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Annual Review

PHYSIOLOGY AND MODELING OF MECHANISMS OF SILVER UPTAKE AND TOXICITY IN FISH

CHRIS M. WOOD,† RICHARD C. PLAYLE,*‡ and CHRISTER HOGSTRAND§
†Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada
‡Department of Biology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada
§T.H. Morgan School of Biological Sciences and Graduate Center for Toxicology, University of Kentucky, 101 Morgan Building, Lexington, Kentucky 40506-0225, USA

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Abstract—In this review, we outline the physiological and toxicological effects of silver (Ag) in freshwater and marine fish. For freshwater fish, the acute toxicity of Ag appears to be caused solely by ionic Ag^+ interacting at the gills, inhibiting basolateral Na^+,K^+-ATPase activity. Disruption of this enzyme inhibits active Na^+ and Cl^- uptake and therefore osmoregulation by the fish. Silver is much less toxic to marine fish, mainly because ionic Ag^+ is complexed by Cl^-, but the mechanisms of acute toxicity and the toxic species of Ag are poorly understood. Osmoregulatory failure occurs in marine fish exposed to high concentrations of Ag, and the intestine appears to be a primary toxic site of action, perhaps along with a gill component. Modeling approaches to calculate Ag interactions at biological surfaces are used to illustrate the effects of water chemistry on Ag speciation and therefore toxicity to freshwater and marine fish. In these models, the most important components affecting Ag speciation are the complexing agents Cl^- and dissolved organic matter followed by the competing agents Na^+ and Ca^{2+}, although a particulate component may be important to incorporate into the models in future. More precise knowledge of the actual toxic sites of Ag is necessary if we are to fully understand the effects of waterborne Ag in the environment.

Keywords—Silver Fish Physiology Toxicology Models

INTRODUCTION

Sources and nature of waterborne silver

Although most silver (Ag) in surface waters originates from natural leaching, elevated concentrations are usually associated with anthropogenic activities such as mining and photographic processing. In recent years, Ag recovery from photoprocessing effluent has become increasingly efficient because of environmental concerns and because economic demand for Ag encourages its reclamation from waste [1,2]. Total Ag concentrations in direct effluent from photographic processing are typically in the low milligram per liter range, most of it in the form of Ag thiosulfate. Virtually all effluent undergoes substantial dilution with other sewage then passes to sewage treatment plants where extraction efficiency from the mixed influent is typically ≥94%, a much higher relative removal than for most other metals [3].

Final discharges of total Ag to the environment vary from below picogram per liter to the low microgram per liter range relative to natural background concentrations in the low nanogram per liter range. Most of this Ag is bound to particles, organic colloids, thiosulfate, sulfide, dissolved organic matter (DOM), and chloride, with the latter two representing the major forms of Ag in oxic natural waters where fish live. The proportion of total Ag existing in the water column as uncomplexed ionic Ag (Ag^+) is normally a very small percentage of the total [2,4].

Overview of silver toxicity in freshwater and seawater

Considerable progress has been made in the last few years in describing the toxicology and bioavailability of Ag to fish, but there remain significant gaps in our understanding of the exact mechanisms of Ag uptake and toxicity (reviewed in [5]). In freshwater, the acute toxicity of Ag appears to be caused solely by ionic Ag^+, which binds to specific sites on or in fish gills. Active uptake of Na^+ and Cl^- is reduced, primarily through the inhibition of Na^+,K^+-ATPase activity, causing a net loss of ions from the blood plasma, circulatory failure through collapse of fluid volume regulation, and ultimately death of the fish [5].

In seawater, the mechanism of acute toxicity and the toxic species of Ag are poorly understood. However, osmoregulatory failure is again involved; fish appear to die from elevated plasma Na^+ and Cl^- concentrations plus dehydration. Here the intestine appears to be the primary toxic site of action. Marine teleosts must drink to preserve fluid balance; the presence of Ag in seawater reduces drinking rates and interferes with the intestinal uptake of water, perhaps through the inhibition of active ion uptake processes that drive water flux by osmosis. Interference with salt excretion through the gills may also contribute to Ag toxicity, but as yet there is no direct evidence for this effect.

The acute toxicity of Ag to fish is much lower in seawater than in freshwater (up to three orders of magnitude less [5–8]). Part of this difference may reflect the different site of toxic effect (gut vs gill), but more importantly it results from the fact that virtually all ionic Ag^+ is bound and rendered unavailable by the high concentrations of Cl^- in seawater. Indeed toxicity is least at intermediate salinities (1/10–1/3 seawater) because an insoluble Ag chloride complex predominates (cerargyrite; $AgCl_{(s)}$) so little Ag remains in solution [8].

These diverse relationships of Ag toxicity with water salinity were summarized in the final figure of the review by

* To whom correspondence may be addressed (rplayle@mach1.wlu.ca).

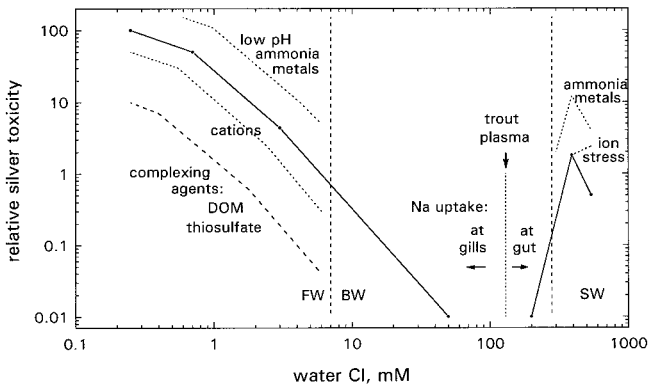

Fig. 1. The relative acute toxicity of silver to teleost fish such as rainbow trout in freshwater (FW), brackish water (BW), and seawater (SW), as a function of water chloride concentration (solid lines). Note that both axes have logarithmic scales. Silver (Ag, added as $AgNO_3$) is most toxic in very low ionic strength water and becomes progressively less toxic as salinity increases, mainly because Cl^- complexes ionic Ag^+. The dashed lines represent other modifiers of Ag toxicity and are described fully in the text. The conversion formula for Cl^- (in millimolar concentrations) to ppt salinity is 0.063 × [Cl], so 550 mM Cl^- is about 35 ppt seawater.

