mortality in these fish. This relationship provides evidence, albeit indirect, of a relationship between short-term gill silver accumulation and acute toxicity. Finally, it now appears very likely that the neutral complex $AgCl_{aq}$ can enter gills (Wood et al., 2002) without causing toxicity (McGeer and Wood, 1998; Bury et al., 1999a; Wood et al., 1999), so studies which attempt to link silver burden with effect at varying Cl^- levels may not show clear cut relationships.

With this background in mind, the objective of the present study was to evaluate, under flow-through conditions at constant, low Cl^- concentration, whether there is a relationship between short-term silver accumulation on the gills (3 h or 24 h) and acute toxicity in terms of 96 h mortality. If such a relationship exists, it would provide direct, experimental support for the BLM approach for silver. To accomplish this objective rainbow trout were exposed to a series of water silver concentrations and monitored for gill silver accumulation at 3 and 24 h and mortality at 96 h. We also investigated possible relationships between gill silver accumulation and inhibition of Na^+ uptake, as well as inhibition of gill Na^+K^+-ATPase activity, as physiological endpoints that may be predictive of toxicity.

Materials and methods

Experimental animals and acclimation

Juvenile rainbow trout (*Oncorhynchus mykiss*, mean weight 6.30 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and held for two weeks in a 200 L polyethylene tank supplied with flowing, aerated, dechlorinated Hamilton city tap water (approximate ionic composition in mM: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}] and 0.05 [K^+]; pH 7.8-8.0; DOC ~3 mg C/L; hardness ~140 mg/L as $CaCO_3$; temperature 9°C). Fish were then acclimated over a four week period to synthetic soft water which was generated by reverse osmosis of Hamilton city dechlorinated tap water (Culligan Aqua-Cleer Reverse Osmosis System, Toronto, ON, Canada). Over the first two weeks, the ratio of soft water to tap water that supplied the tank was increased until the desired ionic composition was achieved (approximate ionic composition in mM: 0.05 [Na^+], 0.05 [Cl^-], 0.05 [Ca^{2+}], 0.02 [Mg^{2+}] and 0.02 [K^+]; pH 7.0; DOC ~0.7 mg C/L; hardness ~10 mg/L as $CaCO_3$; temperature 10 ±2 °C). Fish were maintained in this soft water for at least an additional two weeks before experiments were started. During initial holding and soft water acclimation, fish were fed to satiation once daily with commercial trout pellets (Martin Mills, Tavistock, ON, Canada). Feeding was suspended for one day before and during the experiments to minimize silver binding to organic matter in uneaten food and waste products during the exposure.

Experimental design

Fish were exposed to six different concentrations of silver, including a zero silver control. For each test at a particular concentration, synthetic soft water (composition as

above) was delivered to a primary header tank that overflowed into two separate secondary header tanks, all of which were vigorously aerated. Silver (as $AgNO_3$, Sigma-Aldrich, Canada), was added to these two secondary header tanks by a peristaltic pump from a single, light shielded stock bottle. Each secondary header tank directed soft water at a rate of 130 ml/min to an 80 L polyethylene exposure tank that contained 32 fish in 20 L of aerated, synthetic soft water (i.e. identical loading density in each tank). The overflow from each exposure tank drained to waste. The walls of the exposure tanks were pre-equilibrated with $AgNO_3$ at the exposure concentration for at least 24 h to ensure saturation of silver binding sites on the tank walls.

Fish from one of the exposure tanks were used for all physiological measurements. At 3 h and 24 h, eight fish were sampled for gill silver accumulation and whole body Na^+ uptake (via ^{24}Na). An additional eight fish from the same tank were sampled at each time point for gill Na^+K^+-ATPase activity. Sampled fish were washed in a silver-free solution containing NaCl (2.9 g/L) to remove any loosely bound silver and/or radioisotope and were euthanized by an overdose of MS-222 (1 g/L). In the case of trout used for silver accumulation and Na^+ uptake measurements, the gills were then immediately dissected from the fish and the gills and carcass counted separately for ^{24}Na radioactivity (and the cpm summed) so as to determine whole body Na^+ uptake. The gills were then analyzed for gill silver accumulation. In the case of trout used for measurement of Na^+K^+-ATPase activity, the gills were immediately dissected, frozen in liquid nitrogen and stored at -80°C for later analysis.

The second exposure tank was monitored for mortality over 96 h. The criterion used for mortality was cessation of opercular movement. Dead fish were removed continuously over the exposure. At 1, 3, 24, 48, 72 and 96 h of exposure, a 5 ml non-filtered (for determination of total silver) and a 5 ml filtered (for determination of dissolved silver, Acrodisc polyethersulfone 0.45 micron syringe filters, Pall Gelman Laboratory, Ann Arbor, MI, USA) water sample were taken from each exposure tank and acidified with 1% HNO_3 (trace-metal grade, Fisher Scientific, Canada) for analysis of the total and dissolved silver concentration by graphite furnace atomic absorption spectrophotometry (see below, Varian AA-220, GTA 110, Varian Ltd., Mississauga, ON, Canada).

Gill silver accumulation

Once gills had been counted for ^{24}Na activity (see below), gills were weighed and acidified with five times the sample weight of 1 N HNO_3 (trace-metal grade, Fisher Scientific, Canada). Acidified samples were digested in an oven over a 24 h period at ~60°C, vortexed and allowed to settle. A portion of the supernatant was diluted with distilled, deionized water and analyzed for total silver concentration by graphite furnace atomic absorption spectrophotometry (Varian AA-220, GTA 110, Varian Ltd., Mississauga, ON, Canada) using a certified standard (multi-element standard, Environmental Standard). Water samples were similarly analyzed.

Na^+ uptake measurements

Na^+ uptake measurements lasted a total of 30 min, so 30 min before each sample time, eight fish were removed from the exposure tank and placed in a 600 ml Pyrex glass

beaker containing 300 ml of continuously aerated water to which the fish had been exposed. The beaker was pre-equilibrated in the silver exposure water to ensure saturation of silver binding sites on the beaker walls and a stable silver concentration over the uptake measurement. For Na^+ uptake measurements conducted under control conditions, fish were placed in a beaker that was not pre-equilibrated in the silver exposure water and contained silver-free acclimation water.

