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# The time course of silver accumulation in rainbow trout during static exposure to silver nitrate: physiological regulation or an artifact of the exposure conditions?

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**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) concentrations 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.


*Keywords:* Bioavailability; Biotic ligand model; $Na^+$ uptake; $Na^+K^+$-ATPase; Dissolved organic carbon; Particulate organic carbon

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## 1. Introduction

When present as silver nitrate, silver is one of the most acutely toxic metals to freshwater rainbow trout (*Oncorhynchus mykiss*) with 96 h LC50 values in the range of 6.5–13 $\mu g\,l^{-1}$ in freshwaters that are generally low in organic matter content (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,c; 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,c; 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 the activity of 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., 1999c; 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.

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 concentrations 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., 1993a,b; Hollis et al., 1997; Grosell et al., 1998; Alsop et al., 1999; MacRae et al., 1999). Albeit unusual, the pattern may be characteristic of silver exposure; it is observed in both rainbow trout (*O. mykiss*) and European eel (*Anguilla anguilla*) 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.

## 2. Materials and methods

### 2.1. Experimental animals and acclimation

Juvenile rainbow trout (*O. mykiss*; 1–5 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and held for 2 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 2-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: 0.09 [$Na^+$], 0.09 [$Cl^-$], 0.08 [$Ca^{2+}$], 0.04 [$Mg^{2+}$], 0.04 [$K^+$], 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 2 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.

### 2.2. Experimental design

Silver, as $^{110m}Ag$ labeled $AgNO_3$ (70 μCi; specific activity (SA) 0.92 μCi $\mu g^{-1}$ $Ag^+$; 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 concentrations 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 to 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 $l^{-1}$).

### 2.3. Water analyses

Water samples were taken to determine the concentration of total and dissolved silver during the exposure period. Dissolved silver was measured because it is believed to more closely approximate the bioavailable fraction of metal in the water than does total silver. Silver was referred to as dissolved if it was able to pass through a 0.45 μm 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 of silver in the water was calculated (in 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 (in 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.

### 2.4. 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).

### 2.5. $Na^+$ 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 $\mu g^{-1}$ $Na^+$; 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 $Na^+$ (μmol $g^{-1}$ $h^{-1}$) was then calculated as follows:

$$\text{gill or body } Na^+ \text{ uptake} = \frac{\text{CT}}{\text{SA} \times \text{wt} \times t}$$

where CT is the total counts per minute 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 average specific activity of the water was calculated as follows:

$$\text{SA} = \tfrac{1}{2}((\text{cpm}_\text{i}/[\text{Na}]_\text{i}) + (\text{cpm}_\text{f}/[\text{Na}]_\text{f}))$$

where $\text{cpm}_\text{i}$ represents the $^{24}$Na cpm per ml initially in the water, $\text{cpm}_\text{f}$ represents the final $^{24}$Na cpm per ml in the water, and $[\text{Na}]_\text{i}$ and $[\text{Na}]_\text{f}$ 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.

### 2.6. 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 2 weeks and the samples were counted again to give the $^{110m}Ag$ radioactivity (250 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.

### 2.7. 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 min at room temperature. $Na^+K^+$-ATPase activity (μmol ADP $mg^{-1}$ $h^{-1}$) 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).

### 2.8. Statistical analyses

Data have been expressed as means ± S.E.M. ($n$), 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 the Dunnett's multiple comparison test (SPSS 10 for Windows). Linear regression and non-linear regression analyses were done using SPSS 10 for Windows. A significance level of $P < 0.05$ was used throughout.

## 3. Results

### 3.1. Water chemistry

The mean measured total and dissolved water silver concentrations over the 24 h exposure period were 1.92 ± 0.08 and 0.71 ± 0.11 μg $l^{-1}$, respectively. As explained in the discussion, the large difference between the total and dissolved water silver concentrations is likely due to silver-binding to organic matter produced by the fish.

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. 1A). Approximately, one-third of the decrease in concentration was due to the measured uptake of silver by the fish with the remaining decrease likely due to adhesion of silver to the tank and Pyrex beaker surface. However, the dissolved silver concentration did not remain constant (Fig. 1A). 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. 1B). 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 to 1.9 mg C $l^{-1}$ at 2 h of silver exposure, an increase of over three-fold