Hogstrand and Wood [5] and are presented as Figure 1 here, with modifications in light of new knowledge. Figure 1 assumes a worst case scenario, Ag added to the water in a fully soluble and dissociated form (i.e., as $AgNO_3$). Water Cl^- concentration is plotted on the abscissa as a proxy for salinity, reflecting our belief in the importance of this anion in governing Ag toxicity in natural waters. In essence, Figure 1 illustrates the high acute toxicity of Ag in very low ionic strength water (<0.3 mM Cl^-) to more usual freshwater (<1 mM Cl^-) where appreciable ionic Ag^+ is present and decreasing Ag toxicity as salinity increases from freshwater (FW) to brackish water (BW; solid line at left in Fig. 1). In brackish water, Ag added as $AgNO_3$ is virtually nontoxic to fish because of cerargyrite formation.

Above where plasma and environment osmotic pressures are equal (the isosmotic point, ~130 mM Cl for trout plasma), fish need to drink to avoid dehydration, and the site of acute Ag toxicity probably changes from the gills to the gut (Fig. 1). Moving into the higher salinities representative of seawater (SW), Ag becomes progressively more toxic as fish have to drink more water and excrete more salt to maintain osmotic balance (solid line at right in Fig. 1). The decrease in Ag toxicity in full-strength seawater probably reflects a change in the proportion of the various Ag chloride complexes that are present. Not included in the final figure in [5] are the labeled dashed lines, which will be elaborated upon in this review.

The overall goal of this review is to integrate our knowledge of aquatic chemistry and fish physiology in explaining the relationships summarized in Figure 1. Specifically, we will describe new insights into the mechanisms of Ag uptake and toxicity in freshwater and marine fish and the influence of water chemistry on these mechanisms. In recent years, there has been great interest in developing ligand-binding and tissue-residue models to predict the site-specific toxicity of metals in different water qualities [9–11]. To this end, we will discuss the ''gill modeling approach'' of Janes and Playle [12], both as a general framework for interpreting water chemistry effects on acute Ag toxicity in freshwater and as a possible tool to interpret and explain acute Ag toxicity in seawater.

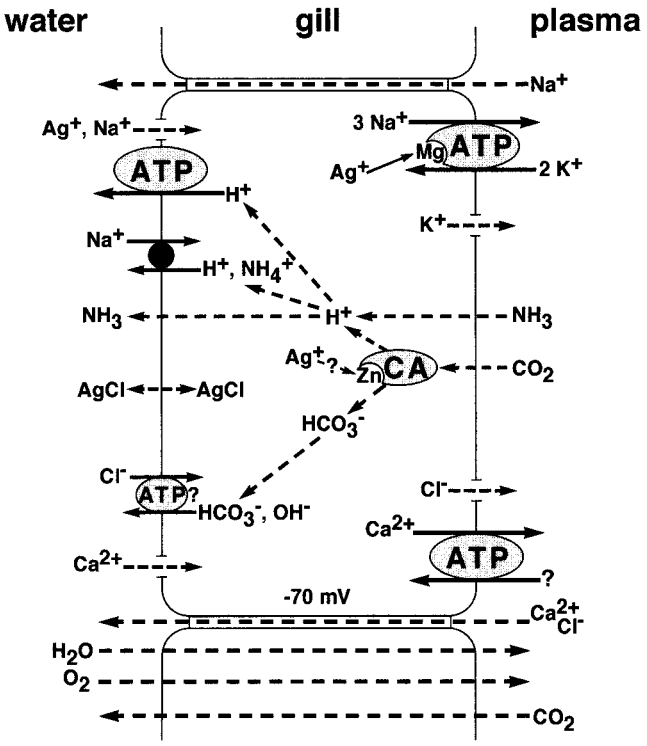

Fig. 2. A schematic diagram of the ionoregulatory and respiratory functions of the freshwater fish gill, with the toxic actions of silver included. Dashed lines represent diffusion; solid lines associated with adenosine 5′-triphosphate represent active transport. CA = carbonic anhydrase. Modified from [13]; see text for details.

SILVER UPTAKE AND TOXICITY IN FRESHWATER FISH

The mechanism of acute silver toxicity

Silver is an ionoregulatory toxicant to freshwater fish, with a very specific mechanism of action that has been elucidated only in the last few years. Plasma osmolality in fish is about 300 mosmol, and that of freshwater ranges from 0.1 mosmol (very low ionic strength water) to about 20 mosmol. Therefore, freshwater fish must actively transport ions such as Na^+, Cl^-, and Ca^{2+} across their gills into their bodies against large electrochemical gradients to maintain proper ion concentrations for physiological processes.

Although certain features of the ion transport model shown in Fig. 2 remain controversial or unproven, there is general agreement that Na^+ is exchanged in some way against ''acid'' (H^+ or NH_4^+) and that Cl^- is exchanged against ''base'' (HCO_3^- or OH^-) at the apical membrane of gill cells involved in ion transport (ionocytes [13–16]). There is also general agreement that basolateral Na^+,K^+-ATPase is the major ''engine'' powering both Na^+ and Cl^- uptake through the ionocytes, whereas high-affinity Ca^{2+}-ATPase is the driving force for Ca^{2+} uptake. These ions are being continually lost by diffusion down their concentration gradients from the fish to the water, whereas water passively enters the fish by osmosis. Freshwater fish therefore drink very little water [17] and excrete large amounts of dilute urine. Fish in very low ionic strength water have a more difficult time ionoregulating because the gradients against active ion uptake and in favor of diffusive loss are higher than in harder water, which increases their sensitivity to toxicants that interact at the gills.

Most evidence to date indicates that it is ionic Ag^+ that is acutely toxic to freshwater fish [18–24] and its toxic effect is

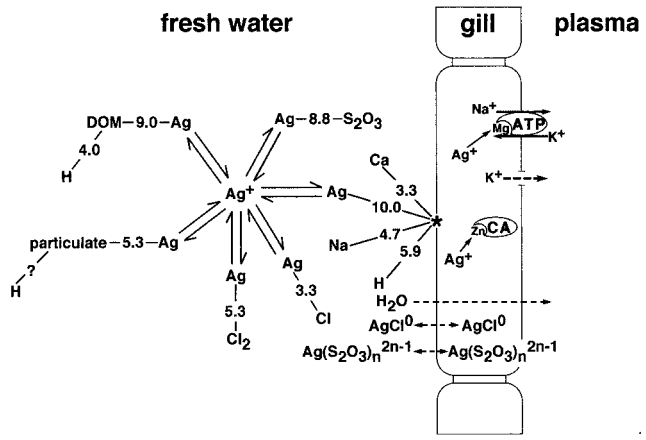

Fig. 3. A schematic diagram of the gill modeling approach. The numbers represent log conditional equilibrium stability constants (*K*) for Ag^+ and other cations binding at the gills (asterisk) and for Ag^+ binding to dissolved organic matter (DOM), thiosulfate ($S_2O_3^{2-}$), and Cl^- in the water column. For Ag^+ complexation by particulate matter, the value 5.3 is an average partition coefficient between particles and river water [52]. See text for more details.

through a noncompetitive inhibition of active gill Na^+ and Cl^- transport [22,23,25,26]. The transport and internal regulation of Ca^{2+} are not affected [27], and there is negligible influence on the passive diffusive losses of Na^+ and Cl^- [25,26] and on diffusive gas transfer [24,27]. The whole sequence of net Na^+ and Cl^- loss, osmoregulatory failure, and death by cardiovascular collapse is set in motion by this highly effective blockade of ion uptake processes (see Fig. 3 in [5]).