Na^+ uptake was determined by the addition of ^{24}Na (6.7 μCi, mean specific activity 0.003 μCi/μg Na^+, McMaster University Nuclear Reactor, Hamilton, ON, Canada) to the beaker. A water sample was taken 5 minutes after the addition of the isotope and again at the end of the measurement for determination of radioactivity by gamma counting (MINAXI Auto-Gamma 5000 gamma counter, Canberra-Packard, Toronto, ON, Canada, corrected for decay to a common reference time) and total Na^+ concentration by flame atomic absorption spectrophotometry (Varian AA-220, Varian Ltd., Mississauga, ON, Canada). At the end of the flux period, the fish were sampled as outlined above and then the gills and body were counted separately for radioactivity (as above), and the counts were combined. The whole body Na^+ uptake was calculated from the formula:

Whole body Na^+ uptake = $CT/(SA \cdot wt \cdot t)$

where CT is the total counts per min (cpm) in the tissue, SA is the measured mean specific activity of the water, wt is the wet weight (g) and t is the time of exposure (h). The specific activity of the water was calculated as follows:

$$SA = [(cpm_i/[Na^+]_i) + (cpm_f/[Na^+]_f)]/2$$

where cpm_i represents the cpm per ml initially in the water, cpm_f represents the final cpm per ml in the water and $[Na^+]_i$ and $[Na^+]_f$ represent the initial and final sodium concentrations of the water, respectively.

Gill Na^+K^+-ATPase activity

The gills frozen for determination of Na^+K^+-ATPase activity were kept on ice throughout sample preparation and the assay. Gills were homogenized in 500 µl of SEID buffer (0.5 g of sodium deoxycholate in 100 ml of SEI; SEI = 150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) using a Teflon-glass homogenizer and then the homogenates were assayed for Na^+K^+-ATPase activity using the microplate method of McCormick (1993). Activity was standardized to protein content. Protein content was measured using the Bradford assay (kit no. B6916, Sigma, Canada) with bovine serum albumin as a standard (Sigma, Canada).

Statistical analyses

Unless otherwise noted, data have been expressed as means ±SEM (N). Percentage data were transformed using the arcsin square root transformation. Linear regression analysis was done using SPSS 10 for windows and the significance of

Pearson's correlation coefficient (r) assessed. The LC50 values and corresponding confidence limits were calculated using the Spearman-Karber method. The LA50 values were calculated using the equation of the regression line for the 96 h mortality logit versus the 3 h and 24 h log gill silver concentration, where the mortality logit = $\log_{10}(M/(1-M))$ and M = the mortality proportion, as outlined by MacRae et al. (1999).

Results and Discussion

96 h LC50 values

The calculated 96 h LC50 values were 13.27 (95% confidence limits: 10.81-16.29) μg total Ag L^{-1} and 3.28 (95% confidence limits: 2.63-4.08) μg dissolved Ag L^{-1}.

A relationship between gill silver accumulation and acute silver toxicity

There were strong positive relationships between silver accumulation on the gills at 3 h and 96 h mortality (Fig. 4-1A, $r = 0.89$, $p<0.05$), as well as between silver accumulation on the gills at 24 h and 96 h mortality (Fig. 4-1B, $r = 0.82$, $p<0.05$), providing the first experimental evidence of a relationship between silver accumulation on the gills and acute silver toxicity. The stronger relationship at 3 h than at 24 h may reflect the difference in exposure time. By 24 h some silver may have become associated with physiologically inert sites at the gills, explaining the weaker correlation with 96 h mortality. Notably, Janes and Playle (1995) employed 3 h exposures (as did Schwartz and Playle, 2001) to determine the original gill binding constants which have been used in setting up both silver BLMs (Paquin et al., 1999; McGeer et al., 2000). The demonstration of a relationship between short-term gill accumulation and 96 h mortality

lends support to the use of gill silver accumulation as an endpoint for predicting acute toxicity in the silver BLM.

Regression of the 96 h mortality logit versus the 3 h or 24 h log gill silver concentration yields a straight line from which 96 h LA50s value for silver can be calculated (Fig. 4-2). The 96 h LA50 values, calculated using the 3 h or 24 h gill silver concentrations were 129 ng g^{-1} (1.19 nmol g^{-1}) and 191 ng g^{-1} (1.77 nmol g^{-1}), respectively. These values are approximately 10 fold lower than the LA50 value utilized in the current toxicological version of the silver BLM of 1834 ng g^{-1} (17 nmol g^{-1}, Paquin et al., 1999) and approximately 5 fold lower than the LA50 value used in the physiological BLM of 702 ng g^{-1} (6.5 nmol g^{-1}, McGeer et al., 2000). Interestingly, the LA50 value used in the current BLM for copper of approximately 650 ng g^{-1} (10 nmol g^{-1}, Santore et al., 2001) is 3 - 5 fold greater than the LA50 values derived in the present study for silver. The difference in LA50 values reflects the difference in LC50 values for the two metals (Davies et al., 1978; Howarth and Sprague, 1978).

Gill silver accumulation and physiological toxicity

There was no significant relationship between gill silver accumulation and inhibition of whole body Na^+ uptake after 3 h of silver exposure ($r = 0.55$, $p>0.05$, Fig. 4-3A). This is likely because by 3 h of exposure the silver that is accumulating at the gills has reached the cytosolic CA but has not yet had enough time for penetration to the basolateral Na^+K^+-ATPase. In fact, the results of Chapters 2 and 3 demonstrate that it can take anywhere from 5 to 24 h for the inhibitory effect of silver on Na^+K^+-ATPase to fully develop. Since inhibition of Na^+K^+-ATPase appears to be responsible for the larger

portion of Na^+ uptake inhibition that occurs in these fish (Chapter 3), this would conceal the relationship between accumulation and Na^+ uptake at 3 h. The time needed for silver penetration to the basolateral membrane could also explain the lack of a significant relationship between gill silver accumulation and inhibition of gill Na^+K^+-ATPase inhibition after 3 h of exposure ($r = 0.42$, $p>0.05$, Fig. 4-4A).