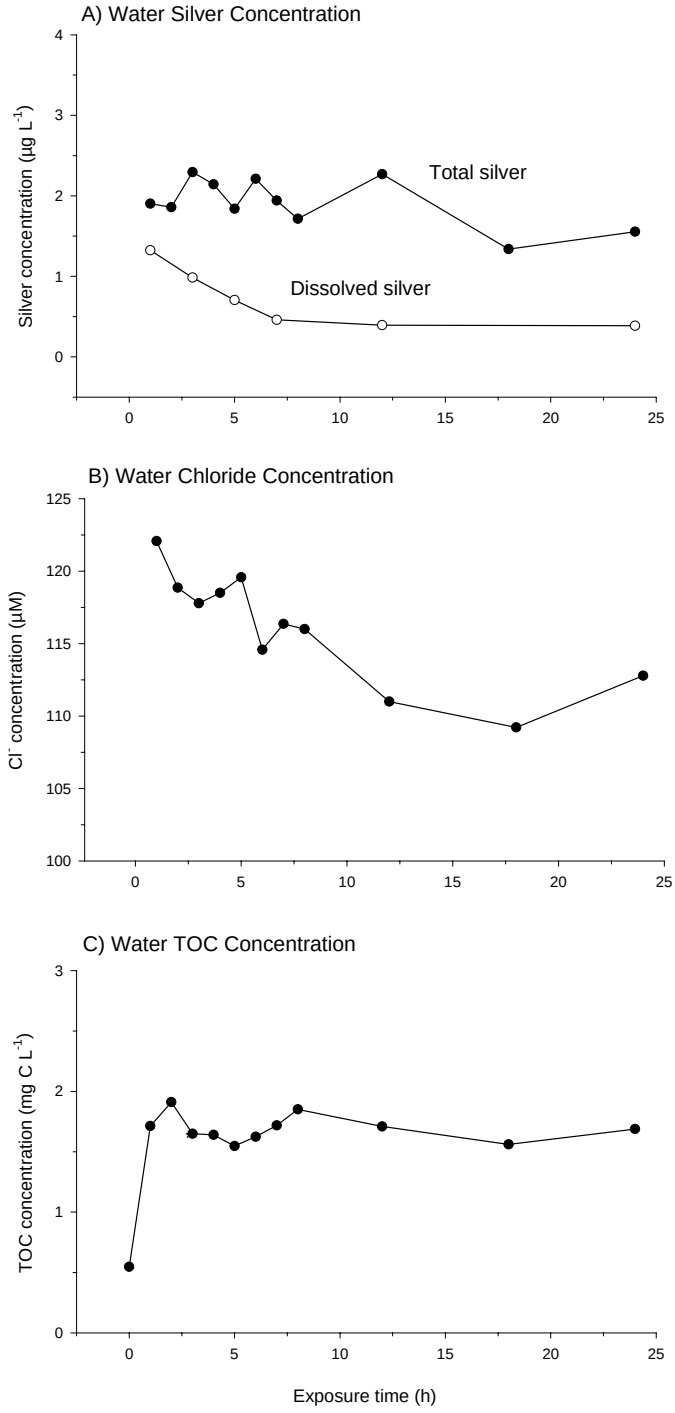



Fig. 1. Changes in the measured water total (filled circles) and dissolved (open circles) silver (A), chloride (B) and TOC (C) concentrations over 24 h of acute exposure of rainbow trout to $AgNO_3$ in synthetic soft water. The dissolved silver concentration is the concentration of silver in the water after passage through a 0.45 μm filter. Values are means ($n = 2$).

(Fig. 1C). TOC concentrations remained elevated relative to the initial value and constant over the following 22 h of exposure.

### 3.2. 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 $g^{-1}$ ww. A gradual decline in gill silver with continued silver exposure followed until a plateau was reached at ~125 ng $g^{-1}$ ww after 12 h of exposure (Fig. 2A).

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

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

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. 3). 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.

### 3.3. $Na^+$ uptake

Silver exposure rapidly inhibited whole body $Na^+$ uptake (Fig. 4A). 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. 4A). 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. 2A). Furthermore, the eventual recovery of $Na^+$ uptake with continued silver exposure corresponded with the decline in gill silver accumulation (compare Figs. 2A and 4A).