Inhibition of Na^+ and Cl^- transport occurs because Ag binds at the Mg^{2+} binding site on the cytoplasmic side of the α-subunit of the basolateral Na^+,K^+-ATPase enzyme, thereby preventing adenosine 5′-triphosphate (ATP) hydrolysis [5,28] (Fig. 2). The importance of Na^+ extrusion across the basolateral membrane by Na^+,K^+-ATPase in driving Na^+ uptake is obvious, but the reason why blockade of this enzyme should also inhibit Cl^- uptake is less clear. Indeed, it is not known exactly how the Na^+,K^+-ATPase energizes Cl^- uptake [14]. Part of the inhibitory mechanism may relate to the acid–base status of the transporting cells because disruption of Na^+ transport will quickly lead to increased intracellular acidity, which in turn will reduce the availability of HCO_3^- or OH^- for Cl^- uptake. In this regard, trout exposed to $AgNO_3$ in freshwater exhibit an accumulation of acidity in the body fluids and a net uptake of ''acid'' from the environment [26,27].

An additional factor of Ag toxicity to freshwater fish may be the partial inhibition by Ag of the carbonic anhydrase enzyme that catalyzes the production of acid and base by the hydration of CO_2 inside the transporting cells [25] (Fig. 2). Silver is one of the most potent metals in inhibiting carbonic anhydrase in fish in vitro [29]. A possible mechanism, as yet unproven, for this partial inhibition is through Ag^+ displacement of Zn^{2+} from the interior of the carbonic anhydrase molecule. Displacement of essential metal ions from biologically important molecules is one mechanism by which metals exert their toxic action [30]. However, under the same exposure conditions to $AgNO_3$ in vivo, inhibition of carbonic anhydrase activity in trout gills was only 28% relative to 85% inhibition of Na^+,K^+-ATPase activity [25]. Because carbonic anhydrase, unlike Na^+,K^+-ATPase, is an enzyme generally considered to be present well in excess of requirements [31], its inhibition is unlikely to be the primary mechanism of Ag toxicity to fish.

The exception to the conclusion that silver specifically interferes with ionoregulation without disturbing respiratory processes appears to be in very low ionic strength water [32,33]. Here the paucity of protective agents of competitition and complexation in the water (Na^+, Cl^-, Ca^{2+}, DOM) may allow so much ionic Ag^+ to bind to the gills that a branchial inflammatory response occurs and excess mucus is produced, interfering with gas transfer. This would explain the decreased arterial O_2 tension, increased coughing rate, and elevated blood lactate concentrations in addition to ionoregulatory disruption reported for trout exposed to ~0.11 μM $AgNO_3$ in ion-poor soft water (~12 μg/L Ag [32,33]). Other studies at similar $AgNO_3$ concentrations in less dilute waters have reported only ionoregulatory effects of Ag [24,26,27].

Influences on silver toxicity

The evidence that ionic Ag^+ is the sole cause of acute Ag toxicity in freshwater fish has been derived from experiments with various complexing agents [12,18–24,27,32–34]. Complexing agents such as Cl^-, DOM, and thiosulfate markedly decrease Ag toxicity (Fig. 1) by binding ionic Ag^+, thereby reducing its availability to interact at Na^+,K^+-ATPase molecules in gill epithelial cells.

Cations in water also reduce Ag toxicity in fish (Fig. 1), and at least three different mechanisms may be involved. First, cations may directly compete with ionic Ag^+ at either initial surface binding sites on the gills (entry sites) or at the Mg^{2+} sites on Na^+,K^+-ATPase molecules (toxicity sites). For example, Janes and Playle [12] demonstrated that high concentrations of Na^+ prevented the binding of Ag^+ to trout gills. Second, an elevation in Na^+ concentration, being the substrate for the Na^+,K^+-ATPase–driven transport, may help overcome the inhibition of active Na^+ uptake caused by Ag^+. Third, the ''hardness cations'' Ca^{2+} and Mg^{2+}, through their well-known effect in reducing the diffusive permeability of the gills [35], may help stave off the ionoregulatory failure caused by Ag^+-poisoning of Na^+,K^+-ATPase, separate from any direct competition with Ag^+ for gill binding sites. For example, Galvez and Wood [20] found that Ca^{2+} (added as either $CaNO_3$ or $CaSO_4$) prolonged the survival of fish exposed to $AgNO_3$, even though Ca^{2+} was only weakly effective in preventing the binding of Ag^+ to trout gills [12] or in preventing the inhibition of Na^+ uptake and of Na^+,K^+-ATPase activity [22].

In general, on a molar basis, the protective effects of cations are much weaker than those of anionic complexation (Fig. 1). Indeed, in standard 96-h tests of concentration causing 50% lethality (LC50), the protective effects of large increases in Ca^{2+} concentration against $AgNO_3$ toxicity were modest [21,23]. In this regard we have questioned elsewhere [5,19,20] the large protective effect ascribed to water hardness by the U.S. Environmental Protection Agency [36] and the omission of complexing agents from the criterion equation.

In addition to complexing agents and cations, a third class of modifying agent is indicated in Figure 1, substances that exacerbate silver toxicity. We emphasize that at present there is no direct evidence for these effects, and their inclusion is to indicate an important direction for future research. Low environmental pH and metals such as copper and aluminum are potent ionoregulatory toxicants that cause net Na^+ and Cl^- losses from freshwater fish (reviewed in [13]). In general, their toxic mechanisms at the gills are different from or are addi-

tional to those of Ag, so their toxicities would likely be additive or perhaps even synergistic in causing ionoregulatory failure. Ammonia is included because it is both an ionoregulatory toxicant and a neurotoxicant in fish [37] and because its endogenous production rate increases in fish under Ag stress as a consequence of cortisol mobilization [26]. A tripling of ammonia production and a 2.5-fold increase in plasma ammonia concentrations was seen in trout subjected to approximately the LC50 concentration of $AgNO_3$ for 6 d [26]. Thus the toxicity of exogenous waterborne ammonia would be compounded by Ag, as was reported for marine fish [6].

