

An in vitro biotic ligand model (BLM) for silver binding to cultured gill epithelia of freshwater rainbow trout (*Oncorhynchus mykiss*)

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Abstract

“Reconstructed” gill epithelia on filter supports were grown in primary culture from dispersed gill cells of freshwater rainbow trout (*Oncorhynchus mykiss*). This preparation contains both pavement cells and chloride cells, and after 7–9 days in culture, permits exposure of the apical surface to true freshwater while maintaining blood-like culture media on the basolateral surface, and exhibits a stable transepithelial resistance (TER) and transepithelial potential (TEP) under these conditions. These epithelia were used to develop a possible in vitro version of the biotic ligand model (BLM) for silver; the in vivo BLM uses short-term gill binding of the metal to predict acute silver toxicity as a function of freshwater chemistry. Radio-labeled silver (^{110m}Ag as AgNO_3) was placed on the apical side (freshwater), and the appearance of ^{110m}Ag in the epithelia (binding) and in the basolateral media (flux) over 3 h were monitored. Silver binding (greater than the approximate range $0\text{--}100\ \mu\text{g l}^{-1}$) and silver flux were concentration-dependent with a 50% saturation point (apparent K_d) value of about $10\ \mu\text{g l}^{-1}$ or $10^{-7}\ \text{M}$, very close to the 96-h LC50 in vivo in the same water chemistry. There were no adverse effects of silver on TER, TEP, or Na^+ , K^+ -ATPase activity, though the latter declined over longer exposures, as in vivo. Silver flux over 3 h was small (<20%) relative to binding, and was insensitive to water chemistry. However, silver binding was decreased by elevations in freshwater Na^+ and dissolved organic carbon (humic acid) concentrations, increased by elevations in freshwater Cl^- and reductions in pH, and insensitive to elevations in Ca^{2+} . With the exception of the pH response, these effects were qualitatively and quantitatively similar to in vivo BLM responses. The results suggest that an in vitro BLM approach may provide a simple and cost-effective way for evaluating the protective effects of site-specific waters.

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Introduction

The gills of freshwater organisms bear negatively charged ligands to which cationic metals can bind and constitute the primary sites for toxicity of most metals, including silver (Bergman and Dorward-King, 1997; Wood, 2001). For silver, acute toxicity is associated with inhibition of sites involved in active Na^+ uptake at the gills, resulting in death from failure of NaCl homeostasis

(Wood et al., 1996). At least in part, silver appears to enter the gill cells via apical Na^+ channels (Bury and Wood, 1999). The basolateral Na^+ , K^+ -ATPase enzyme which powers active Na^+ uptake represents the key target molecule in the gills of both freshwater trout (Bury et al., 1999c; McGeer and Wood, 1998; Morgan et al., 1997) and daphnia (Bianchini and Wood, 2003). Silver salts such as AgNO_3 are routinely used in toxicity tests, and are highly water-soluble and strongly dissociated, yielding uncomplexed cationic silver (Ag^+) which is very toxic in simple laboratory waters, with a 96-h LC50 value for fish in the range of about $3\text{--}70\ \mu\text{g l}^{-1}$ total silver (for review, see Andren and Bober, 2002; Ratte, 1999; Wood et al., 1999). However, in the natural environment, most silver is bound to anions, the most important of which include particulate matter, organic colloids, thiosulphate, sulphide, dissolved organic carbon (DOC), and Cl^- , so

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that free Ag^+ represents only a very small percentage of the total (for review, see Andren and Bober, 2002; Wood et al., 1999).

Water chemistry and associated silver speciation can greatly affect silver toxicity (e.g., Andren and Bober, 2002; Galvez and Wood, 1997; Hogstrand and Wood, 1998; Hogstrand et al., 1996; Janes and Playle, 1995). Naturally occurring cations (e.g., Na^+) can offer protection by competing with Ag^+ for binding sites on the gills, while naturally occurring anions can bind Ag^+ , rendering it poorly available to gill sites. For example, $\text{Ag}(\text{S}_2\text{O}_3)_n$ and $\text{AgCl}_{(n)}$ have 96-h LC50 values in rainbow trout that are >10,000-fold greater than that of Ag^+ (Hogstrand and Wood, 1998; Hogstrand et al., 1996; LeBlanc et al., 1984). Because water chemistry can modify silver toxicity, these factors should be considered when new ambient water quality criteria (AWQC) are proposed for environmental regulations.

In recent years, there has been a great interest to develop ligand-binding and tissue-residue models to predict the site-specific toxicity of metals in different water qualities (Andren and Bober, 2002; Bergman and Dorward-King, 1997; Playle, 1998; Paquin et al., 2000, 2002a, 2002b). One of these approaches is the biotic ligand model (BLM), which provides a link between metal accumulation and its effects, while at the same time integrating the reactions of metals with important organic and inorganic ligands in the water column as well as the biotic ligands (e.g., gill toxic sites) on the organism itself (DiToro et al., 2001; Paquin et al., 2000). The BLM has received great interest among scientific and regulatory agencies because of its potential for use in developing AWQC and performing risk assessments for metals; Paquin et al. (2002a) have recently reviewed the history of BLM development and its application for these purposes.

For silver, Janes and Playle (1995) and Schwartz and Playle (2001) used model anionic ligands and competing cations in combination with a geochemical speciation program to estimate the strength of Ag^+ binding to the gills of live freshwater rainbow trout, and to model how Ag^+ binding changed in natural waters. Comparable data have been collected for copper, and the relationship between directly measured short-term binding of copper to the gills (i.e., 3–24 h, before pathology develops) and acute toxicity (e.g., 96-h mortality) has been documented (MacRae et al., 1999; Playle, 1998; Playle et al., 1993a, 1993b). The latter has facilitated the development of a robust BLM predictive of copper toxicity in natural waters (Santore et al., 2001). A comparable relationship for silver has proven elusive for methodological reasons (Bury and Wood, 1999; Bury et al., 1999a, 1999b, 1999c; McGeer and Wood, 1998; Morgan et al., 2004). Nevertheless, useful BLM approaches for silver have been developed based on other endpoints. For example, Paquin et al. (1999) used assumed gill-binding data, and then calibrated

BLMs for trout, fathead minnow, and daphnia directly to toxicity data in the literature. McGeer et al. (2000) noted that approximately 85% inhibition of branchial Na^+ , K^+ -ATPase activity was associated with 96-h mortality in rainbow trout, and developed a physiologically based BLM to calculate the acute toxicity of silver based on measured stability constants for Ag^+ inhibiting this enzyme. Very recently, Morgan and Wood (2004) demonstrated that when methodological limitations were overcome, a direct relationship could be demonstrated between short-term gill silver binding and 96-h mortality in trout.

There has also been much recent interest in replacing *in vivo* fish toxicity tests with *in vitro* fish cell tests (Castaño et al., 2003). Given that gills are the major sites for silver toxicity, it is conceivable that gill epithelia in primary culture could be used to construct an *in vitro* BLM for silver binding. Several different preparations of gill epithelia grown on filter “inserts” in primary culture have been developed for the freshwater rainbow trout (Fletcher et al., 2000; Wood and Pärt, 1997; Wood et al., 2003). These preparations permit exposure of the apical surface to true freshwater while maintaining blood-like culture media on the basolateral surface—that is, duplicating “asymmetrical” *in vivo* conditions—and have already been used in aquatic toxicology (reviewed by Castaño et al., 2003). The double-seeded insert (DSI) preparation (Fletcher et al., 2000) in particular incorporates both pavement cells (respiratory cells) and chloride cells (mitochondria-rich cells), maintains the typical electrophysiological and permeability characteristics of fish gills *in vivo*, and therefore to date best represents a “reconstructed gill”. The transepithelial resistance (TER) provides a readily measured index of epithelial integrity (for review, see Wood et al., 2002b).