### 3.4. Gill $Na^+K^+$-ATPase activity

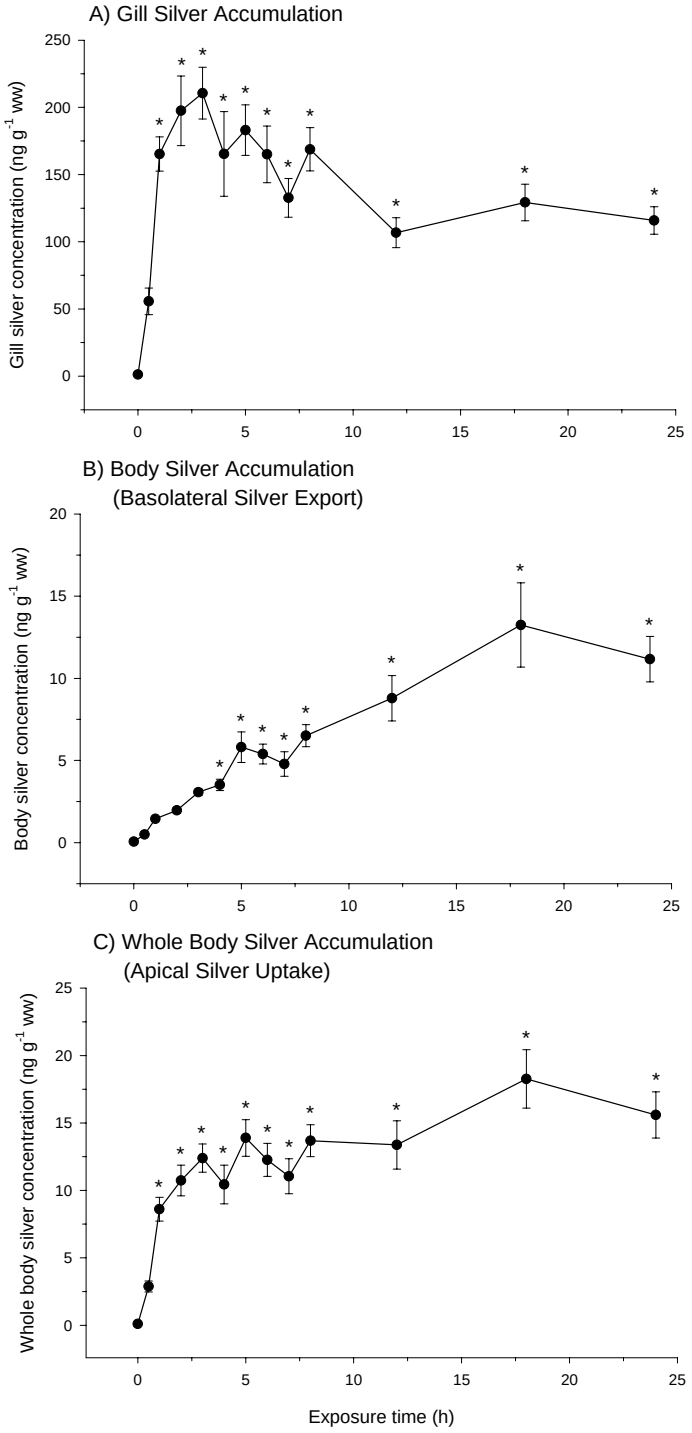
Silver exposure significantly inhibited gill $Na^+K^+$-ATPase activity in rainbow trout by ~40%, but the effect did not occur until 5 h (from 4.2 μmol ADP $mg^{-1}$ protein $h^{-1}$ before silver exposure to 2.6 μmol ADP $mg^{-1}$ protein $h^{-1}$ at 5 h of silver exposure; Fig. 4B). The 5 h delay in enzyme inhibition was in contrast to the rapid inhibition of whole body $Na^+$ uptake (significant at 2 h and maximal at 3 h; Fig. 4A) and rapid accumulation of gill silver (significant at 0.5 h and peaking at 3 h; Fig. 2A). $Na^+K^+$-ATPase activity remained inhibited over the remaining 19 h of exposure except at 12 and 18 h.

## 4. Discussion

### 4.1. 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 equations of the lines of best fit (provided in legend of Fig. 2) for the whole body (apical uptake; Fig. 2C) and body (basolateral export; Fig. 2B; 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. 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. 2A) therefore could be attributed to the


A) Gill Silver Accumulation
Gill silver concentration (ng g-1 ww)
B) Body Silver Accumulation
(Basolateral Silver Export)
Body silver concentration (ng g-1 ww)
C) Whole Body Silver Accumulation
(Apical Silver Uptake)
Whole body silver concentration (ng g-1 ww)
Exposure time (h)

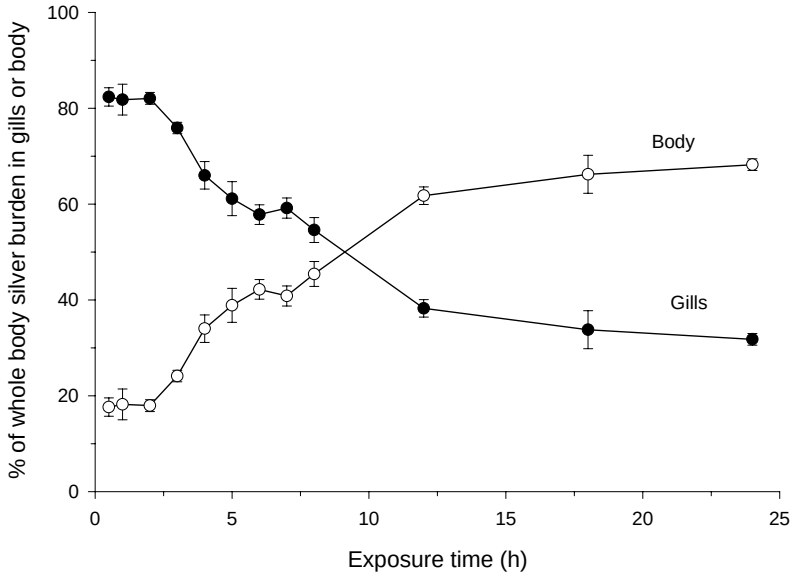



Fig. 3. Gill (filled circles) and body (open circles) silver burden as a percentage of the whole body silver burden in rainbow trout acutely exposed for up to 24 h to 1.92 μg $l^{-1}$ total silver as $AgNO_3$ in synthetic soft water. The body was that portion of the rainbow trout remaining after the gills were excised. Values are means ± S.E.M. ($n = 8$ at each time point).

initially much higher uptake rate than export rate, a phenomenon that rapidly declined (Fig. 5). Basolateral silver export was low but constant over the exposure period (Fig. 5). The steady increase in body silver accumulation over time (Fig. 2B) reflected the constant basolateral export of silver (Fig. 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. 4A). 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.