There was however, a significant positive relationship between gill silver accumulation and inhibition of whole body Na^+ uptake after 24 h of silver exposure ($r = 0.86$, $p<0.05$, Fig. 4-3B). By 24 h silver accumulation at the gills under flow-through conditions has reached a plateau (Chapter 3) and silver has fully inhibited both CA and Na^+K^+-ATPase (likely because it has equilibrated fully to the basolateral membrane). This would yield the observed relationship between accumulation and inhibition of uptake. This result is not surprising in light of other recent studies demonstrating a relationship over the same time frame (Bury et al., 1999a, Chapter 2, Chapter 3). It is puzzling, however, why there was not a significant relationship between gill silver burden and Na^+K^+-ATPase inhibition in the present study at 24 h ($r = 0.49$, $p>0.05$, Fig. 4-4B). However, as noted earlier, some silver may have become associated with physiologically inert sites at the gills by 24 h, explaining the lack of correlation with Na^+K^+-ATPase inhibition. Similarly, McGeer and Wood (1998) were unable to demonstrate a significant correlation between gill silver concentration and disruption of Na^+ balance at 48 h. Again, time may be a factor such that the longer term silver exposure of McGeer and Wood (1998) may have masked the relationship by enabling silver binding to physiologically inert sites on the gills. Regardless, the observed relationship between gill

silver accumulation and the actual toxic mechanism of action of silver (i.e. inhibition of Na^+ uptake) at 24 h illustrates that gill silver accumulation is a good indicator of the physiological impact of silver and lends additional support to the BLM approach of predicting acute silver toxicity from gill silver accumulation.

A relationship between water silver concentration and gill silver accumulation

There was a strong positive relationship between the water total silver concentration averaged over the 96 h exposure period and silver accumulation on the gills after 24 h of exposure ($r = 0.81$, $p<0.05$, Fig. 4-5A). Similarly, a strong positive relationship also existed between the water dissolved silver concentration averaged over 96 h and silver accumulation on the gills after 24 h of exposure ($r = 0.79$, $p<0.05$, Fig. 4-5B). Comparable relationships between average water silver levels and silver accumulation on the gills after 3 h of exposure were not significant. The water dissolved silver concentration is believed to better represent the bioavailable fraction of silver than the total silver concentration, and is now used in the application of US-EPA WQC (US-EPA, 1999).

In essence we have demonstrated a relationship between the water bioavailable silver concentration and gill silver accumulation. Because a fraction of the bioavailable silver concentration represents Ag^+, this relationship is consistent with the BLM which assumes that accumulation of silver occurs at the gills as the result of Ag^+ binding and that accumulation will increase as the Ag^+ concentration increases for a specific water chemistry (Di Toro et al., 2001; Paquin et al., 2002). The relationship between the water dissolved silver concentration and silver accumulation on the gills after 24 h of exposure

(Fig. 4-5B) and the 96 h LA50 value determined from the gill silver accumulation after 24 h (Fig. 4-2B) can be used to calculate a conditional equilibrium binding constant (K) for Ag^+ binding to the gills of rainbow trout. First, the water dissolved silver concentration at the 96 h LA50 value (191 ng g^{-1}) was determined using the equation of the regression line of Fig. 4-5B (dissolved [Ag] = 4.26 μg L^{-1}). Second, the ionic silver concentration at the LA50 value was calculated using the aquatic chemistry program MINEQL+ (version 4.0, Schecher and McAvoy, 1992) based on the known chemistry of the water ([Na^+] 0.05 mM, [Cl^-] 0.05 mM, [Ca^{2+}] 0.05 mM, [Mg^{2+}] 0.02mM, [K^+] 0.02 mM, [DOC] 0.7 mg C L^{-1}) and appropriate binding constants from Janes and Playle (1995). The water ionic silver concentration ([Ag^+] = 1.02 μg L^{-1}) was converted to a conditional equilibrium binding constant by taking the negative logarithm of the molar value. The conditional equilibrium binding constant determined by this procedure (log $K_{Ag\text{-}gill}$ = 8.0) is higher than the conditional equilibrium binding constant used in the current toxicological version of the BLM (log $K_{Ag\text{-}gill}$ = 7.3, Paquin et al., 1999), being approximately 5 fold higher, but closer to the value used in the physiological version of the BLM (log $K_{Ag\text{-}gill}$ = 7.6, McGeer et al., 2000) for rainbow trout. Inasmuch as silver is 3- to 5-fold more toxic than copper to trout (Davies et al., 1978; Howarth and Sprague, 1978), it makes sense that the log $K_{Ag\text{-}gill}$ should be 3- to 5-fold higher than the log $K_{Cu\text{-}gill}$ which is 7.4 in the current copper BLM (Santore et al., 2001; Paquin et al., 2002).

For accurate prediction of acute silver toxicity, the binding constant determined in the present study may be more appropriate for use in the silver BLM as this value was determined experimentally and is a directly measured toxicological value associated with

short-term gill silver load (LA50) at the 96 h LC50 value. In contrast, the binding constant used in the current toxicological silver BLM was determined by fitting the BLM to silver toxicity data (Paquin et al., 1999) and thus represents a toxicity based binding constant without direct gill measurements. Utilization of the current toxicological BLM binding constant may underestimate the amount of Ag^+ bound to the gill, leading to an overestimation of the LC50 value. However, it should be noted that the high LA50 value of Paquin et al. (1999) may compensate for the low log *K* in their model, providing a model which can still accurately predict acute silver toxicity.

Conclusions

We have provided the first experimental evidence of a relationship between gill silver accumulation and acute silver toxicity in terms of mortality. We have also demonstrated a relationship between gill silver accumulation and inhibition of Na^+ uptake, the eventual cause of mortality in rainbow trout. With the results of Chapter 3 which show that an equilibrium level of silver accumulation on the gills is reached over time, these results justify the use of gill silver accumulation as an endpoint to predict the acute toxicity of silver to juvenile rainbow trout as is done in the current toxicological version of the BLM. The demonstration of a relationship between the water dissolved silver concentration and gill silver accumulation provides additional support to the BLM which assumes that at a particular water chemistry an increase in the ionic silver concentration will result in an increase in gill silver accumulation. Together, these results may help promote use of the toxicological silver BLM by regulatory agencies such as the

US-EPA in the assessment of the toxicity of silver to aquatic organisms and in the generation of WQC.