Influences on silver uptake

Although we have so far emphasized only the toxic action of ionic Ag^+ at the gills, it is important to note that waterborne Ag also readily enters fish, accumulating first in the blood [26,33,34] and then in the kidney and especially the liver, where it acts as a very potent inducer of metallothionein synthesis [19,27,34,38]. To date there is no evidence that internalized Ag causes any acute toxicity [5], but whether it contributes to sublethal, chronic toxic effects has not yet been investigated and is an important topic for future research.

Silver is readily accumulated in blood, kidneys, and liver from solutions where ionic Ag^+ predominates, indicating that this form passes easily through the gills. At present, the mechanism of penetration is unresolved, although preliminary evidence suggests that Ag^+ may enter through the same channel or porter as Na^+ on the apical surface of the ionocytes (N.R. Bury and C. M. Wood, unpublished results). The basolateral exit step is problematical, although it is not impossible that Ag^+ could inhibit at the Mg^{2+} site while simultaneously being transported at the Na^+ site on the Na^+,K^+-ATPase (Fig. 2).

It is important to emphasize that binding of Ag by Cl^- and by thiosulfate does not necessarily prevent the accumulation of Ag on the gills or its entry into the fish [5,12,19,22,23,33,34]. Again, such entry appears to cause no acute toxicity. Under these circumstances, the Ag–ligand complexes, rather than dissociated, ionic Ag^+ from the complexes, may be the nontoxic forms that enter by diffusion. The $AgCl^0$ complex is small and neutral and likely diffuses easily across the gill and into a fish (Fig. 2).

It may seem illogical that $AgCl^0$ entering the gill tissue would have any different effect than Ag entering as ionic Ag^+. After all, once the silver has passed the apical membrane, the speciation of Ag in the external water should be irrelevant, and a new equilibrium with intracellular ligands should be established. The concentration of Cl^- in gill cells is likely above 10 mM [14], so even if Ag enters as Ag^+ it would be expected to complex to proteins and Cl^- inside gill cells. The same would undoubtedly happen to Ag that enters as $AgCl^0$.

It is probably not so much how $AgCl^0$ behaves in the gill as where it enters that makes it different from ionic Ag^+. As noted above, the uptake of Ag^+ likely occurs by specific transport proteins sitting in the apical membrane of gill ionocytes. These cells normally occupy no more than 5 to 10% of the total gill surface area [14,39]. In contrast, uptake of the uncharged $AgCl^0$ complex probably takes place passively by diffusion across the entire apical surface of the gill. Thus, we believe that one reason for the extreme toxicity of ionic Ag^+ is that, in contrast to $AgCl^0$, it is internalized specifically at the very site of toxic action, the ionocytes.

Entry per unit concentration is much lower for Ag-thiosulfate than for $AgCl^0$ (see analysis in [5]), presumably because the Ag-thiosulfate complex is larger and is negatively charged. However, at extremely high exposure concentrations such as the ~280 μM Ag-thiosulfate used by Wood et al. [34], substantial amounts of Ag did enter the fish, presumably by diffusion as Ag-thiosulfate. With respect to Ag–DOM complexes, the limited available evidence suggests that these forms do not readily enter fish gills [12,22,32; B. Bertram and R. Playle, unpublished results], presumably because of their even larger size and more anionic nature. For copper (Cu), it has been shown that Cu–DOM complexes do not enter trout gills over 9 d [40].

Modeling approach

Janes and Playle [12] developed a Ag-gill binding model, in which the gill is considered a negatively charged ligand to which ionic Ag^+ can bind. This work was a continuation of modeling Cu and cadmium binding to fish gills [41,42], which was a logical extension of approaches presented by Pagenkopf [43], Morel [44], and Morel and Hering [45]; see also [10,46]. The Ag-gill model [12] was based on conditional equilibrium stability constants (K) determined for ionic Ag^+ binding to trout gills ($\log K_{\text{Ag-gill}} = 10.0$), Ag^+ binding to DOM ($\log K_{\text{Ag-DOM}} = 9.0$), plus Na^+, Ca^{2+}, and H^+ binding at the Ag^+ binding sites ($\log K_{\text{Na-gill}} = 4.7$, $\log K_{\text{Ca-gill}} = 3.3$, $\log K_{\text{H-gill}} = 5.9$). These values, along with their binding site numbers, were inserted into a chemical equilibrium program, MINEQL$^+$ (Environmental Research Software, Hallowell, ME, USA [47]), to calculate and predict the amount of Ag binding on or in fish gills [12].

The gill modeling approach has two potential advantages over simply speciating Ag in the water column. First, it takes into account competition between cations for Ag^+ binding sites on the gills. Second, it takes into account the high binding affinity of fish gills for ionic Ag^+ so that loosely bound Ag^+ may be stripped from low-affinity ligands and then attach to the gills (Fig. 3). In essence, the contribution of cationic competition and anionic complexation in reducing Ag toxicity depends on the relative binding strengths of cations to the negatively charged gills and on the relative binding strengths of Ag^+ to anions in the water column plus the concentrations of the cations and anions.

When the Ag-gill model was being developed and published in 1995, evidence was accumulating that Ag caused disruption in ionoregulation in freshwater fish, but few details were available. Today we know the exact site of Ag^+ toxicity and a great deal more about the physiological processes of Ag toxicity and accumulation on the gills. In light of this new knowledge and with more toxicological data available, it is appropriate to reexamine the Ag-gill model. How valid is the model today, and how does it fit with current knowledge? The model may work well as a conceptual framework of metal toxicity that incorporates explicitly the ''three Cs'' (concentration, complexation, and competition [10]), but how well does it fare in interpreting new knowledge and answering remaining questions regarding Ag toxicity in freshwater fish? The following points are of key importance:

(i) The original data used to create the model (gill silver burdens) were obtained after exposures of rainbow trout to $AgNO_3$ for 2 to 3 h. The model contains no kinetic component and assumes that thermodynamic equilibrium is achieved in this time. Furthermore, the original model was designed to predict short-term Ag-gill binding not to predict Ag toxicity.