Therefore, the main objectives of our study were to use the cultured DSI gill epithelia of trout to (1) investigate tolerance of the preparation to silver exposure; (2) determine whether concentration-dependent silver binding could be detected; (3) characterize the individual effects of water chemistry changes (e.g., Na^+ , Ca^{2+} , Cl^- , DOC, and pH) on silver binding to the epithelia; and (4) compare the results with recent *in vivo* data used in silver BLM development. Radiolabeled silver ($^{110\text{m}}\text{Ag}$) was used to provide greatest sensitivity and accuracy in silver-binding measurements (cf. Bianchini and Wood, 2003; Bury and Wood, 1999).

Materials and methods

Fish and preparation of double-seeded inserts (DSI). Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). The fish (85–200 g) were held in dechlorinated Hamilton tap water [in mM: Na^+ =

0.55; $\text{Cl}^- = 0.70$; $\text{Ca}^{2+} = 1.00$; $\text{Mg}^{2+} = 0.15$; $\text{K}^+ = 0.05$; pH 7.8–8.0; dissolved organic carbon (DOC) = 3.0 mg l^{-1}] at seasonal temperatures (12–15 °C).

The double-seeded insert (DSI) preparations were prepared following the procedure originally outlined by Fletcher et al. (2000) and described in detail by Kelly et al. (2000). In brief, gill cells were excised from gill filaments by two consecutive cycles of tryptic digestion at room temperature (Gibco Life Technologies, 0.05% trypsin in phosphate-buffered saline [PBS], pH 7.7 with 5.5 mM EDTA). The cells were resuspended in cold culture medium (Leibovitz L-15 supplemented with 5% foetal bovine serum [FBS], 100 iu ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, $200 \mu\text{g ml}^{-1}$ gentamycin) and seeded at a density of $3 \times 10^6 \text{ cells cm}^{-2}$ on the upper surface of Falcon cell inserts (Cyclopore polyethylene terephthalate “filters”, Becton-Dickinson, Franklin Lakes, NJ, USA; pore density: $1 \times 10^6 \text{ pores cm}^{-2}$; pore size $0.45 \mu\text{m}$; growth area: 0.9 cm^2). One day after seeding, new cells identically prepared from a second fish were seeded onto the cell layer of each insert at a density of $2 \times 10^6 \text{ cells cm}^{-2}$; this date was termed day 1. L15+5% FBS culture medium was present on both apical and basolateral surfaces (symmetrical conditions, L15/L15) and was replaced every second day, and was antibiotic-free beginning on day 4. The culture temperature was 18 °C, and all experiments were conducted at this temperature.

Electrophysiological measurements. Transepithelial resistance (TER) across the epithelium was monitored with STX-2 chopstick electrodes connected to an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) which was custom-modified to read in the range 0–100 $\text{K}\Omega \text{ cm}^2$. Transepithelial potential (TEP) was measured with an agar/salt bridge (3 M KCl in 4% agar) connected to Ag/AgCl electrodes (WPI) using a pH meter (Radiometer pHM 84, Copenhagen, Denmark) as a high-impedance electrometer. All TEP measurements were expressed relative to the apical side as 0 mV. Corrections for junction potential and for blank TER of vacant inserts were performed as described by Kelly and Wood (2001). All epithelia were allowed to develop in the normal manner under symmetrical conditions (L15/L15) to a stable TER of 25–30 $\text{K}\Omega \text{ cm}^2$ (plateau phase), which was typically reached in 7–9 days, and were then used for silver exposure experiments. During all tests, TER was measured at the beginning, middle, and end of the experimental period, and TEP was measured at the same time, except in the pH and DOC series (see below), where TEP was not monitored. Reported TER and TEP values are the means of measurements taken at these three times.

Fresh water and silver exposure. After TER had reached the plateau phase, the apical medium was replaced by

fresh water (FW), and the preparation was allowed to acclimate for 3 h to these asymmetrical conditions (FW/L15). FW was sterilized through a $0.22\text{-}\mu\text{m}$ Acrodisc filter. The FW used, depending on the particular experiment (see Results), was either the acclimation water (AW: Hamilton tap water [in mM: $\text{Na}^+ = 0.55$; $\text{Cl}^- = 0.70$; $\text{Ca}^{2+} = 1.00$; $\text{Mg}^{2+} = 0.15$; $\text{K}^+ = 0.05$; pH 7.8–8.0; dissolved organic carbon (DOC) = 3.0 mg l^{-1}]) or a synthetic water (SW: ion-poor soft water [$\text{Na}^+ = 0.04$; $\text{Cl}^- = 0.04$; $\text{Ca}^{2+} = 0.04$; $\text{Mg}^{2+} = 0.02$, $\text{K}^+ = 0.02$; DOC = 0.3 mg l^{-1} ; pH = 7.0]. The latter was designed to duplicate the ion- and DOC-poor soft water used by Janes and Playle (1995) and Morgan and Wood (2004) in developing some of the BLM relationships in vivo, and was made by mixing reverse-osmosis treated water with dechlorinated Hamilton tap water. At changeover, the apical compartment was rinsed three times with FW (each time with 2 ml) to ensure that there was no culture medium contamination on the apical side.

Silver exposure periods were typically 3 h, as for in vivo binding tests used in BLM development (e.g., Janes and Playle, 1995; Morgan and Wood, 2004), and started at 3 h after the initial exposure to FW. This regime was selected based on our recent studies with a variety of freshwater exposure regimes (Fletcher et al., 2000; Kelly and Wood, 2001, 2003; Wood et al., 2002b, 2003; Zhou et al., 2003) so as to provide a 3-h test period during which TER, TEP, and both transcellular and paracellular conductance would remain stable. This test period follows the period of regulatory cell volume decrease after hypotonic exposure (Leguen and Prunet, 2001; Leguen et al., 2001). At 3 h after the changeover to FW, the apical FW was changed again to a FW solution (start of a test), containing the appropriate amount of the agent of interest (see below) plus radiolabeled silver ($^{110\text{m}}\text{Ag}$ as AgNO_3 , Risø National Laboratory, Roskilde, Denmark) to achieve an approximate desired concentration, which was later verified by measurement. In practice, because significant amounts of silver were lost from FW to contact surfaces other than the epithelium itself (e.g., side walls of beakers and the insert itself), it was difficult to set exact values, and therefore critical to directly measure the concentrations of silver in the apical FW during the binding tests. Samples from both apical and basolateral sides were collected at the beginning (time 0 h) and at the end of the experiment (3 h) to measure radioactivity and total silver concentration. Typically, apical silver values decreased by less than 20% during a 3-h test, and were consistent among replicates, so mean measured exposure values are reported. At the end of a 3-h test, the FW on the apical side was removed, the epithelium was rinsed four times with silver-free FW to remove loosely bound silver, and was then removed by cutting the filter support from the insert walls and dispatched for γ -counting (for measurement of silver binding), or in some cases for determination of Na^+ , K^+ -ATPase activity.

Silver binding was calculated as:

$$\text{Binding (pg cm}^{-2}\text{)} = \text{CPM}_e / \text{SA} \times \text{Area} \quad (1)$$

where CPM_e represents the epithelial membrane count, SA is the mean measured specific activity (average of time 0 h and 3 h values) of total silver on the “hot” apical side, and Area of an insert = 0.9 cm^2 .

The flux rate of silver from the apical to the basolateral compartment was calculated as:

$$\text{Flux Rate (pg cm}^{-2}\text{h}^{-1}\text{)} = \Delta\text{CPM}_{\text{bl}} / \text{SA} \times T \times \text{Area} \quad (2)$$

where $\Delta\text{CPM}_{\text{bl}}$ is the net increase in total counts in the basolateral compartment from time 0 to 3 h, T (time) is 3 h, and the SA and Area are the same as in Eq. (1).

Tests for silver binding by the filter membranes and basolateral well walls. The loss of silver from the apical FW to the walls of the apical compartment mentioned above raised the possibility that silver might also bind to the walls of the basolateral well and/or to the polyethylene terephthalate filters themselves. The former would reduce apparent apical-to-basolateral silver flux, and the latter would do the same while increasing apparent silver binding to the cultured epithelium, in both cases representing artifacts. To address these possibilities, several series of methodology tests were performed.