### 4.2. 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

Fig. 2. 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 ± S.E.M. ($n = 8$ 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 the Dunnett's multiple comparison test; $P < 0.05$). The equations of the lines of best fit for (B) and (C) are gill silver accumulation $= -70.50 + 82.15/[(1 + e^{(-(\text{exposure time} - 15.71)/0.33)}]^{0.003}$ and gill silver accumulation $= 13.74 \times \text{exposure time}/(0.89 + \text{exposure time}) + 0.14 \times \text{exposure time}$, respectively.

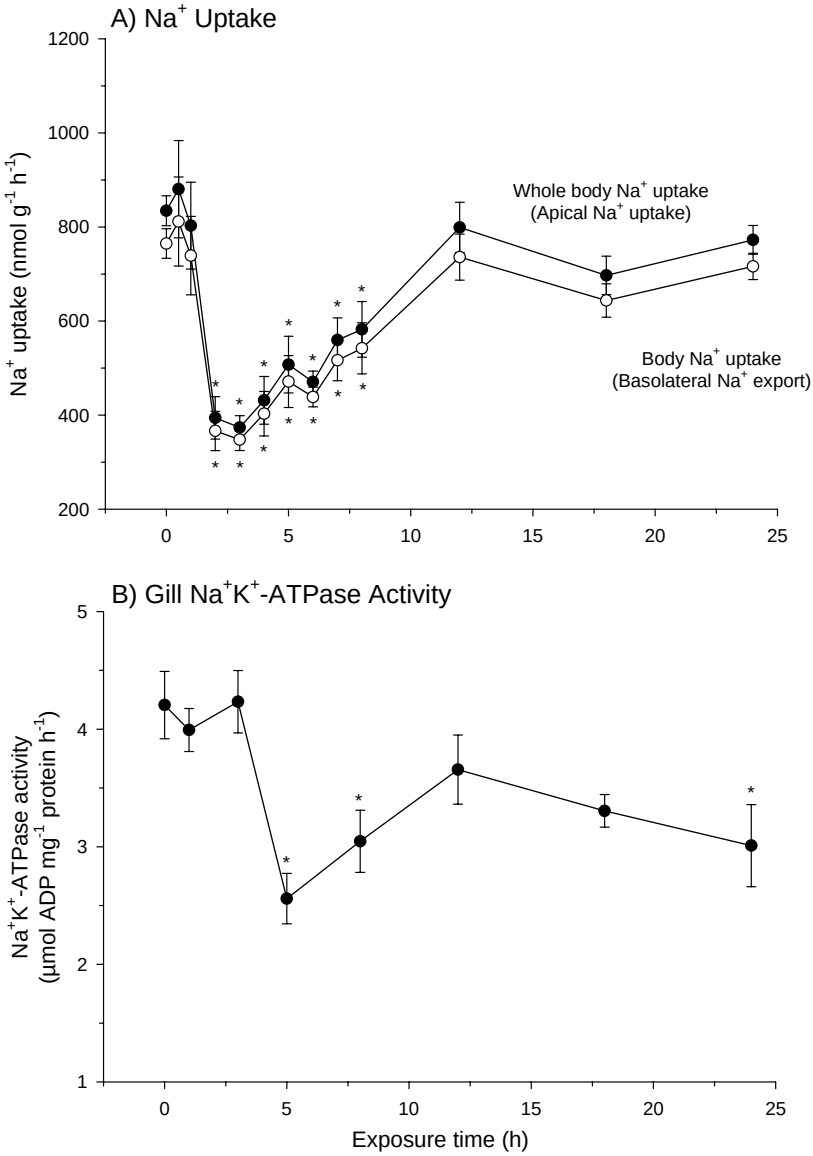



Fig. 4. Time course of inhibition of whole body (filled circles) and body (open circles) $Na^+$ uptake (A) and gill $Na^+K^+$-ATPase activity (B) of rainbow trout acutely exposed to 1.92 $\mu g\,l^{-1}$ total silver as $AgNO_3$ over 24 h in synthetic soft water. Values are means ±S.E.M. ($n = 8$ at each 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 $n = 7$). $*$ indicate significant differences from initial values (before silver exposure; ANOVA followed by the Dunnett's multiple comparison test; $P < 0.05$).

inhibited by ∼55% (Fig. 4A). 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 $\mu g\ Ag\,l^{-1}$ 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. 4B), 2 h after maximal inhibition of $Na^+$ uptake. If physiological regulation of silver movement through inhibition of $Na^+K^+$-ATPase activity could explain the