(ii) Recent physiological studies show that it takes 4 to 10

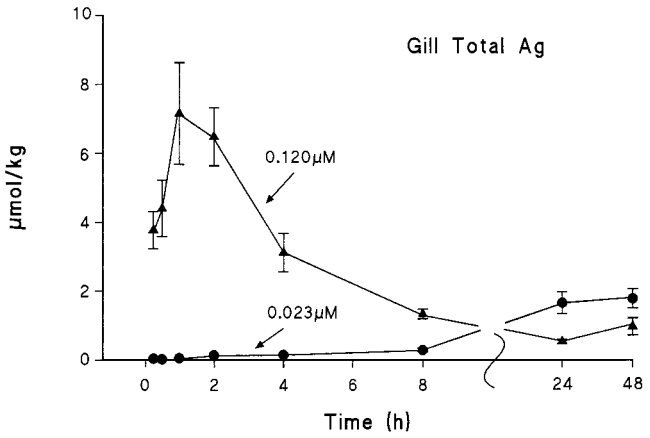

Fig. 4. Changes in gill silver (Ag) load over 48 h in juvenile rainbow trout continuously exposed to either 0.120 or 0.023 µM Ag (added as $AgNO_3$) in synthetic soft water (30 µM Na^+, 30 µM Cl^-, 20 µM Ca^{2+}, 0.3 mg C/L dissolved organic matter, pH 6.5). Means ± 1 SE (n = 5–8). (N.R Bury, C.M. Wood unpublished results.)

h for the inhibitory effects of $AgNO_3$ exposure on Na^+ uptake to develop fully [22,25], presumably the time taken for Ag^+ penetration to the basolateral Na^+,K^+-ATPase. Furthermore, it can take up to 6 or 7 d for the fish to die from the resulting ionoregulatory failure [19,27].

(iii) Two recent studies on the time course of Ag accumulation in trout gills demonstrate that the gill Ag load is constantly changing over time and the pattern of change depends on the concentration of Ag used in the exposure (C. Hogstrand, C.M. Wood, M. Grosell, and H. Hansen, unpublished results; N.R. Bury and C. M. Wood, unpublished results). For example, Fig. 4 illustrates that in synthetic soft water similar to that used by Janes and Playle [12] in the original model formulation, with a continuous exposure to a similar $AgNO_3$ concentration (0.120 µM; 13 µg/L), gill Ag burden peaks at 1 to 2 h and then declines to about 15% of the peak value by 48 h; this pattern was also seen in [12]. However, at a much lower Ag concentration (0.023 µM; 2.5 µg/L) there is no initial peak but rather a gradual rise over time such that the value at 48 h is virtually the same as that in the 0.120-µM exposure at 48 h. At present, the explanations for these patterns are unclear, but a threshold phenomenon for a depuration response (increased clearance from gill to blood or water) may be involved.

(iv) At least over periods longer than 6 h, high gill Ag loads can accumulate under conditions where there is no acute toxicity, i.e., where most of the Ag^+ is complexed as $AgCl^0$ or as Ag-thiosulfate [19,34; C. Hogstrand et al., unpublished results].

(v) In accord with points iii and iv, there was no relationship between the gill Ag load, measured at 48 h, and the inhibition of Na^+,K^+-ATPase activity in trout exposed to $AgNO_3$ (0.030 µM; 3.2 µg/L) at different water Cl^- concentrations (Fig. 5A [24]). However, in a similar study (0.034 µM $AgNO_3$) with a more extensive range of water chemistry manipulations and measurements at 6 h rather than 48 h, there was a reasonable relationship between the gill Ag load and the inhibition of Na^+,K^+-ATPase activity when only the data from complexing agents (Cl^-, DOM) were considered [22]. This relationship broke down when data from competing agents (Na^+ and Ca^{2+}) were also considered. However, in both studies, for all data, there were very strong relationships between the ionic Ag^+ concentration, as calculated by MINEQL$^+$, and the inhibition of Na^+,K^+-ATPase activity (Fig. 5B).

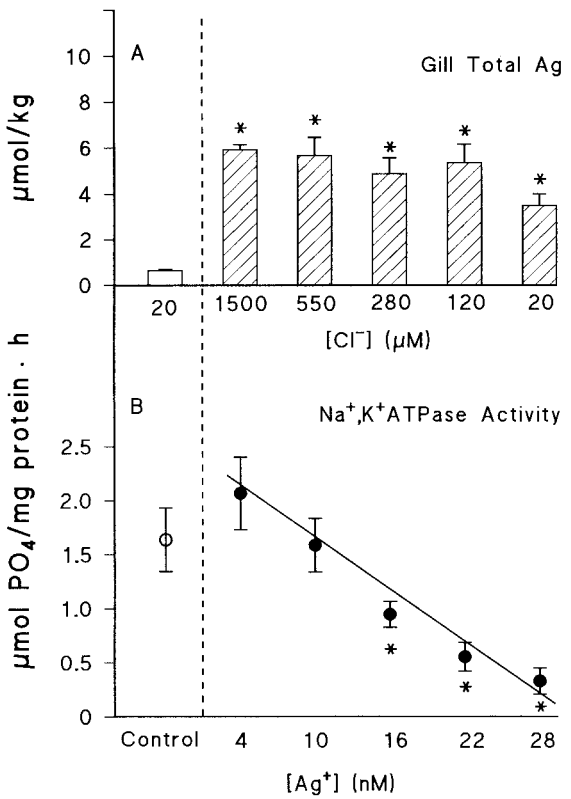

Fig. 5. Gill silver (Ag) load in juvenile rainbow trout exposed for 48 h to 0.030 µM Ag in synthetic soft water (30 µM Na^+, 0.3 mg C/L DOM, pH 7.0) at different Cl^- concentrations (**A**). Water Cl^- was adjusted using KCl. Gill Na^+,K^+-ATPase activity in these same fish versus calculated ionic Ag^+ concentration (**B**). Means ±1 SE (n = 6, except for the nonexposed control group where n = 4). Asterisks indicate means significantly different from the unexposed control group ($p \leq 0.05$). Data are from [24]. Note the lack of correlation of 48 h gill Ag burden (**A**) with 48 h Na^+,K^+-ATPase inhibition (**B**).

Evaluating the models

A reasonable conclusion from this new knowledge is that, if the original Ag-gill binding model [12] is successful in predicting acute toxicity from water chemistry, it is because the short-term gill Ag load correlates with the longer-term inhibition of Na^+,K^+-ATPase activity and ionoregulatory failure, which kills the fish. It is not because the model predicts the gill Ag load at equilibrium, and it does not imply that there is any relationship between the longer-term gill Ag load and toxicity. An alternative conclusion is that a more practical approach would be to simply calculate the ionic Ag^+ concentration in the water column from the water chemistry via MINEQL$^+$ and use this parameter to directly predict toxicity (e.g., the ''free ion activity model'' [48]; see also [49]). This simplified approach would omit the gill entirely from the model, thereby considering only ''two Cs'' (concentration and complexation) while ignoring competition.