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Fig. 4-1

The relationship between 96 h mortality of rainbow trout and silver accumulation on the gills after (A) 3 h and (B) 24 h of exposure to $AgNO_3$ in synthetic soft water. Lines indicate linear regressions with (A) $r = 0.89$, $p<0.05$ and (B) $r = 0.82$, $p<0.05$.

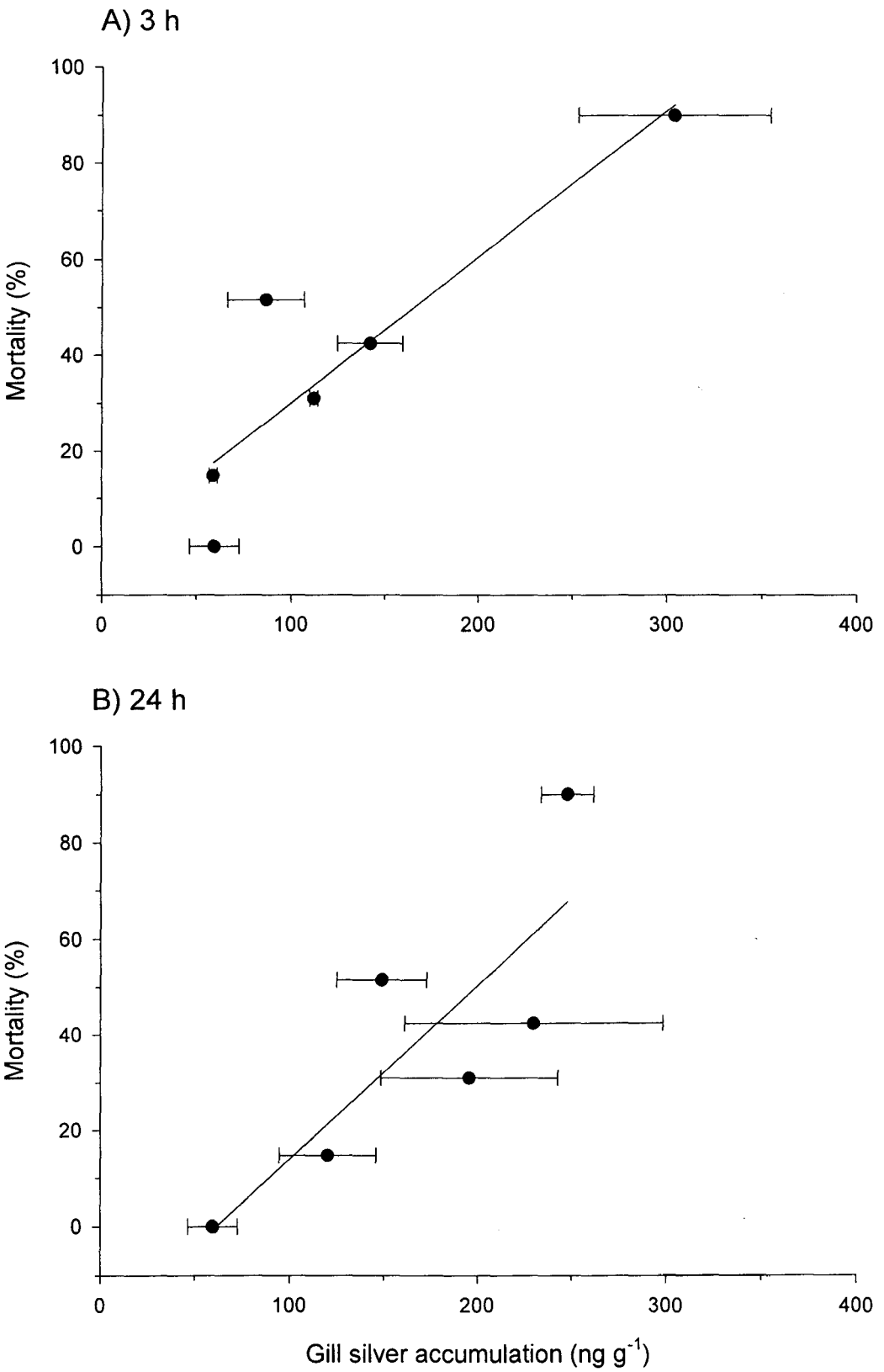

A) 3 h
Mortality (%)
100
80
60
40
20
0
0
100
200
300
400
B) 24 h
Mortality (%)
100
80
60
40
20
0
0
100
200
300
400
Gill silver accumulation (ng g-1)

Fig. 4-2

Plot of 96 h mortality logit as a function of log gill silver accumulation determined at (A) 3 h and (B) 24 h of silver exposure. A regression of the plot yields a straight line from which a 96 h LA50 (lethal accumulation yielding 50% mortality) can be calculated. The regression equation is (A) $\log_{10}(M/(1-M)) = \log_{10}$gill silver concentration*2.17-4.57, r = 0.91, and (B) $\log_{10}(M/(1-M)) = \log_{10}$gill silver concentration*3.45-7.85, r = 0.71 yielding a LA50 of (A) 129 ng g^{-1} and (B) 191 ng g^{-1}.