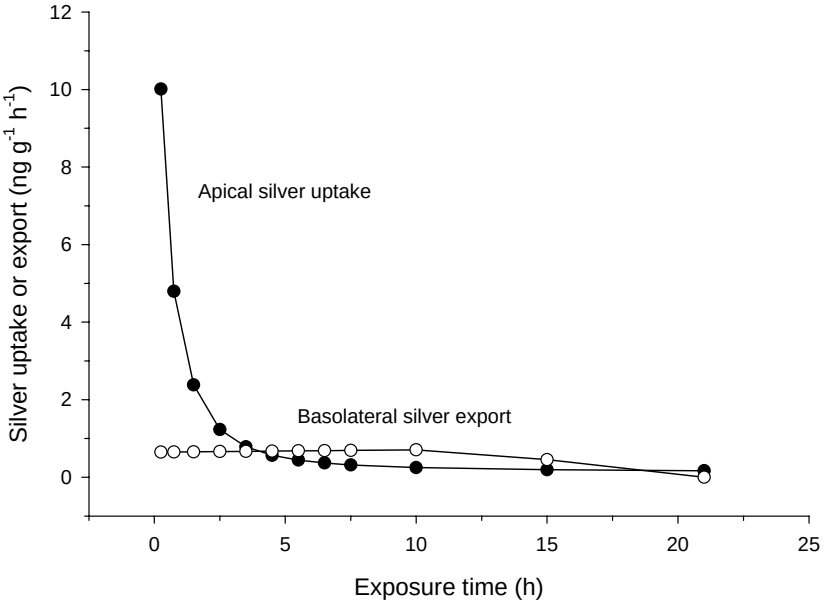



Fig. 5. Apical silver uptake (filled circles) and basolateral silver export (open circles) across the gills of rainbow trout acutely exposed to 1.92 $\mu g\,l^{-1}$ total silver as $AgNO_3$ for 24 h in synthetic soft water. Silver uptake was determined by first calculating silver accumulation at each time point based on the equations of the lines of best fit for the whole body and body silver accumulation data and then dividing the change in silver accumulation over each time interval by the length of the time interval.

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. 4A), 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 and Moczydlowski, 1991; Ravindran et al., 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.

### 4.3. 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 (Hogstrand et al., 1996; Galvez and Wood, 1997; Bury et al., 1999a; Karen et al., 1999). Complexation of silver by DOC or $Cl^-$ decreases the concentration 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 $Ag^+$, Ratte, 1999) 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,c; 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. 1B). However, there was also a large decrease in the dissolved silver concentration over the course of the exposure (~70% decline over 24 h; Fig. 1A). 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. 1C). The change in water TOC content is consistent with the earlier study results of Janes and Playle (1995), where TOC levels also increased during static gill silver accumulation studies. Their 2–3 h static uptake studies had biomass densities in the range of 6–18 g/l (versus 11 g/l in our study) and the TOC content in their lab reverse osmosis water increased from approximately 0.5–0.7 to 1.7 or 2.4 mg C/L TOC.

Although we were unable to interpret filtered water samples taken for DOC concentration analysis due to contamination of the samples with organic carbon from the 0.45 μm filters used, an increase in the DOC content of the exposure water might have 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., 1999b), 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$^+$ (Schecher and McAvoy, 1992) using

measured total silver concentrations and TOC values and appropriate binding constants from Janes and Playle (1995). Note that the DOC-binding constant taken from Janes and Playle (1995) is the binding constant for natural humic substances. It is possible that the constant may be different for mucus-like material derived from fish because of the higher protein content relative to natural humic substances. Therefore, speciation calculations should be interpreted with caution.

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. 5), these results suggest that altered $Ag^+$ bioavailability could explain the pattern of gill silver accumulation noted in these experiments.

Contradictory to this theory of altered silver bioavailability is the observation that gill silver accumulation continued to increase up to 3 h of exposure even though the TOC concentration was maximal by 2 h of silver exposure. However, if the kinetics of complexation of silver with TOC are slow, silver bioavailability and hence gill silver accumulation may not be affected until later in the exposure, once sufficient time has passed for complexation. There is no evidence to suggest that the rate of reaction between silver and TOC is slow, however Bowles et al. (2002) showed that it takes up to 3 h before silver interacts with another anionic ligand, sulfide. Furthermore, Ma et al. (1999) showed that increasing the reaction time between copper and humic acid reduces the toxicity of copper. The physiological and toxicological similarities between copper and silver suggest the same kinetic limitation may exist between silver and TOC. This idea of a kinetic limitation is consistent with the observation that initially during silver exposure whole body $Na^+$ uptake fell, but with continued silver exposure uptake later increased to pre-silver exposure values (Fig. 4A). As time passed and there was complexation of silver by TOC, the bioavailability of $Ag^+$ would decrease and 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, lending further support to the bioavailability theory.

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.

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

For the past 6 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. 4A and B) 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.