To evaluate these two possibilities, we modeled an exposure of trout to 0.1 µM $AgNO_3$ in two ways. We entered available water chemistry parameters for Ag toxicity (from Fig. 1) into the Ag-gill binding model [12] to predict short-term gill Ag load and into MINEQL$^+$ to predict ionic Ag^+ concentrations in the water column. Dissolved organic matter was held con-

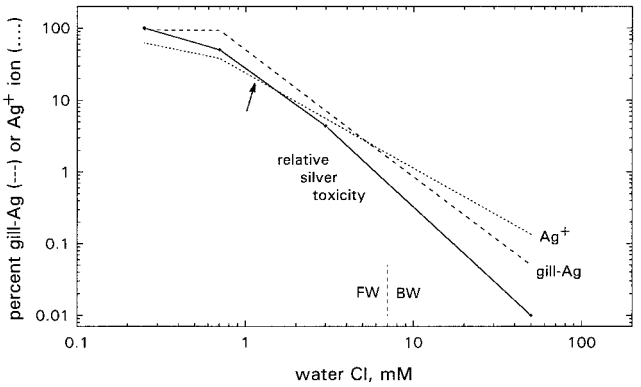

Fig. 6. Modeling results using the silver (Ag)-gill binding model [12] to calculate gill-Ag burden, as a percentage of all gill Ag binding sites filled by Ag and using water chemistry alone (via MINEQL$^+$) to calculate the percent ionic Ag^+ in the bulk water. See text for details.

stant in this simulation, so the principal factors that changed were Cl^-, Na^+, Ca^{2+}, pH, and ionic strength. Water chemistry used was 0.1 μM Ag (11 μg/L, as $AgNO_3$) for Playle's laboratory (50 μM Ca^{2+}, 700 μM Na^+, 250 μM Cl^-, pH 6.8, 11°C; 100% relative Ag toxicity), Wood's laboratory (1,000 μM Ca^{2+}, 500 μM Na^+, 700 μM Cl^-, pH 8.0, 15°C; 50% relative Ag toxicity), and Wood's laboratory water plus 50 mM NaCl (0.01% relative Ag toxicity, [19]). The results were superimposed onto the Ag toxicity curve for freshwater from Fig. 1. Gill Ag load is expressed as a percentage of the gill Ag binding sites filled, as defined in [12], and ionic Ag^+ is expressed as a percentage of the total Ag added.

Both models work reasonably well, that is, predicted short-term gill Ag load and predicted water ionic Ag^+ concentrations both track the available toxicity data (Fig. 6). The scales are logarithmic and the data on which the Ag toxicity curve was based are sparse, both of which must temper any conclusions, but two differences are worthy of comment. At low Cl^- concentrations (<0.8 mM) the free Ag^+ model parallels the toxicity data, whereas the gill-Ag model does not, because the former predicts that increasing the Cl^- concentration causes a drop in uncomplexed Ag^+, and therefore a decline in toxicity, whereas the latter predicts that Cl^- should not much affect Ag binding to the gills in this concentration range because the gills can still out-compete Cl^- for ionic Ag^+ (Fig. 3). At higher salinity representative of brackish water (~50 mM Cl^-), the gill-Ag model more closely parallels the sharp decline in toxicity observed because it takes into account the protective competitive effects of increasing Na^+ and Ca^{2+} concentrations, whereas the free Ag^+ model does not.

New perspectives in modeling

It is clear that modeling approaches, with their emphasis on geochemical speciation, have advanced our understanding of Ag toxicity considerably in the past few years. Most importantly, they have illustrated that regulatory approaches for acute toxicity based simply on measuring total Ag concentrations in water or effluent [36,50] are not meaningful. However, the models to date are as yet immature and require further development and validation before they are incorporated into regulations. Several ideas are outlined.

An important requirement is to incorporate particulate-bound Ag. Particulate matter could be added into both the Ag-gill model and into MINEQL$^+$ modeling of ionic Ag^+ concentrations, as was done for Ag–DOM binding [12] (Fig. 3). However, to do so it will be necessary to overcome the same problems associated with modeling DOM, mainly determining the number of binding sites on heterogeneous particles [51]. Suspended particulate matter varies between 0.08 and 38 g/L in river water, and DOM varies between 2 and 100 mg C/L [51]. Particulate matter could be important in either model, especially for riverine systems, because Ag has a relatively high affinity for particles; the log partition coefficient between the particulate and aqueous phase in rivers is between 5.0 and 5.5, depending on filter pore size [52,53], although Ag is relatively easy to displace from particulate matter under acidic conditions (e.g., pH 5.0 [54,55]).

A limitation of both the gill-Ag model and the free Ag^+ model is that neither considers binding at gill Na^+,K^+-ATPase molecules. However, it should now be possible to build such a model using a ''black-box'' experimental approach similar to that used by Janes and Playle [12], but using a longer time period and an endpoint of Na^+,K^+-ATPase inhibition rather than gill Ag load. Indeed, we already have some information as to the general parameters for such a model. From the data of McGeer and Wood [24], 50% inhibition of trout gill Na^+,K^+-ATPase activity (IC50) at 48 h exposure in vivo occurs at a calculated Ag^+ concentration of about 16 nM (1.7 μg/L; arrow in Fig. 6, corresponding with about 30% gill sites filled by Ag). The data of Bury et al. [22] yield an almost identical IC50 value at 6 h. Thus, the conditional equilibrium stability constant for water Ag^+ binding to gill Na^+,K^+-ATPase (log $K_{Ag\text{-}ATPase}$) would be approximately 7.8. This value is about 100 times weaker than the log $K_{Ag\text{-}gill}$ value of 10.0 used in the gill-Ag model [12] and may explain why freshwater Cl^- concentrations, despite their relatively weak stability constants (log $K_{Ag\text{-}Cl}$ = 3.3; log $K_{Ag\text{-}Cl2}$ = 5.3) are much more effective in preventing toxicity [5,20] than anticipated by the gill-Ag model.

It is particularly interesting that these IC50 values (~16 nM Ag^+) are similar to conditional IC50 values recently determined in vitro for purified mammalian Na^+,K^+-ATPase preparations (9–40 nM [28,56,57]) and about three orders of magnitude lower than for total Ag when $AgNO_3$ is added in vitro to a crude trout gill homogenate [25]. It is as though waterborne ionic Ag^+ has a direct ''pipeline'' access to the basolateral Na^+,K^+-ATPase, avoiding complexation by intracellular Cl^-, thiols, proteins, and other intracellular constituents! The basis of this phenomenon is not known, but it is clear that the Na^+,K^+-ATPase has a tremendous affinity for Ag. A K_i value for binding to the Mg^{2+} site of the enzyme has been determined to be about 70 pM (log K_i = 10.1; E. Ferguson and C. Hogstrand, unpublished results). This value should not be confused with the conditional log K values that are determined in vivo or in vitro in the presence of physiological intracellular free Mg^{2+} concentrations (e.g., 15.2 mM [28]).