To assess possible silver binding to the walls of the basolateral well, at the end of several routine 3-h apical silver exposure tests, the basolateral wells were rinsed once with fresh L-15 media + 5% FBS, then cut out of the plate and dispatched for individual γ -counting.

To assess possible binding to the polyethylene terephthalate filters on which the cultured epithelium was supported, typical 3-h silver binding exposures were performed at two different levels of apical silver corresponding to about $30 \mu\text{g l}^{-1}$ and $100 \mu\text{g l}^{-1}$. At the end of 3 h, half of the cultured epithelial preparations were processed exactly as described above with the filter support plus attached epithelium being rinsed, then removed intact by cutting the filter support from the insert walls, and finally dispatched for γ -counting. The other half, matched to the same TER values, were similarly rinsed, removed intact, but then placed into individual glass vials, each containing 1 ml of trypsin solution (Gibco Life Technologies, 0.05% trypsin in phosphate-buffered saline [PBS], pH 7.7 with 5.5 mM EDTA) to remove the cells from the underlying filter support. During this 3- to 4-min period, the glass vial was continuously agitated, with periodic inspections under the microscope to monitor cell removal. The filter was then removed to a second vial and agitated for a further 3–4 min with 2 ml of FW (final rinse water), again with periodic microscopical observation to ensure that cell

removal was complete. The filter, the trypsin solution (containing the majority of the cells), and the final rinse water (containing a few cells) were then dispatched for separate γ -counting. Naked filter membranes (no cells) were similarly exposed and processed.

Experimental design. Because TER and other characteristics of cultured epithelia tend to vary seasonally and with fish from different batches (Kelly et al., 2000; Wood et al., 2002b), experiments were typically run using DSI preparations generated from a few fish within days of one another, with $N = 6\text{--}10$ DSI epithelia per treatment. Experiments were repeated several times to ensure that observed trends were not “batch-specific”, but different experiments were not averaged because of sometimes large differences in absolute values.

The water to which the fish had been acclimated (AW) was used as the standard FW in the apical compartment in most tests. However, in several of the cation competition experiments where no effects were seen in AW, it was suspected that the background levels of the particular cation were already too high in AW—that is, above the effective response range. In these cases, the experiments were repeated using synthetic water (SW, ion-poor soft water, see above for composition) as the apical FW.

Tolerance to silver and concentration-dependent silver binding. A series of concentrations of radiolabeled silver ($^{110\text{m}}\text{AgNO}_3$, nominally in the range of $2\text{--}100 \mu\text{g l}^{-1}$) was added to the apical FW. Tolerance of cultured epithelia to silver was assessed by changes in TER and TEP over the 3-h exposure period, and in a few trials, by changes in Na^+ , K^+ -ATPase activity. In these cases, the exposures were extended up to 24 h.

Effects of water chemistry (Na^+ , Ca^{2+} , Cl^- , DOC, and pH) on silver binding. Experiments investigating the effects of water chemistry were initially performed using AW, and those evaluating the influence of Na^+ and Ca^{2+} were repeated with SW. Na^+ tests were carried out with both Na_2SO_4 and NaNO_3 in the range $0.3\text{--}33 \text{ mM}$ (as Na^+). Ca^{2+} tests were carried out with $\text{Ca}(\text{NO}_3)_2$ in the range $0.2\text{--}30 \text{ mM}$ (as Ca^{2+}). With the knowledge of the effects of the Na^+ and Ca^{2+} cations in hand, the influence of Cl^- on silver binding was tested using NaCl and CaCl_2 (0.8 to 13 mM , as Cl^-). To investigate the effects of DOC on silver binding, a humic acid (Aldrich) stock solution was made by dissolution in double-distilled water and filtered through a $0.45\text{-}\mu\text{m}$ glass microfiber filter (GD/X Syringe Filter, Whatman) to ensure that only dissolved material was present, and tested in the range 3 to 20 mg C l^{-1} . To determine whether lower pH affected silver binding, the pH of the apical solution (AW) was adjusted to 7.8, 7.0, 6.0, and 5.5 using 1% HNO_3 .

In all cases, $^{110\text{m}}\text{AgNO}_3$ was added to a batch of the appropriate freshwater (AW or SW) to create a common stock solution at the intended silver concentration (20–40 $\mu\text{g l}^{-1}$), and then the required amount of the agent of interest (as a concentrated stock) was added to subsamples to be used as the apical exposure solutions. In the case of DOC, the solutions were allowed to equilibrate for 3 h with the silver before testing. Actual concentrations of Cl^- and DOC were measured in the exposure media, while pH, which tended to rise, was adjusted at 30-min intervals with 1% HNO_3 . In our experience, DOC and Cl^- concentrations were labile (probably due to adsorption to test chamber walls), whereas Na^+ and Ca^{2+} concentrations were stable, allowing the use of preset nominal values for these cations.

Analytical techniques. Radioactivity of $^{110\text{m}}\text{Ag}$ was counted in a γ -counter (MINAXI Auto-gamma 5000, Canberra-Packard) according to the procedures of Hansen et al. (2002). The silver concentration was measured by graphite furnace atomic absorption (Varian AA-1275 with GTA-95 atomizer) using certified standards (Fisher). Na^+ and Ca^{2+} concentrations were measured by flame atomic absorption (Varian AA-1275) and Cl^- by the colorimetric mercuric thiocyanate method (Zall et al., 1956) using certified standards (Fisher Scientific, Radiometer-Copenhagen). Total and inorganic carbon concentrations were measured using a total organic carbon analyzer (Shimadzu TOC-5050A, Tokyo, Japan). Organic carbon concentrations were calculated automatically by subtracting inorganic carbon from total carbon, and are reported as DOC (in mg C l^{-1}), because the Aldrich humic acid had been 0.45 μm filtered. Na^+ , K^+ -ATPase activities were determined by the microplate method of McCormick (1993) according to methods outlined by Kelly and Wood (2001) for cultured gill epithelia. Results were expressed as specific activities (per unit total protein) with protein measured by the Bradford method (Sigma) using bovine serum albumin (Sigma) as a standard.

Speciation of silver in the exposure water was calculated using the MINEQL+ aquatic geochemistry program, version 3.01 (Schecher and McAvoy, 1992), using measured chemistry, constants for DOC from Janes and Playle (1995), and other constants as in the program. Conditional stability constants for cations (log K values) binding to the same sites as Ag^+ were estimated from the lowest concentrations which were effective in reducing silver binding to the gills, as described by Janes and Playle (1995), taking into account the estimated affinity of the gill sites for Ag^+ (see Results) and the measured silver levels in the exposure.

Statistical analysis. All data are expressed as means \pm SEM (N), where N represents the number of inserts. A one-way analysis of variance (ANOVA) was used, followed by either Dunnett's multiple comparison test

(for repeated measures) or Newman–Keuls multiple comparison test (for independent measures) to determine significant differences. Unpaired Student's t test was used to test certain differences between controls and experimental treatments. Non-linear or linear regressions were conducted with SigmaPlot software (Version 7.0). The level of statistical significance for all analyses was $P < 0.05$.

Results

Silver binding by the filter membranes and basolateral well walls

All tests demonstrated that losses of silver from the basolateral media to contact surfaces (the basolateral well walls) were negligible, probably because the plasma-like L15 media + 5% FBS strongly complexed the silver, unlike the apical FW. When naked polyethylene terephthalate filter membranes (no cells present) were exposed to silver in apical FW, considerable silver bound to them, up to 2–3 times as much as measured on the cultured epithelia plus filter supports. However, the 3-h tests to determine whether silver binding to the filter supports also occurred when they were covered with a cultured epithelium demonstrated that this was not a problem. In the high concentration tests (approximately 100 $\mu\text{g l}^{-1}$, $N = 6$), $97.7 \pm 0.9\%$ of the $^{110\text{m}}\text{Ag}$ cpm were in the cellular fraction (removed by trypsin) and only $2.3 \pm 0.9\%$ were bound to the filter. In the low concentration tests (approximately 30 $\mu\text{g l}^{-1}$; $N = 4$), 100% of the $^{110\text{m}}\text{Ag}$ cpm were in the cellular fraction. Total $^{110\text{m}}\text{Ag}$ cpm recovered did not differ significantly from those of TER-matched preparations (cultured epithelia covering filter supports) which were processed in the normal manner and not subjected to trypsinization ($N = 10$ pairs).