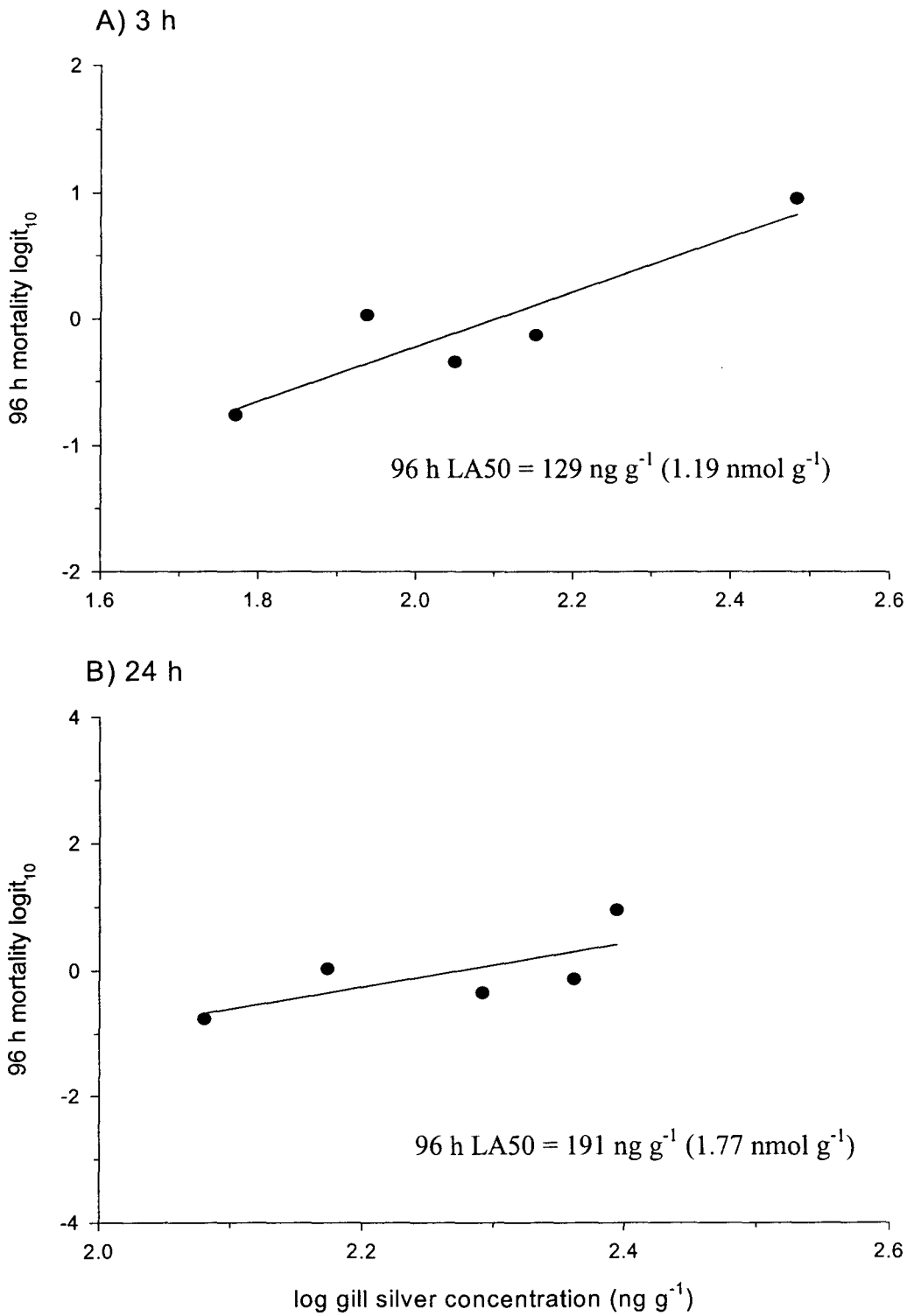

A) 3 h
96 h mortality logit10
2
1
0
-1
-2
1.6
1.8
2.0
2.2
2.4
2.6
96 h LA50 = 129 ng g-1 (1.19 nmol g-1)
B) 24 h
96 h mortality logit10
4
2
0
-2
-4
2.0
2.2
2.4
2.6
96 h LA50 = 191 ng g-1 (1.77 nmol g-1)
log gill silver concentration (ng g-1)

Fig. 4-3

The relationship between percent inhibition of whole body Na^+ uptake and silver accumulation on the gills of rainbow trout after (A) 3 h and (B) 24 h of exposure to $AgNO_3$ in synthetic soft water. Lines indicate linear regressions with (A) r = 0.55, $p>0.05$ and (B) r = 0.86, $p<0.05$.