### 4.5. A relationship between gill silver accumulation and physiological mechanism of 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 (Figs. 2A and 4A). Indeed, regression analysis revealed a significant negative relationship between the two ($r^2 = 0.64$; $P < 0.01$; Fig. 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 US 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 (Figs. 2A and 4B), an observation that is supported by regression analysis ($r^2 = 0.08$; $P > 0.05$; Fig. 6B). This corroborates the findings of other investigators (McGeer and Wood, 1998; Bury et al., 1999c). 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.

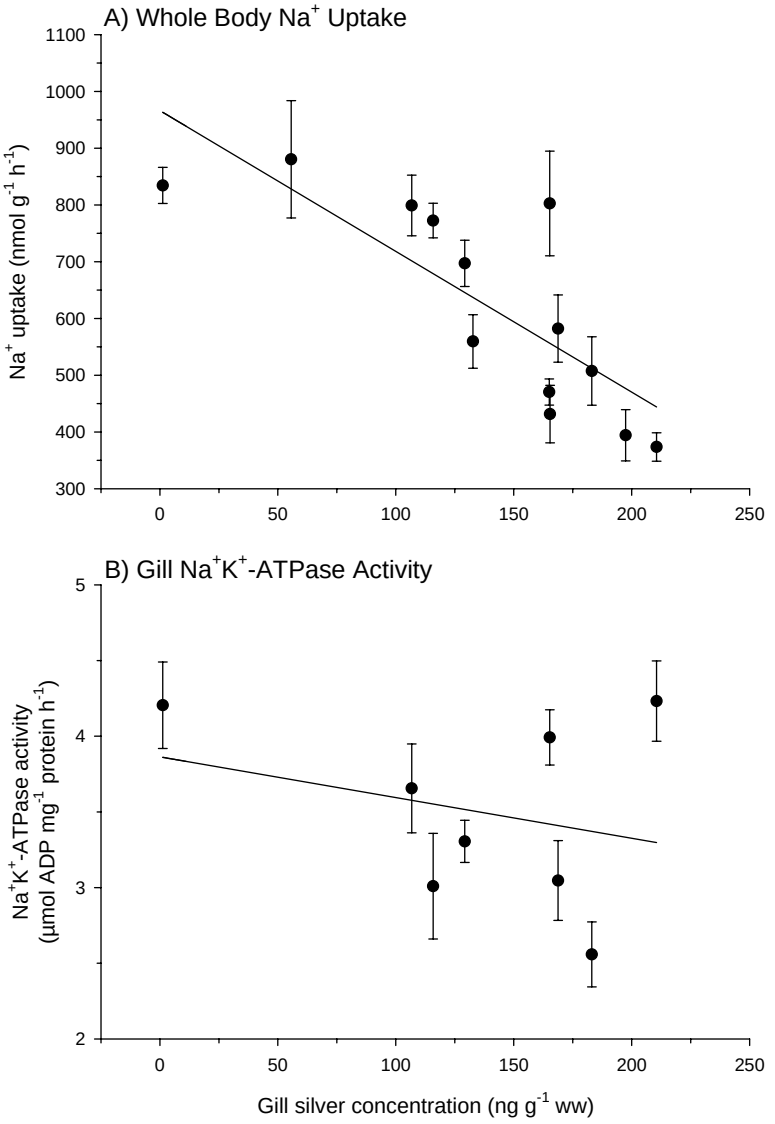



Fig. 6. Plot of whole body $Na^+$ uptake (A) and gill $Na^+K^+$-ATPase activity (B) as a function of gill silver concentration. Whole body $Na^+$ uptake was inversely correlated to the gill silver concentration by the following equation: whole body $Na^+$ uptake $= 966.4 - 2.5 \times$ gill silver concentration, 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 ($r^2 = 0.08$; $P > 0.05$).

## 5. 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. With regards to a role for carbonic anhydrase inhibition, a time course study of $Na^+$ uptake together with measurements of CA activity and $Na^+K^+$-ATPase activity are required to test this hypothesis. 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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## References

Alsop, D.H., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water. Environ. Toxicol. Chem. 18, 1014–1025.

Bowles, K.C., Bianchini, A., Brauner, C.J., Kramer, J.R., Wood, C.M., 2002. Evaluation of the effect of reactive sulfide on the acute toxicity of silver(I) to *Daphnia magna*. Part 1. Description of the chemical system. Environ. Toxicol. Chem. 21, 1286–1293.

Bury, N.R., Wood, C.M., 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled $Na^+$ channel. Am. J. Physiol. 277, R1385–R1391.

Bury, N.R., Galvez, F., Wood, C.M., 1999a. Effects of chloride, calcium and dissolved organic carbon on silver toxicity: comparison between rainbow trout and fathead minnows. Environ. Toxicol. Chem. 18, 56–62.

Bury, N.R., Grosell, M., Grover, A.K., Wood, C.M., 1999b. ATP-dependent silver transport across the basolateral membrane of rainbow trout gills. Toxicol. Appl. Pharmacol. 159, 1–8.