Tolerance to silver; concentration-dependent silver binding, and flux rates

There was no effect of apical silver levels up to 87 $\mu\text{g l}^{-1}$ on the TER (Fig. 1A) or TEP (Fig. 1B) of cultured gill epithelia either during the 3-h exposure period itself, or relative to unexposed controls (Fig. 1). The observed TER was typically around 25 $\text{K}\Omega \text{ cm}^2$ while TEP was about -4 mV (with reference to the apical side as 0 mV). Neither parameter differed among groups, indicating no acute toxicity of silver exposure during the experimental period. This result was reinforced by the finding of unchanged Na^+ , K^+ -ATPase activity at 3 h (Fig. 2). However, by 6 and 24 h, significant decreases in Na^+ , K^+ -ATPase activity were seen in SW in two separate experiments (Fig. 2). In these longer exposures, TER was not significantly altered relative to simultaneous control measurements (e.g., 17.5 ± 9.2 , 16.2 ± 1.9 , and $19.1 \pm 1.2 \text{ K}\Omega \text{ cm}^2$ in control, 28, and

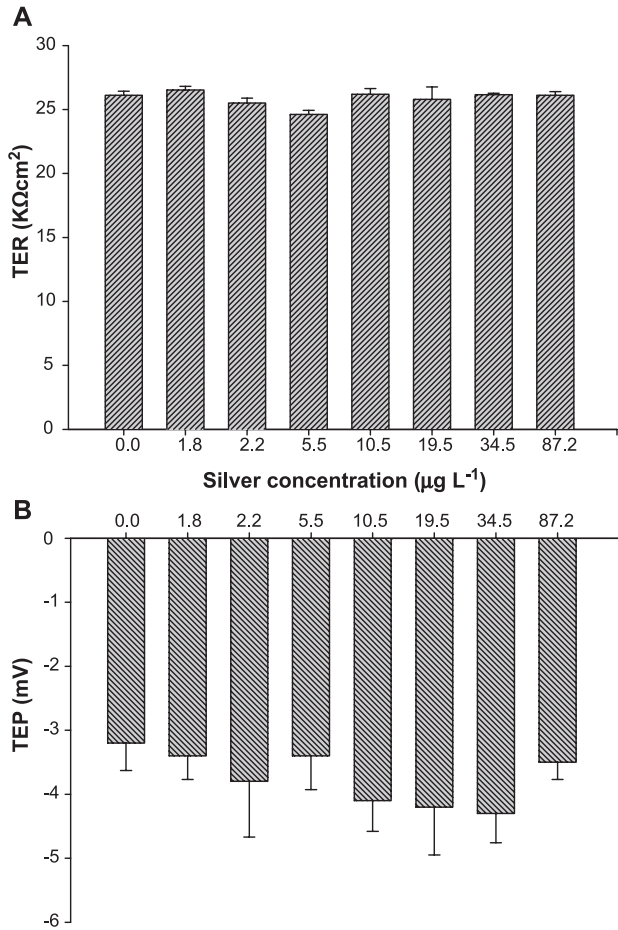


Fig. 1. (A) Transepithelial resistance (TER) and (B) transepithelial potential (TEP, with reference to apical surface as 0 mV) of cultured gill epithelia after exposure to different levels of silver in apical acclimation water (AW) for 3 h (means \pm SEM, $N = 6$ at each concentration). Different concentrations of silver (as AgNO_3) were added to the apical side, and TER and TEP were monitored at the beginning (T_0), middle ($T_{1.5h}$), and end (T_{3h}) of each 3-h experiment. The reported TER and TEP values are the respective means of these three measurements. There were no significant differences in TER or TEP associated with the different concentrations of silver.

60.5 $\mu\text{g l}^{-1}$ silver at 24 h); these slightly lower values reflect the normal decline in TER that occurs by this time. TEP remained unchanged.

Cultured gill epithelia exhibited concentration-dependent silver binding during the 3-h exposure. While absolute values of binding differed among different experimental series, the patterns were qualitatively similar; non-linear regressions revealed saturable binding with a 50% saturation point (apparent K_d) value of about 10 $\mu\text{g l}^{-1}$ or 10^{-7} M (Fig. 3A). By MINEQL+ speciation analysis, this represents about 1.7×10^{-9} M as free Ag^+ , or a log K value of 8.8 (i.e., the log of the inverse of K_d).

Flux rates of silver from the apical to basolateral compartments were detectable in all experiments, though rather variable, and increased significantly with apical concentration in a comparable saturable manner. However, the

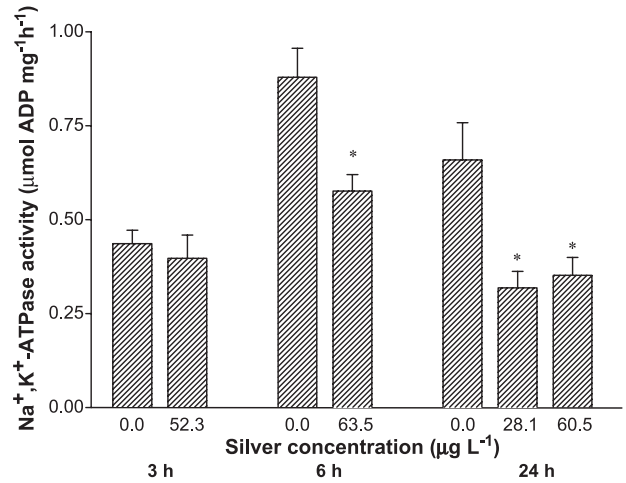


Fig. 2. The influence of exposure to silver in apical FW for various time periods (3-h test in AW, 6- and 24-h tests in soft water [SW]) on Na^+ , K^+ -ATPase activities of cultured gill epithelia (means \pm SEM, $N = 6-8$). Asterisks indicate significant differences from the corresponding control values determined at the same time in the absence of silver.

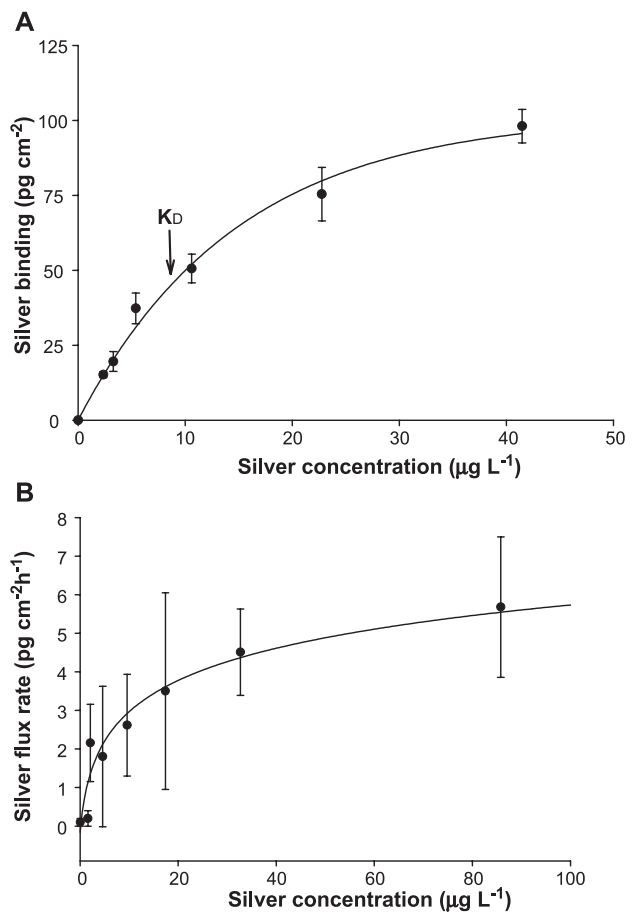


Fig. 3. (A) Concentration-dependent silver binding to cultured gill epithelia after 3-h exposures in apical AW. 50% saturation occurred at approximately 10 $\mu\text{g l}^{-1}$, or 10^{-7} M. (B) Concentration-dependent silver flux rates from apical to basolateral media over the same time periods in a separate series. For both, $N = 6-8$ at each silver concentration.

amount of silver moving into the basolateral compartment over the 3-h experiment was typically only a small fraction (<20%) of that bound to the epithelium. For example, in the series illustrated in Fig. 3B, flux rates at approximately $40 \mu\text{g l}^{-1}$ Ag were about $4 \text{ pg cm}^{-2} \text{ h}^{-1}$, amounting to about 12% over 3 h relative to the silver binding at 3 h of 100 pg cm^{-2} (Fig. 3A).