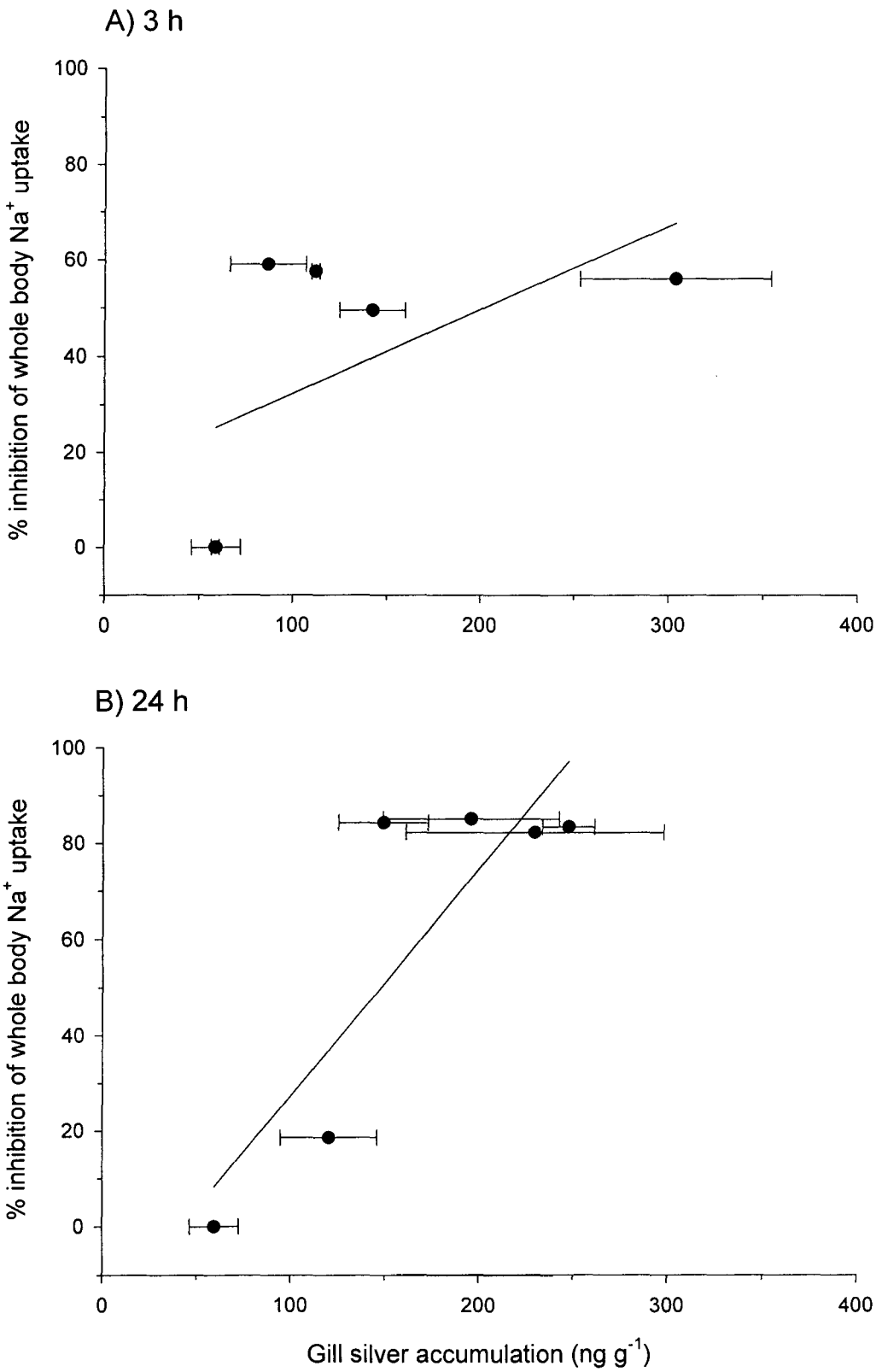

A) 3 h
% inhibition of whole body Na+ uptake
100
80
60
40
20
0
0
100
200
300
400
B) 24 h
% inhibition of whole body Na+ uptake
100
80
60
40
20
0
0
100
200
300
400
Gill silver accumulation (ng g-1)

Fig. 4-4

The relationship between percent inhibition of Na^+K^+-ATPase activity and silver accumulation on the gills after (A) 3 h and (B) 24 h of exposure of rainbow trout to $AgNO_3$ in synthetic soft water. Lines indicate linear regressions with (A) r = 0.42, $p>0.05$ and (B) r = 0.49, $p>0.05$.