To create a Ag-ATPase model, it is also essential to know the concentration of Ag binding sites. Again, some information is already available. In vitro experiments with mammalian Na^+,K^+-ATPase in artificial liposomes have shown that each ATPase molecule binds one to two Ag^+ ions [57]. However, as these authors pointed out, binding of Ag to the enzyme does not necessarily mean reduced activity unless the binding occurs at a functionally important site. Enzyme kinetic data strongly suggest that Ag^+ inhibits the Na^+,K^+-ATPase by binding to one single site, the Mg^{2+} site [28], but it is possible that two monovalent Ag^+ ions could be clustered at this divalent bind-

ing site. Thus, from the mammalian data we will make the assumption that one to two Ag^+ ions bind to each Na^+,K^+-ATPase molecule in fish.

The concentration of Na^+,K^+-ATPase in the gills of several teleost species has been measured based on the number of radiolabeled ouabain-binding sites [58–62]. The data are fairly uniform, ranging from 1 to 7 μmol ATPase molecules per kilogram wet gill tissue, with the lowest value occurring in freshwater juvenile coho salmon [62], the most closely related species to trout. If this conservative value (1 μmol/kg gill × [1–2] Ag^+ binding sites per ATPase molecule) is applied to the trout data presented in Figure 5, it yields another surprising conclusion. At the lowest Cl^- concentration tested (20 μM, Fig. 5A), where the water Ag^+ ion concentration was greatest (28 nM) and most of the Na^+,K^+-ATPase activity was inhibited (Fig. 5B), about 34 to 69% of the 48-h gill Ag load (~2.9 μmol Ag per kilogram above background, Fig. 5A) would have been bound to Na^+,K^+-ATPase, depending on whether one or two binding sites per ATPase molecule are assumed (e.g., 1/2.9–2/2.9, expressed as percentages). Again this suggests that Ag^+ entering from the water is directly targetted by a ''pipeline'' to the basolateral enzyme.

Once the Ag-ATPase model is fully formulated, it should be possible to experimentally determine which model works best in a series of *natural* fresh waters, that is, does toxicity (assessed as 96-h LC50 values for juvenile trout) correlate best with predicted and measured short-term binding of Ag to the gills, with calculated ionic Ag^+ concentration or with predicted and measured gill Na^+,K^+-ATPase inhibition? Based on measured water characteristics, calculations of short-term gill Ag burdens (from the gill-Ag model) of water Ag^+ concentrations (from MINEQL$^+$) and of Na^+,K^+-ATPase activities (from the Ag-ATPase model) would be made *before* the toxicity results and measured values were known, analogous to the original approach of Janes and Playle [12]. Together, these comparisons would determine which model has the most predictive power with respect to acute Ag toxicity to fish.

A time course experiment should also be run in which both Na^+,K^+-ATPase activity and gill Ag concentrations are measured over 2 to 48 h in water supplemented with various concentrations of Cl^-. This experiment would yield information on the temporal relationships between gill Ag burden and Na^+,K^+-ATPase activity and would provide much-needed information on the diffusion rate of $AgCl^0$ relative to the active uptake of ionic Ag^+ into fish gills. Overall, these experiments should determine which modeling approach is most sensitive and therefore most relevant to the regulation of acute Ag toxicity in the field.

SILVER UPTAKE AND TOXICITY IN MARINE FISH

Mechanisms of acute silver toxicity

Plasma osmolality in a marine teleost is about 300 mosmol, and seawater is about 1,000 mosmol, so water tends to leave the fish by osmosis, and ions tend to enter the fish along their concentration gradients. Thus, marine fish must drink to avoid dehydration and must excrete ions such as Na^+, Cl^-, Ca^{2+}, and Mg^{2+} to maintain physiological concentrations of electrolytes. Uptake of water against the osmotic gradient from seawater requires energy, but there are no energetically coupled transporters for water itself. Instead ions are moved actively through the intestinal epithelium, and water follows passively along the generated osmotic gradient from the intestinal lumen into the blood (Fig. 7). In essence, Na^+ is transported across the basolateral membrane by Na^+,K^+-ATPase, which provides the driving force not only for the transepithelial movement of Na^+ but also for transport of other ions (e.g., Cl^- and K^+), nutrients (e.g., amino acids and glucose), and water from the gut into the body.

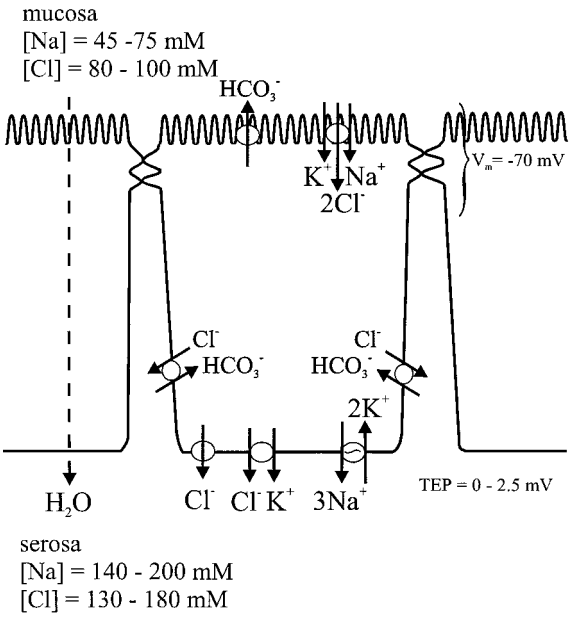

Fig. 7. A current model for water uptake by osmosis in the marine teleost intestine. Basolateral Na^+,K^+-ATPase pumps Na^+ into the plasma, which drives the apical Na^+, K^+, $2Cl^-$ symport system. Basolateral Cl^- channels and coupled K^+, Cl^- secretion move Cl^- into the plasma. By osmosis water follows the net uptake of Na^+ and Cl^- ions. See text for more details.

Several other ion transporters are involved in setting up the osmotic gradient that pulls water from the intestine into the blood. Of particular importance are the apical $Na^+,K^+,2Cl^-$ cotransporter and HCO_3^- channel and, on the basolateral side, Cl^- channels, a Cl^-/HCO_3^- exchanger, and a K^+,Cl^- cotransport system [16,63,64] (Fig. 7). Water passes across biological membranes without the presence of specific transporters. However, relatively recently it has been acknowledged that in many systems water channels, called aquaporins, facilitate the diffusion of water at critical sites and regulate the permeability of epithelia for water (reviewed in Verkman et al. [65]). Although the Na^+,K^+-ATPase is known to be sensitive to ionic Ag^+, inhibition of any step involved in water uptake would disturb ionic and osmotic balance within the animal.