Effects of water chemistry (Na^+ , Ca^{2+} , Cl^- , DOC, and pH) on silver binding

These tests were run at measured apical silver concentrations which were typically 2- to 4-fold higher than the K_d concentration so that the epithelial binding sites were close to saturation. None of the treatments had any effect on TER or TEP over 3-h exposures, indicating unchanged epithelial integrity. However, TEP was not measured in the DOC and pH series.

When Na_2SO_4 and NaNO_3 were tested in AW (background $[\text{Na}^+] = 0.55 \text{ mM}$), there were no significant effects on silver binding at Na^+ concentrations up to 33 mM (data not shown). However, when the experiments were repeated in SW (ion-poor soft water, background $[\text{Na}^+] = 0.04 \text{ mM}$), silver binding was significantly reduced at a concentration of 1.0 mM or above by both Na_2SO_4 (Fig. 4A) and NaNO_3 (Fig. 4B), suggesting a

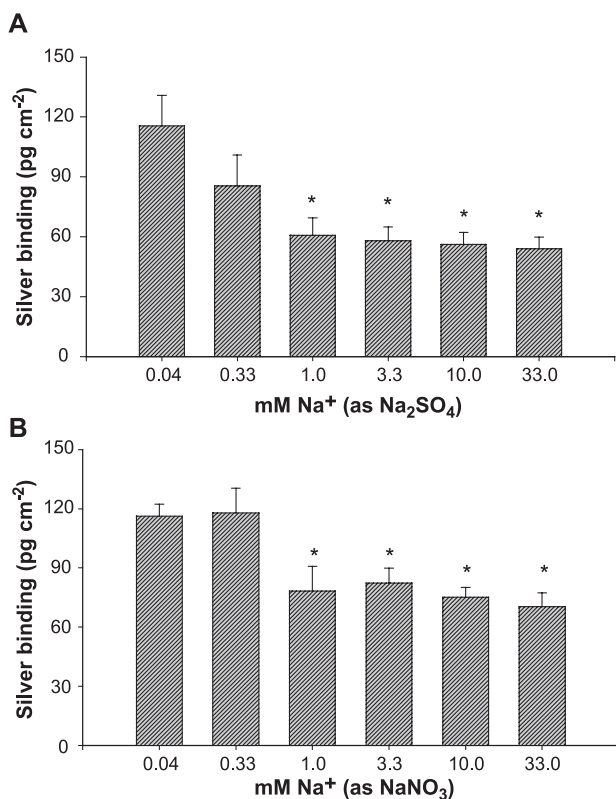


Fig. 4. The influence of Na^+ in apical SW (ion-poor soft water) on silver binding ($11.2 \mu\text{g l}^{-1}$ as $^{110\text{m}}\text{Ag}$ -labeled AgNO_3) to cultured gill epithelia. (A) Na_2SO_4 ; (B) NaNO_3 (means \pm SEM, $N = 7-8$). Asterisks indicate significant differences from controls (lowest Na^+ concentration).

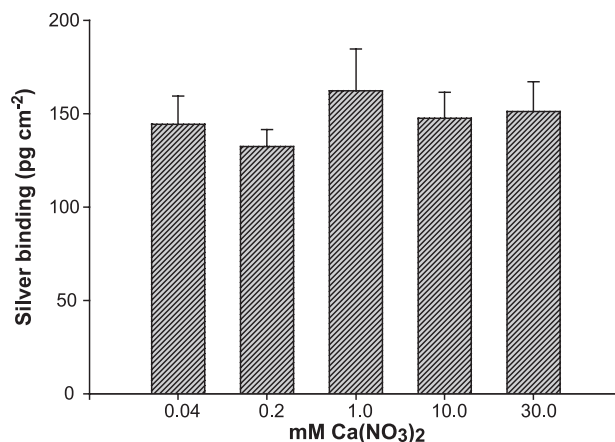


Fig. 5. The influence of Ca^{2+} [added as $\text{Ca}(\text{NO}_3)_2$] in apical SW (ion-poor soft water) on silver binding ($34.4 \mu\text{g l}^{-1}$ as $^{110\text{m}}\text{Ag}$ -labeled AgNO_3) to cultured gill epithelia (means \pm SEM, $N = 7$). There were no significant differences.

log K value for Na^+ around 4.8 at these silver binding sites. The decrease in silver load was about 30–40% and did not change at higher Na^+ concentrations, indicating the additional presence of higher affinity silver binding sites, which were refractory to Na^+ over the concentration range tested.

When $\text{Ca}(\text{NO}_3)_2$ was tested in AW (background $[\text{Ca}^{2+}] = 1 \text{ mM}$), there were no significant effects of Ca^{2+} up to 30 mM (data not shown), similar to the situation for Na^+ . However, in contrast to Na^+ , when the experiments were repeated in SW (background $[\text{Ca}^{2+}] = 0.04 \text{ mM}$), the addition of up to 30 mM Ca^{2+} still did not decrease silver binding to the epithelia (Fig. 5). This result indicates a very low affinity of the silver binding sites for Ca^{2+} , with a log K value less than 3.8.

The Cl^- anion has log K values for silver of about 3.2–5.5 in geochemical speciation programs (e.g., Schecher and McAvoy, 1992), so Cl^- would be expected to react with Ag^+ to form silver chloride complexes, thereby decreasing Ag^+ binding to the gill epithelium, and thereby preventing toxicity. However, in practice, this has not been the case in *in vivo* tests where Cl^- decreases toxicity without necessarily decreasing Ag^+ binding to the gills, which has been a problem in the theoretical development of the BLM for silver (see Discussion). *In vitro*, exposure of the cultured gill epithelium to increasing concentrations (0.8 to 25 mM) of Cl^- as either NaCl (Fig. 6A) or CaCl_2 (Fig. 6B) resulted not in a decrease, but rather in an increase in silver binding. This trend was more pronounced for CaCl_2 , where the effect became significant at 2.4 mM Cl^- , whereas for NaCl , the effect first became significant at 13.2 mM Cl^- . This quantitative difference likely reflects the competitive influences of the accompanying cations: Na^+ was at least ten-fold more effective than Ca^{2+} in displacing silver from gill binding sites (Fig. 4 vs. Fig. 5).