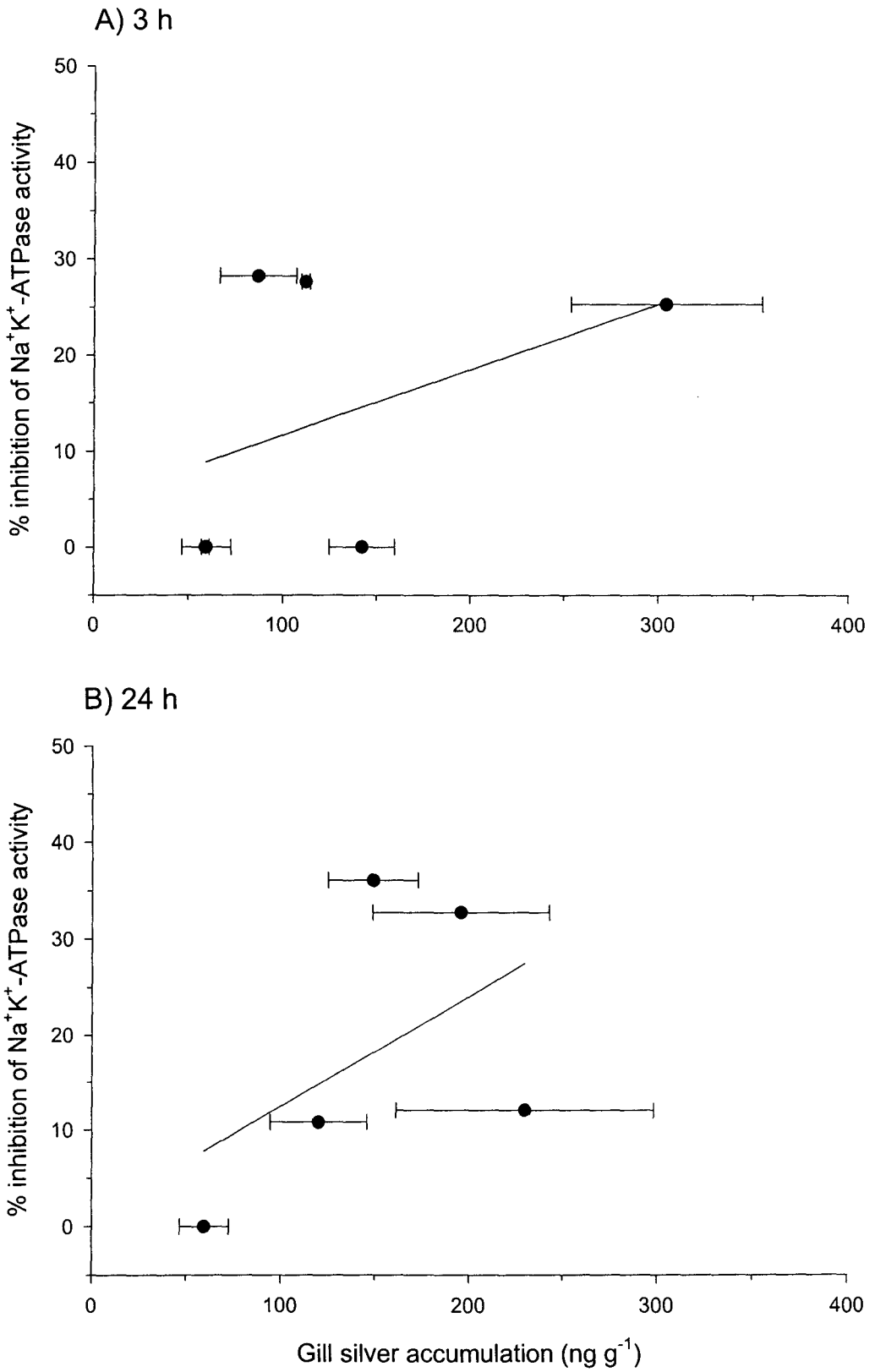
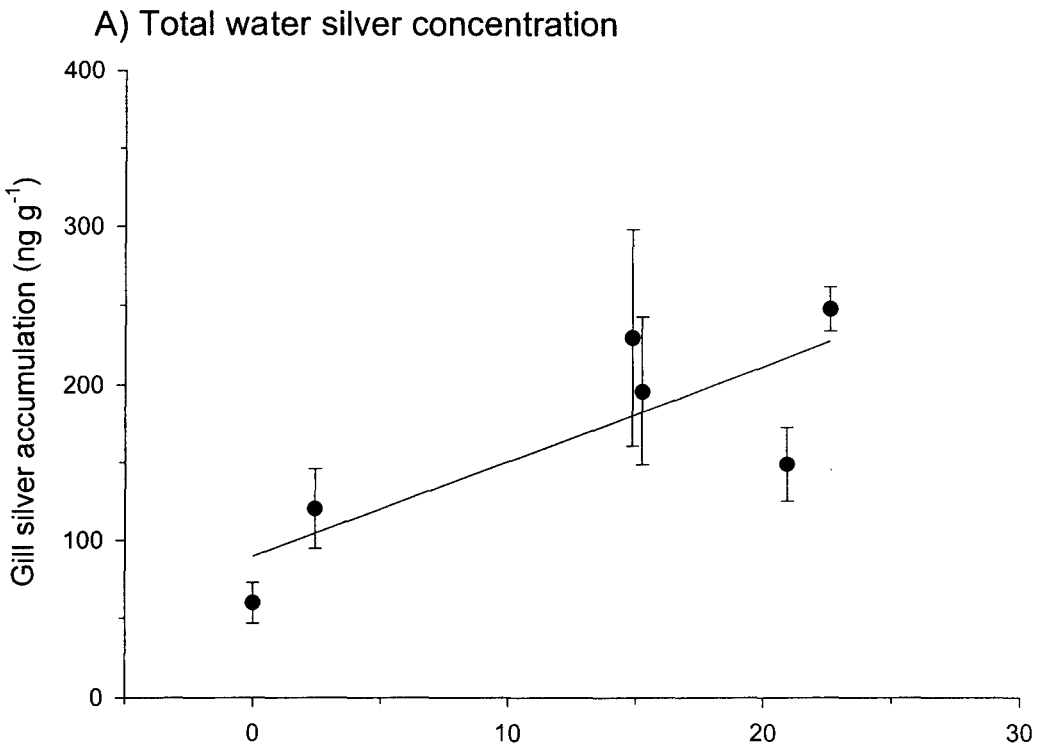
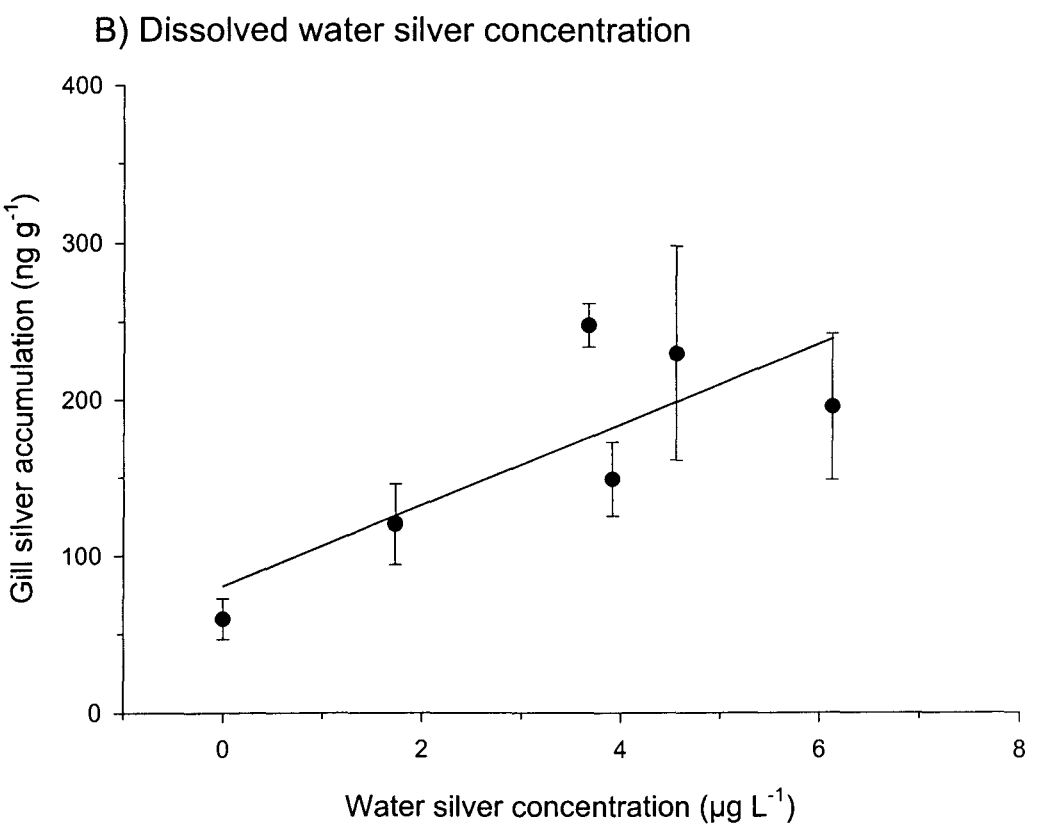

A) 3 h
% inhibition of Na+K+-ATPase activity
50
40
30
20
10
0
0
100
200
300
400
B) 24 h
% inhibition of Na+K+-ATPase activity
50
40
30
20
10
0
0
100
200
300
400
Gill silver accumulation (ng g-1)

Fig. 4-5

Rainbow trout 24 h gill silver accumulation as a function of the (A) total and (B) dissolved water silver concentration averaged over the 96 h exposure period. Lines indicate linear regressions with (A) $r = 0.81$, $p<0.05$ and (B) $r = 0.79$, $p<0.05$.