Bury, N.R., McGeer, J.C., Wood, C.M., 1999c. Effects of altering freshwater chemistry on physiological responses of rainbow trout to silver exposure. Environ. Toxicol. Chem. 18, 49–55.

Christensen, G.M., Tucker, J.H., 1976. Effects of selected water toxicants on the in vitro activity of fish carbonic anhydrase. Chem. Biol. Interact. 13, 181–192.

Cooper, C.F., Jolly, W.C., 1970. Ecological effects of silver iodide and other weather modification agents: a review. Water Resour. Res. 6, 88–98.

Davies, P.H., Goettl, J.P., Sinley, J.R., 1978. Toxicity of silver to rainbow trout (*Salmo gairdneri*). Water Res. 12, 113–117.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. Part 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2396.

Doyle, D.D., Guo, Y., Lustig, S.L., Satin, J., Rogart, R.B., Fozzard, H.A., 1993. Divalent cation competition with [$^3$H] saxitoxin binding to tetrodotoxin-resistant and -sensitive sodium channels. A two-site structural model of ion/toxin interaction. J. Gen. Physiol. 101, 153–182.

Erickson, R.J., Brooke, L.T., Kahl, M.D., Vende Venter, F., Harting, S.L., Markee, T.P., Spehar, R.L., 1998. Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. Environ. Toxicol. Chem. 17, 572–578.

Galvez, F., Wood, C.M., 1997. The relative importance of water hardness and chloride levels in modifying the acute toxicity of silver to rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 16, 2363–2368.

Giles, M.A., 1988. Accumulation of cadmium by rainbow trout, *Salmo gairdneri*, during extended exposure. Can. J. Fish. Aquat. Sci. 45, 1045–1053.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Grosell, M., Hogstrand, C., Wood, C.M., 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 38, 257–276.

Grosell, M., Hansen, H.J.M., Rosenkilde, P., 1998. Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. Comp. Biochem. Physiol. C. 120, 295–305.

Grosell, M., Hogstrand, C., Wood, C.M., Hansen, H.J.M., 2000. A nose-to-nose comparison of the physiological effects of exposure to ionic silver versus silver chloride in the European eel (*Anguilla anguilla*) and the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 48, 327–342.

Hansen, H.J.M., Grosell, M., Jacobsen, U., Jorgensen, J.C., Hogstrand, C., Wood, C.M., 2002. Precautions in the use of $^{110m}$Ag as a tracer of silver metabolism in ecotoxicology: preferential bioconcentration of $^{109}$Cd by trout gills after $^{110m}$Ag exposure. Environ. Toxicol. Chem. 21, 1004–1008.

Henry, R.P., 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annu. Rev. Physiol. 58, 523–538.

Hisatome, I., Kurata, Y., Sasaki, N., Morisaki, T., Morisaki, H., Tanaka, Y., Urashima, T., Yatsuhashi, T., Tsuboi, M., Kitamura, F., Miake, J., Takeda, S.-i., Taniguchi, S.-i., Ogino, K., Igawa, O., Yoshida, A., Sato, R., Makita, N., Shigemasa, C., 2000. Block of sodium channels by divalent mercury: role of specific cysteinyl residues in the P-loop region. Biophys. J. 79, 1336–1345.

Hogstrand, C., Wood, C.M., 1998. Toward a better understanding of the bioavailability, physiology and toxicity of silver in fish: implications for water quality criteria. Environ. Toxicol. Chem. 17, 547–561.

Hogstrand, C., Galvez, F., Wood, C.M., 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. Environ. Toxicol. Chem. 15, 1102–1108.

Hollis, L., Muench, L., Playle, R.C., 1997. Influence of dissolved organic matter on copper binding, and calcium on cadmium binding, by gills of rainbow trout. J. Fish Biol. 50, 703–720.

Janes, N., Playle, R.C., 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 14, 1847–1858.

Karen, D.J., Ownby, D.R., Forsythe, B.L., Bills, T.P., La Point, T.W., Cobb, G.B., Klaine, S.J., 1999. Influence of water quality on silver toxicity to rainbow trout (*Oncorhynchus mykiss*), fathead minnows (*Pimephales promelas*) and water fleas (*Daphnia magna*). Environ. Toxicol. Chem. 18, 63–70.

Kellenberger, S., Schild, L., 2002. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. Physiol. Rev. 82, 735–767.

Laurén, D.J., McDonald, D.G., 1986. Influence of water hardness, pH and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. Can. J. Fish Aquat. Sci. 43, 1488–1496.

Ma, H., Kim, S.D., Cha, D.K., Allen, H.E., 1999. Effect of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 18, 828–837.

MacRae, R.K., Smith, D.E., Swoboda-Colberg, N., Meyer, J.S., Bergman, H.L., 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gills: implications for assessing bioavailable metal. Environ. Toxicol. Chem. 18, 1180–1189.

McCormick, S.D., 1993. Methods for nonlethal gill biopsy and measurement of $Na^+$, $K^+$-ATPase activity. Can. J. Fish Aquat. Sci. 50, 656–658.