DOC is anionic at circumneutral pH and might be expected to complex with silver, thereby reducing its

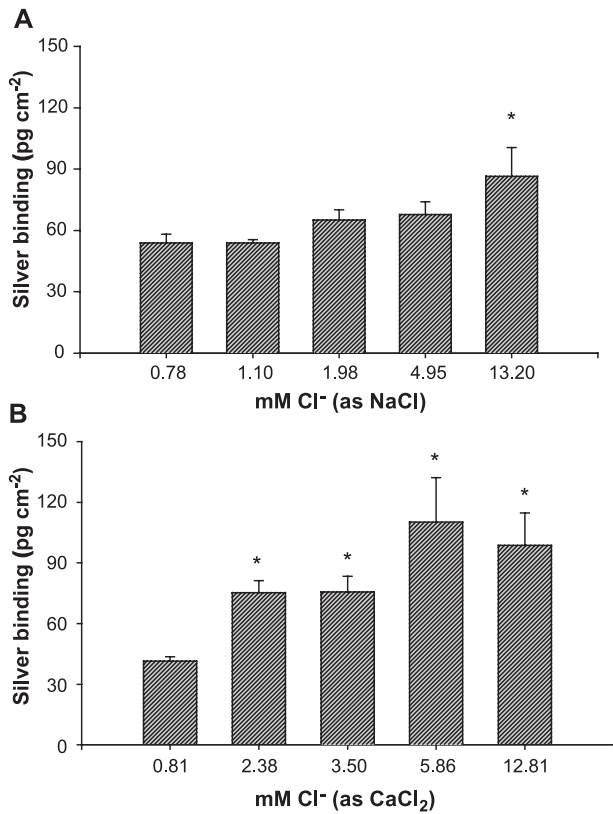


Fig. 6. The influence of Cl⁻ in apical AW on silver binding (39.5 μg l⁻¹ as ^{110m}Ag-labeled AgNO₃) to cultured gill epithelia. (A) NaCl; (B) CaCl₂ (means ± SEM, N = 7–8). Asterisks indicate significant differences from controls (lowest Cl⁻ concentration).

availability to gill sites. There was no significant effect of increasing DOC concentrations up to about 14 mg C l⁻¹ on silver binding to the epithelium in vitro, but the highest concentration tested (17.6 mg C l⁻¹) caused about 50% reduction of silver binding (Fig. 7).

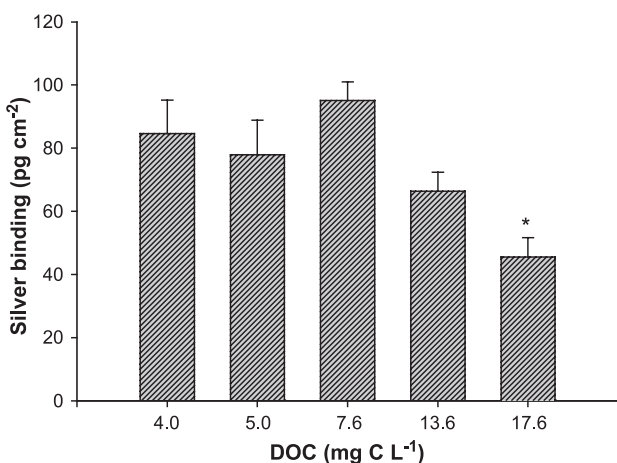


Fig. 7. The influence of DOC (as Aldrich humic acid) in apical AW on silver binding (24.5 μg l⁻¹ as ^{110m}Ag-labeled AgNO₃) to cultured gill epithelia (means ± SEM, N = 8). Asterisks indicate significant differences from controls (lowest DOC concentration).

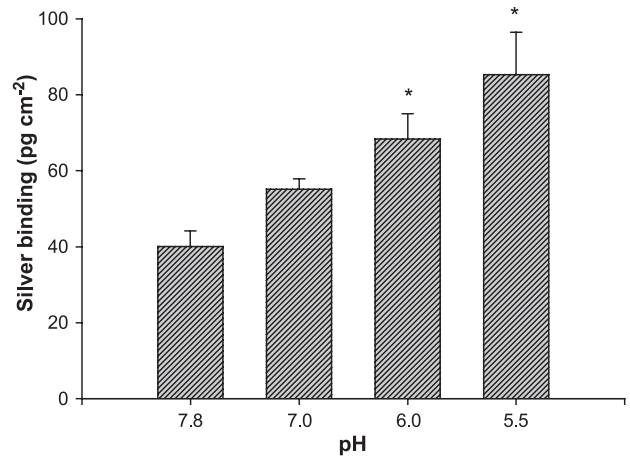


Fig. 8. The influence of pH (adjusted with 1% HNO₃) in apical AW on silver binding (33.3 μg l⁻¹ as ^{110m}Ag-labeled AgNO₃) to cultured gill epithelia (means ± SEM, N = 10). Asterisks indicate significant differences from controls (highest pH).

Decreases in AW pH resulted in increases in silver binding to the cultured gill epithelium, an effect that first became significant at pH 6.0, and by pH 5.5, the amount of silver bound was two-fold higher than the control value at pH 7.8 (Fig. 8). Over this range, [H⁺] increased 200-fold, so there was no evidence of competition of this cation for silver binding sites, but rather an indication that lowered pH increased silver binding. MINEQL+ analysis (data not shown) indicated no marked change in silver speciation over this pH range (note that NO₃⁻, originating from the HNO₃ used for acidification, does not bind silver), so the effect of H⁺ on silver binding was likely indirect.

Effects of water chemistry (Na⁺, Ca²⁺, Cl⁻, DOC, and pH) on silver flux rates

Despite substantial changes in silver binding in some treatments (see above), none of the alterations in water chemistry had any significant effect on the flux rates of silver from the apical to basolateral compartments (data not shown). Flux rates remained low (<8 pg cm⁻² h⁻¹) and rather variable, and over 3 h represented at most 20% of the silver bound to the gills during that period.

Discussion

To our knowledge, this is the first study to use cultured epithelia to study silver binding in vitro, and is the first to characterize the binding of any metal from apical FW to a cultured gill epithelium from a freshwater fish (Castaño et al., 2003). Tests demonstrated that there were negligible artifacts from silver binding to the filter support or to the basolateral well. Several important conclusions may be drawn, all supporting the use of this preparation as an in vitro approach to the BLM. First, the preparation is highly

tolerant of silver, sustaining up to $87 \mu\text{g l}^{-1}$ without change in TER or TEP (Fig. 1), and $52 \mu\text{g l}^{-1}$ without change in Na^+ , K^+ -ATPase activity (Fig. 2) during the short-term (3 h) exposure. The high and stable TER, stable negative TEP, and unchanged Na^+ , K^+ -ATPase activity after a 3-h exposure indicated that the cultured epithelium was able to maintain its integrity, indicating no acute toxicity. The second conclusion is that silver binding to the cultured epithelium could be readily measured and showed a saturable binding pattern, in accord with the assumptions of the BLM. The third conclusion is that the individual effects of water chemistry (e.g., Na^+ , Ca^{2+} , Cl^- , and DOC) on silver binding to the epithelium were in general accord with reported in vivo data for rainbow trout used in BLM development, with the single exception of pH effects (Bury et al., 1999a, 1999c; Janes and Playle, 1995; McGeer and Wood, 1998; McGeer et al., 2000; Wood et al., 1999, 2002a). These points are elaborated below.

The lack of toxic response over the 3-h exposure period to silver, i.e., stable TER, TEP (Fig. 1), and Na^+ , K^+ -ATPase activity (Fig. 2), is a fundamental assumption of the BLM in vivo—that binding is measured before the development of pathology because the latter can greatly change metal binding (Paquin et al., 2002a). By 24 h, pathology had started to develop in the silver-exposed epithelia with significant inhibition of Na^+ , K^+ -ATPase activity (Fig. 2), as observed in vivo (Bury et al., 1999c; Morgan et al., 1997; McGeer and Wood 1998; Morgan et al., 2004). While this inhibition was not pursued in detail in the present study, detailed characterization of its concentration-dependency and sensitivity to water chemistry in the future might form the basis for an in vitro version of the physiologically based BLM developed by McGeer et al. (2000).