A) Total water silver concentration
Gill silver accumulation (ng g-1)
400
300
200
100
0
0
10
20
30


B) Dissolved water silver concentration
Gill silver accumulation (ng g-1)
400
300
200
100
0
0
2
4
6
8
Water silver concentration (µg L-1)

Chapter 5

Summary of Results and Conclusions

The mechanism behind the pattern of gill silver accumulation during static silver exposures

Physiological regulation of silver movement across the gill epithelium as the result of Na^+K^+-ATPase inhibition by silver cannot explain the pattern of gill silver accumulation demonstrated in rainbow trout during waterborne static silver exposure. Time course analysis of the pattern of whole body Na^+ uptake inhibition and gill Na^+K^+-ATPase inhibition during static silver exposure demonstrated that whole body Na^+ uptake was inhibited before Na^+K^+-ATPase activity. It appears that the mechanism behind the pattern of silver accumulation involves changes in the bioavailability of Ag^+ due to complexation by organic matter produced by the fish, a phenomenon associated with the use of a static exposure system. Static exposure resulted in a build-up of organic carbon and a decrease in the bioavailability of Ag^+ because the exposure water was not replaced over the exposure period, causing a decline in apical silver uptake. Together with constant basolateral silver export, this led to a peak and decline in gill silver accumulation. In contrast, flow-through exposure was associated with constant Ag^+ bioavailability because the exposure water and any Ag^+ lost by complexation was replaced by the inflowing water and organic matter was washed away. As a result, apical

silver uptake did not decline, leading to an increase in gill silver accumulation to a plateau over time.

Toxicological implications of static silver exposures

In addition to the time course of gill silver accumulation, the time course of Na^+ uptake inhibition was different between the static and flow-through exposures. During static exposure, whole body Na^+ uptake fell but recovered despite continued silver exposure. During flow-through exposure, Na^+ uptake fell in two phases and there was no sign of recovery of uptake with continued exposure. The difference in the time course of Na^+ uptake between static and flow-through tests, together with the difference in the time course of gill silver accumulation between tests emphasizes the importance of considering the exposure conditions when assessing the acute toxicity of silver to fish in the laboratory for possible application of the results to natural freshwater environments. Flow-through tests are more representative of natural freshwater lotic environments and as such, results of static exposures may underestimate the acute toxicity of silver in the natural environment because they show a decline in gill silver accumulation and recovery of gill function with continued silver exposure.

The mechanism by which silver causes the early decline in Na^+ uptake during silver exposure

In contrast to previous beliefs, the inhibition in Na^+ uptake that occurs early during silver exposure is not due to an inhibition of Na^+K^+-ATPase activity because whole body Na^+ uptake declined before enzyme inhibition occurred. The delay in Na^+K^+-ATPase inhibition likely reflects the time needed for silver penetration to the

basolateral membrane. The mechanism responsible appears to involve CA inhibition. Evidence for this comes from the observation that CA activity was inhibited early during silver exposure when Na^+ and Cl^- uptake were declining but Na^+K^+-ATPase activity was not inhibited. In addition, the similar time courses of inhibition of apical Na^+ uptake and basolateral Na^+ export, as well as, the similar time courses of inhibition of apical Cl^- uptake and basolateral Cl^- export implicate CA inhibition. The similar time coursed imply that the rate limiting step in the movement of Na^+ and Cl^- across the gill epithelium is the movement across the apical membrane, and CA powers ion movement across the apical membrane through the generation of H^+ and HCO_3^- which are exchanged against Na^+ and Cl^-. Additional evidence for a role of CA inhibition comes from the observation that the time course of Cl^- uptake inhibition and the degree of Cl^- uptake inhibition were quantitatively similar to the time course and degree of Na^+ uptake inhibition. A simultaneous and equal inhibition of Na^+ and Cl^- uptake is expected if CA inhibition is responsible for the early decline in Na^+ uptake because, as outlined above, CA activity provides H^+ to the proton pump-coupled Na^+ channel and HCO_3^- to the Cl^-/HCO_3^- exchanger which are involved in apical exchange of Na^+ and Cl^- respectively.

Carbonic anhydrase inhibition explains the early decline in Na^+ and Cl^- during silver exposure but the effect of Na^+K^+-ATPase inhibition by silver on Na^+ uptake remains important later during silver exposure, once silver has traversed the apical membrane and cytosol of the gill cell and reached the basolateral membrane.

Implications for the silver BLMs

Short term gill silver accumulation is an appropriate endpoint for the prediction of acute silver toxicity in freshwater fish. First, an equilibrium level of silver accumulation at the gills is achieved over time during flow-through exposures. Although an equilibrium level is not achieved during static exposure, flow-through exposures are believed to better represent the natural freshwater environment than static exposures, justifying the use of flow-through results. Second, a relationship exists between short term gill silver accumulation (at progressive times up to 24 h) and inhibition of Na^+ uptake. Because inhibition of Na^+ uptake is the primary toxic physiological response to acute silver exposure which eventually leads to mortality in fish, this relationship suggests a relationship between gill silver accumulation and mortality. A relationship between gill silver accumulation and inhibition of Na^+ uptake has not been demonstrated before because previous studies looked at the relationship after longer exposure times (i.e. 48 h) when silver may have become associated with physiologically inert sites on the gills, obscuring the relationship between the two parameters. Third, a relationship exists between silver accumulation on the gills after 3 h, as well as after 24 h, and 96 h mortality. Together, these results indicate a relationship between short term gill silver accumulation and acute silver toxicity in freshwater fish, lending support to the current toxicological version of the BLM. These results, together with the incorporation of the LA50 and log *K* values directly determined in these studies may help promote use of the toxicological BLM by regulatory agencies in the generation of site-specific WQC for silver.

With regards to the physiological BLM, the predictive capabilities of the model could be improved by using Na^+ uptake inhibition rather than Na^+K^+-ATPase activity inhibition as an endpoint to predict acute silver toxicity. CA inhibition plays an important role in the acute toxicity of silver by way of its effects on Na^+ uptake and Na^+ uptake inhibition as an endpoint includes the effect of CA inhibition on Na^+ uptake, whereas an endpoint of Na^+K^+-ATPase activity inhibition does not.