McGeer, J.C., Wood, C.M., 1998. Protective effects of water $Cl^-$ on physiological responses to waterborne silver in rainbow trout. Can. J. Fish Aquat. Sci. 55, 2447–2454.

McGeer, J.C., Playle, R.C., Wood, C.M., Galvez, F., 2000. A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to rainbow trout in freshwaters. Environ. Sci. Technol. 34, 4199–4207.

Morgan, I.J., Henry, R.P., Wood, C.M., 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill $Na^+$ and $Cl^-$ transport. Aquat. Toxicol. 38, 145–163.

Moschen, K., Schweizer, K., Wagner, C.A., Geis-Gerstorfer, J., Lang, F., 2001. Effects of gallium and mercury ions on transport systems. J. Dent. Res. 8, 1753–1757.

Nebeker, A.V., McAuliffe, C.K., Mshar, R., Stevens, D.G., 1983. Toxicity of silver to steelhead and rainbow trout, fathead minnows and *Daphnia magna*. Environ. Toxicol. Chem. 2, 95–104.

Paquin, P.R., Di Toro, D.M., Santore, R.C., Trivedi, D., Wu, B., 1999. A biotic ligand model of the acute toxicity of metals. III. Application to fish and Daphnia exposure to silver. EPA 822-E-99-001.

Perry, S.F., Fryer, J.N., 1997. Proton pumps in the fish gill and kidney. Fish Physiol. Biochem. 17, 363–369.

Playle, R.C., Dixon, G.D., Burnison, K., 1993a. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Can. J. Fish Aquat. Sci. 50, 2667–2677.

Playle, R.C., Dixon, G.D., Burnison, K., 1993b. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modelling of metal accumulation. Can. J. Fish Aquat. Sci. 50, 2678–2687.

Rahim, S.M., Delaunoy, J.-P., Laurent, P., 1988. Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. Histochemistry. 89, 451–459.

Ratte, H.T., 1999. Bioaccumulation and toxicity of silver compounds: a review. Environ. Toxicol. Chem. 18, 89–108.

Ravindran, A., Moczydlowski, E., 1991. Competitive binding interaction between $Zn^{2+}$ and saxitoxin in cardiac $Na^+$ channels. Evidence for a sulfhydryl group in the $Zn^{2+}$/saxitoxin binding site. Biophys. J. 59, 523–537.

Ravindran, A., Schild, L., Moczydlowski, E., 1991. Divalent cation selectivity for external block of voltage-dependent $Na^+$ channels prolonged by batrachotoxin. $Zn^{2+}$ induces discrete substrates in cardiac $Na^+$ channels. J. Gen. Physiol. 97, 89–115.

Richards, B.D., Fromm, P.O., 1970. Sodium uptake by isolated-perfused gills of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Phys. 33, 303–310.

Rose-Janes, N.G., Playle, R.C., 2000. Protection by two complexing agents, thiosulphate and dissolved organic matter, against the physiological effects of silver nitrate to rainbow trout (*Oncorhynchus mykiss*) in ion-poor water. Aquat. Toxicol. 51, 1–18.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S., 2001. Biotic ligand model of the acute toxicity of metals. Part 2. Application to acute copper toxicity in freshwater fish and *Daphnia*. Environ. Toxicol. Chem. 20, 2397–2402.

Schecher, W.D., McAvoy, D.C., 1992. MINEQL$^+$: a software environment for chemical equilibrium modeling. Comput. Environ. Urban Syst. 16, 65–76.

Schild, L., Moczydlowski, E., 1994. Permeation of $Na^+$ through open and $Zn^{2+}$-occupied conductance states of cardiac sodium channels modified by batrachotoxin: exploring ion-ion interactions in a multi-ion channel. Biophys. J. 66, 654–666.

Schild, L., Ravindran, A., Moczydlowski, E., 1991. $Zn^{2+}$-induced subconductance events in cardiac $Na^{+}$ channels prolonged by batrachotoxin. Current-voltage behavior and single-channel kinetics. J. Gen. Physiol. 97, 117–142.

Skou, J.C., 1990. The energy coupled exchange of $Na^{+}$ for $K^{+}$ across the cell membrane. The $Na^{+}$, $K^{+}$-pump. FEBS Lett. 268, 314–324.

Webb, N.A., Wood, C.M., 1998. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 17, 579–588.

Wood, C.M., Hogstrand, C., Galvez, F., Munger, R.S., 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). Part 1. The effects of ionic $Ag^{+}$. Aquat. Toxicol. 35, 93–109.

Wood, C.M., Grosell, M., Hogstrand, C., Hansen, H., 2002. Kinetics of radiolabelled silver uptake and depuration in the gills of rainbow trout (*Oncorhynchus mykiss*) and European eel (*Anguilla anguilla*): the influence of silver speciation. Aquat. Toxicol. 56, 197–213.

Zall, D.M., Fisher, D., Garner, M.D., 1956. Photometric determination of chlorides in water. Anal. Chem. 28, 1665–1678.