The silver-binding relationships exhibited a saturable pattern with an apparent K_d of about $10 \mu\text{g l}^{-1}$ as total silver in AW (Fig. 3). This value corresponds remarkably well to the 96-h LC50 value reported for rainbow trout in identical water quality (Hogstrand et al., 1996: $11.8 \mu\text{g l}^{-1}$; Galvez and Wood, 2002: 7.6 to $15.1 \mu\text{g l}^{-1}$). Thus, the LA50 (accumulation at 3 h predictive of 50% mortality at 96 h in vivo) corresponds to binding at the K_d concentration in vitro, thereby fulfilling another requirement of the BLM. Taking silver speciation and water chemistry into account, this translates to about 1.7×10^{-9} M as free Ag^+ , or a log K value of 8.8. As a competing agent (Na^+ , 0.55 mM) was present in appreciable concentration during the determination, the true log K value may actually be higher. The present in vitro estimate of log K (8.8) may be compared with in vivo estimates of 10.0 for directly measured 3-h gill silver binding (Janes and Playle, 1995), 8.0 for directly measured 24-h gill silver binding predictive of 50% mortality at 96 h (Morgan and Wood, 2004), 7.6 for the degree of inhibition of Na^+ , K^+ -ATPase activity predictive of 50% mortality at 96 h (McGeer et al., 2000), and 7.3 by back-calculation from 96-h toxicity data alone (Paquin et al., 1999). Clearly, there is some variation in the literature, but it

is encouraging that the present in vitro value lies in the mid-range of reported in vivo values.

It must be remembered that all of these log K values are conditional, and likely reflect both the assumptions behind them and the water chemistry in which they were determined. Furthermore, in BLM calculations, higher log K values may be compensated by lower binding site density numbers (and vice versa). As Morgan and Wood (2004) have recently pointed out, site density estimates for silver binding sites on trout gills differ by more than an order of magnitude among these studies, in a reciprocal manner with log K values, so that discrepancies in BLM predictions are actually reduced. It is not possible to directly compare site densities (approximately $1\text{--}2 \text{ pmol cm}^{-2}$, measured per unit surface area in the present study) in the cultured epithelium with in vivo estimates ($1\text{--}40 \text{ nmol g}^{-1}$ measured per g of gill tissue). A very approximate calculation using the gill area data for trout of Hughes and Morgan (1973) and assuming the gills account for about 1% of body weight suggests that the in vivo site densities are higher ($5\text{--}200 \text{ pmol cm}^{-2}$) than the present in vitro values ($1\text{--}2 \text{ pmol cm}^{-2}$).

Flux rates of silver from the apical to basolateral compartments were very low in magnitude compared to epithelial silver burden, and were notably insensitive to apical water chemistry in all experiments. TEP was similarly unresponsive to variations in apical chemistry and silver concentration, so TEP did not confound the flux results. The potential of about -4 mV would have favored silver uptake equally in all treatments. In vivo, this would correspond to uptake into the bloodstream, which is similarly negative with respect to the environment (Potts, 1994; Wood, 2001), with resulting silver accumulation in the fish body. This uptake step has been demonstrated to be similarly slow relative to gill binding (Bury and Wood, 1999; Morgan et al., 2004). The rate-limiting step is probably transport of silver across the basolateral membranes of the gill cells, which is energy-requiring and occurs via an as yet uncharacterized P-type ATPase (Bury et al., 1999b). However, flux rates of silver were concentration-dependent (Fig. 3b). By way of analogy, Na^+ influx also exhibits concentration-dependence, and occurs via the H^+ -coupled Na^+ channel on the apical surface (in competition with silver) and the basolateral Na^+ , K^+ -ATPase (presumably separate from silver) in freshwater animals (Potts, 1994). Water chemistry effects on gill silver binding are likely exerted on surface binding and the apical entry step (at least in part the apical Na^+ channels; Bury and Wood, 1999), and thus would have little influence on the slower, rate-limiting, basolateral export step.

If silver (presumably as Ag^+) and Na^+ compete for the same entry step, then Na^+ would be expected to decrease silver binding to the gill epithelium, as observed in the present study (Fig. 4). Inhibition by Na^+ was independent of the anion, and complete at 1.0 mM, indicating a log K value for Na^+ around 4.8 for the BLM. By way of comparison,

Janes and Playle (1995), using a similar silver exposure concentration, reported that 1.6 mM Na⁺ was ineffective whereas 16 mM Na⁺ was highly effective in keeping silver off the gills in vivo. Because their log *K* for gill silver binding (10.0) was higher than in our study (8.8), their final estimate for the log *K* value for Na⁺ competition (4.8) was identical to our estimate (4.8). Other BLMs use lower log *K* values for Na⁺ (2.3: Paquin et al., 1999; 2.9: McGeer et al., 2000) which are essentially fitted values for the documented protective effects in the literature of water Na⁺ against 96-h toxicity and physiological disturbance in vivo, respectively. For a more detailed examination of Na⁺ vs. silver interactions, see Paquin et al. (2002b).

While the Na⁺ response was in good general accord with the in vivo BLM literature, an unusual feature was the observation that only about 40% of silver binding to the cultured epithelium was sensitive to Na⁺ competition over the concentration range tested (Fig. 4). This effect was not seen by Janes and Playle (1995), but Bury and Wood (1999) reported that only about 60% of whole-body silver uptake was sensitive to Na⁺ competition or pharmacological blockade of apical Na channels. The phenomenon is not completely understood, but it is noteworthy that Grosell and Wood (2002) recently presented evidence that the apical uptake of copper (which is chemically similar to silver) in trout gills in vivo occurs both by Na⁺ channels and by a Na⁺-insensitive pathway.

We found that apical water hardness, increased by raising Ca²⁺ up to 30 mM (Fig. 5), had no competitive effect on silver binding to the cultured epithelium. This is in agreement with the findings of Janes and Playle (1995) that Ca²⁺ (tested up to 10.6 mM) had no effect on gill silver binding in vivo, of Bury and Wood (1999) that Ca²⁺ (tested up to 10 mM) did not inhibit whole-body silver uptake in vivo, of Wood et al. (1996) that silver exposure did not influence whole-body Ca²⁺ uptake, and the generally weak (or non-existent) protective effects of water hardness reported in toxicity tests in vivo (e.g., Bury et al., 1999a; Davies et al., 1978; Erickson et al., 1998; Galvez and Wood, 1997). The present data and those of Janes and Playle (1995) both yield a log *K* value for Ca²⁺ binding to silver sites of less than 3.8, and fitted values to toxicity data (2.3; Paquin et al., 1999) and physiological endpoints (2.3; McGeer et al., 2000) are even lower in current in vivo BLMs. In reality, such protective effects as do exist are likely indirect because Ca²⁺ is known to stabilize the gill membranes against diffusible outward leakage of Na⁺ and Cl⁻ (Hunn, 1985; McDonald, 1983), thereby extending survival time under silver exposure (for a detailed discussion, see Paquin et al., 2002b). This relative lack of importance of water hardness is noteworthy, inasmuch as current U.S. EPA regulations for silver AWQC (U.S. EPA, 1980) rely on a “hardness equation” which allows consideration of hardness as the one and only factor modifying silver toxicity, and attributes major weight to it. Widespread recognition that the relationship is flawed (e.g., Andren and Bober, 2002; Hogstrand

et al., 1996) has provided a major impetus for development of BLM approaches that give appropriate weights to all components of water chemistry.

The presence of increasing Cl⁻ clearly elevated silver binding to the cultured gill epithelium, with thresholds of 2.4 mM Cl⁻ (for CaCl₂; Fig. 6B), and 13.2 mM Cl⁻ (for NaCl; Fig. 6A). The Cl⁻ effect was more pronounced with CaCl₂ (Fig. 6B) because Ca²⁺ alone had no counteracting competitive effect (Fig. 5), but it was also seen at higher concentrations of NaCl, showing that the Cl⁻ effect was large enough to overwhelm competition by Na⁺ against Ag⁺ for binding sites (Fig. 4). Calculated silver speciation for the present Cl⁻ exposures is given in Table 1, indicating that [Ag⁺] decreased while [AgCl_(aq)] and [AgCl₂⁻] increased at the higher Cl⁻ concentrations. Thus, silver binding to the cultured epithelium increased despite reduction in the availability of free Ag⁺ ion, providing support for the idea that silver chloride complexes can enter the epithelium. However, there was no effect of Cl⁻ on silver flux rates from apical to basolateral compartments in vitro, suggesting that this facilitation occurs only on the apical entry step.

In vivo, the role of the Cl⁻ anion has been a major problem in the theoretical development of the BLM for silver because Cl⁻ clearly provides protection against both physiological disturbances (e.g., ion loss, Na⁺, K⁺-ATPase inhibition) and 96-h toxicity (e.g., Bury et al. 1999a, 1999c; Galvez and Wood, 1997; Hogstrand and Wood, 1998; Hogstrand et al., 1996; LeBlanc et al., 1984; McGeer and Wood, 1998), yet does not clearly protect against gill silver binding. Various studies have reported that increasing Cl⁻ levels increase (McGeer and Wood, 1998; Wood et al., 2002a, 2002b), have no effect on (Bury et al., 1999c; Hogstrand et al., 1996), or reduce (Bury et al., 1999a; Janes and Playle, 1995) gill silver burdens, while in all cases reducing toxicity.

These disparate results can be rationalized if we assume that the neutral silver chloride complex (AgCl_(aq)), and perhaps other silver chloride species, can enter the gills without

Table 1
Silver speciation (nM) at different Cl⁻ concentrations (mM)

[Cl ⁻]	Measured concentration		MINEQL+ calculated concentration		
	Total [Ag]	[AgCl ₂ ⁻]	[Ag ⁺]	[AgCl _(aq)]	[AgDOC]
<i>A</i>					
0.78	238.0	0.0	29.0	45.8	160.0
1.10	227.0	0.0	23.2	51.5	158.0
1.98	221.0	0.0	15.0	59.0	153.0
4.95	218.0	8.8	6.8	64.4	138.0
13.20	209.0	43.9	2.6	60.5	102.0
<i>B</i>					
0.81	273.0	4.0	44.4	63.1	162.0
2.38	295.0	35.1	12.7	96.8	150.0
3.50	251.0	40.4	6.0	70.8	134.0
5.86	268.0	11.7	21.9	76.2	158.0
12.81	192.0	118	0.6	33.4	33.3

A: NaCl; B: CaCl₂.

causing toxic effects. Recently, Wood et al. (2002a) provided evidence that AgCl_{aq} may in fact enter more readily than Ag^+ , and suggested that because the AgCl_{aq} complex is small and neutral, it may diffuse easily across the entire apical surface of the gill epithelium, whereas the uptake of Ag^+ would be restricted to the (relatively few) apical Na^+ channels. Endocytotic or pinocytotic entry of the silver chloride complex is another possibility. Current versions of the in vivo BLMs do not address this problem, but simply assume that such silver chloride complexes are not bioavailable and therefore benign (McGeer et al., 2000; Paquin et al., 2002b).

DOC was effective in reducing silver binding to the cultured epithelium (Fig. 7), but only at higher concentrations (significant only at 17.6 mg C l^{-1}). This result may be compared to findings that $15\text{--}24 \text{ mg C l}^{-1}$ DOC (Janes and Playle, 1995) or $24\text{--}35 \text{ mg C l}^{-1}$ DOC (Rose-Janes and Playle, 2000) were required to prevent silver binding to trout gills in vivo, whereas Bury and Wood (1999) reported that only 2.5 mg C l^{-1} DOC were needed to cause this effect. The result is also consistent with demonstrations that DOC reduces physiological disturbances (e.g., ion loss, Na^+ , K^+ -ATPase inhibition) and 96-h silver toxicity to trout and fathead minnows in vivo, though again, the effective levels varied greatly among studies (Bury et al., 1999a, 1999b, 1999c; Erickson et al., 1998; Karen et al., 1999; Rose-Janes and Playle, 2000; VanGenderen et al., 2003). In all cases, the effect was attributed to anionic groups on DOC which bind silver strongly, reducing the available free Ag^+ concentration, and forming large complexes which are not taken up by fish gills. Recent results indicate that quantitative variation among studies may result from both differences in the source of DOC (VanGenderen et al., 2003) and in the DOC-silver equilibration times used (C. Glover, R.C. Playle, and C.M. Wood, unpublished results); Aldrich humic acid and 3-h equilibration were used in the present study. Similar phenomena have been reported for copper-DOC interactions (e.g., Ma et al., 1999). Using the same assumptions as to binding site density on DOC as those of Janes and Playle (1995; i.e., approximately 35 nmol binding sites per mg C), our data (Fig. 7) indicate a relatively high $\log K$ value of about 8.2 for DOC-silver interaction, indicating that DOC is highly protective on a molar binding-site basis.

The increased silver binding of the cultured trout gill epithelium in response to decreases in water pH (Fig. 7) was the only data set that did not agree with in vivo silver binding. In vivo BLM's assign a protective role to H^+ , assuming competition with Ag^+ at gill sites, with fitted $\log K$ values of 4.3 (Paquin et al., 1999) or 5.9 (McGeer et al., 2000). In reality, the in vivo data are unclear. Changes in pH over the range used here have been reported to have no effect on 3-h silver binding (Janes and Playle, 1995), whereas 96-h toxicity for fathead minnow increased with a decline in pH (Erickson et al., 1998), in accord with the current data. Furthermore, silver speciation, as calculated using MINEQL+ from the water chemistry data in our in vitro exposures, was not appreciably altered by the decrease in

pH. In vivo, the gill microenvironment is well buffered by NH_3 efflux at low ambient pH (Playle and Wood, 1989). This NH_3 efflux raises gill boundary layer pH in vivo so that it would likely undergo little change over the first few hours in response to the ambient water pH shifts imposed here. Indeed, in the present in vitro experiments, we adjusted apical pH back down to the desired value with 1% HNO_3 every 30 min (see Materials and methods) to overcome this phenomenon. Thus, in vitro, the cultured epithelium did not have the benefit of this buffering protection to any great extent, so the real effects of the lowered water pH were probably revealed. These effects due to H^+ may include inflammation, structural damage, and increased production of mucus, all of which have been reported at even lower pHs in vivo (e.g., Daye and Garside, 1976; Jagoe and Haines, 1983; Wood, 2001), and all of which may tend to increase silver binding and silver toxicity in a non-specific fashion. Notably, using confocal microscopy, we have recently demonstrated that mucous cells are present in this cultured DSI preparation, in addition to pavement cells and chloride cells (J.M. Wilson, B. Zhou, C.M. Wood, unpublished results).

In summary, the in vitro approach to a silver BLM appears to hold great promise, and could be used to derive apparent K_d values for other metals, as well as $\log K$ values for their competing cations. The approach is in accord with recent legislation and public sentiment to replace whole-animal toxicity testing with tests on cell systems or other models (Castaño et al., 2003). While this move has been largely driven by ethical considerations, other advantages of an in vitro approach to BLM development include a reduction of the number of animals used in experiments (two fish yield approximately 35 DSI preparations; Wood et al., 2003), and a reduction in time (3-h BLM tests versus 96-h toxicity tests) and space requirements (culture plates versus a battery of fish tanks). However, we believe the most important advantage of the in vitro approach is the ability to work with much smaller volumes of natural water samples (a few ml versus 100s of l for in vivo tests), for the following reason. The U.S. EPA (1994, 2001) currently relies on the Water Effect Ratio (WER) approach to grant deviations from its AWQC (U.S. EPA, 1980). In brief, this procedure involves running a battery of live animal toxicity tests with the metal of interest. The tests are performed side-by-side in site water and reference laboratory water of known chemistry, involving large volumes of site water and large expenditures of labor, both of which are often expensive to procure. If toxicity is significantly lower in the site water (reflecting its protective chemistry), then a less stringent AWQC may be granted. An in vitro BLM approach, comparing metal binding to the cultured gill epithelia incubated with known concentrations of metals in reference water and in site water, may provide a simple and cost-effective alternative to the current cumbersome procedure, as well as a way for quickly screening small volumes of previously uncharacterized waters and proposed ameliorative agents.

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