As a result of this mechanism to take up water, marine teleosts continually receive a large inward net flux of monovalent ions across the gastrointestinal tract. Excess Na^+ and Cl^- ions are excreted across the gills using a basolaterally located Na^+,K^+-ATPase to produce the driving force for the uphill transport of both Na^+ and Cl^- from the blood back to the sea. The transport model at the gills is now well described [16,66] and will not be elaborated here in view of our current lack of knowledge regarding the effects of Ag on ion transport at the gills of marine fish.

Indeed, the overall physiological effects of Ag on marine fish remain poorly understood. Recently it has been shown that the acute toxicity of Ag in seawater involves osmoregulatory disturbances, with increases in plasma Na^+ and Cl^- concentrations and increases in intestinal and branchial Ag concentrations [5,67]. Based on tissue Ag residues, it appears that

there may be species differences in the importance of the gill as an avenue of Ag uptake. All marine teleosts examined accumulated substantial amounts of Ag in the intestine, but two flatfish, the English sole (*Parophrys vetulus*) and the starry flounder (*Platichthys stellatus*), accumulated little Ag on the gills compared with the intestine at reasonable exposure concentrations (below 2.3 μM Ag; <248 μg/L Ag), whereas other species such as the tidepool sculpin (*Oligocottus maculosus*) and seawater-adapted rainbow trout (*Oncorhynchus mykiss*) accumulated more Ag on the gills than in the intestinal tissue on a weight-specific basis. Elasmobranchs accumulate even higher levels of Ag in the gills [67,68], but these fish will be dealt with separately. Overall, the mechanisms of acute Ag toxicity appear to be more complex than in freshwater fish.

The activity of gill Na^+,K^+-ATPase has been measured in a few marine fish species during exposures to Ag. Exposure to 0.13 and 0.46 μM Ag (sublethal concentrations, 14 and 50 μg/L) in 32 ppt seawater over 21 d markedly reduced the gill Na^+,K^+-ATPase activity of the plainfin midshipman (*Porichthys notatus* [67]). In contrast, these same or higher concentrations of Ag generally caused increased gill Na^+,K^+-ATPase activity in tidepool sculpin, rainbow trout, and starry flounder [5,67,68] (N.A. Webb et al., unpublished results). This upregulation likely increased branchial Na^+ and Cl^- excretion to offset increased plasma osmolarity, which in turn was caused by reduced water uptake. In all cases, the concentrations of Ag in the gills were similar to those seen in freshwater fish exposed to much lower concentrations of Ag (e.g., Fig 5A).

Silver inhibits drinking in several marine fish species by up to 50% by an as-yet unexplained mechanism [67,68]. The mechanism appears to be very sensitive to Ag because inhibited drinking was evident in tidepool sculpins exposed to only 0.014 μM Ag (1.5 μg/L, added as $AgNO_3$) for 8 d in 18 ppt saltwater [67]. It could be that fish avoid drinking water containing Ag because of its taste, because of intestinal distension by water that is not absorbed, or because of any other discomfort that ingested Ag may cause. Another possibility is that Ag somehow interferes with the drinking reflex. Similar to mammals, the drinking reflex in marine fish is activated hormonally by the renin–angiotensin system [69] in response to a lowered blood volume (hypovolemia) and is inhibited by a C-type natriuretic peptide [70]. It is conceivable that Ag might disrupt this endocrine control so that the drinking reflex becomes suppressed.

In addition to reduced drinking, water uptake in marine fish is directly impaired by Ag through a reduction of the active transport of Na^+ and Cl^- from the intestinal lumen to the blood. This inhibition of salt transport has now been demonstrated in the English sole in vivo, and the accompanying reduction in inward water flux has been demonstrated in isolated intestinal sacs in vitro (M. Grosell, G. De Boeck, O.E. Johannsson, and C.M. Wood, unpublished results).

It is tempting to conclude that Ag blocks ion and water uptake by inhibiting Na^+,K^+-ATPase in the intestinal ion-transporting cells (enterocytes). However, analysis of Na^+,K^+-ATPase activities in intestinal mucosa of Ag-exposed marine fish has provided inconclusive data. In tidepool sculpin exposed to sublethal Ag concentrations in 30 ppt seawater, there was a dose-related decrease in the Na^+,K^+-ATPase activity of the intestinal mucosa [67]. In contrast, at 18 ppt salinity the same sublethal Ag exposure seemed to stimulate the activity of the enzyme in the intestine [67], which is peculiar because Ag toxicity to tidepool sculpin has been shown to be inversely related to salinity [6]. However, the activity of the Na^+,K^+-ATPase in unexposed fish was lower at 18 ppt than at 30 ppt salinity, so it is possible that at the lower salinity there was spare capacity to increase the number of active enzymes as compensation for enzyme inhibition and reduced drinking. No reduction in intestinal Na^+,K^+-ATPase activity was found in intestines of starry flounder or plainfin midshipman exposed to sublethal concentrations of waterborne Ag at 32 ppt salinity [67,68].

Thus, to date it has not been demonstrated that inhibition of Na^+,K^+-ATPase is responsible for the impaired ion and water uptake in the intestine of marine fish. However, it should be emphasized that neither intestinal nor gill Na^+,K^+-ATPase activities have been measured at acutely lethal concentrations of Ag in seawater. It is also possible that one or several of the other transport proteins involved in ion and water uptake are more sensitive targets. In this regard, as well as blocking Na^+ uptake, Ag also reduced the portion of intestinal Cl^- absorption that was coupled to HCO_3^- secretion in English sole (M. Grosell, G. De Boeck, O.E. Johannsson, and C.M. Wood, unpublished results), suggesting an effect on the mucosal HCO_3^- channel or serosal Cl^-/HCO_3^- exchanger (Fig. 7). However, there may also be a role for carbonic anhydrase in the as-yet poorly understood coupling of Cl^- uptake to HCO_3^- secretion [71,72]. Thus, an effect of Ag on carbonic anhydrase [29], as indicated for the freshwater gill [25], may also have contributed to the disruption of water uptake in the intestine.

In the marine species investigated so far, effects of waterborne Ag (added as $AgNO_3$) on osmoregulatory function have been demonstrated down to 0.014 μM Ag (inhibited drinking in tidepool sculpin [67]). The tidepool sculpin is not unusually sensitive to Ag; the 96-h LC50 values are 3.06 and 6.11 μM Ag in 25 and 32 ppt saltwater, respectively (330 and 660 μg/L, [7]). At present, it is not known if inhibited drinking at this Ag concentration has any detrimental consequences in terms of health or fitness, but the issue deserves attention. It should be noted that, although this Ag concentration (0.014 μM, 1.5 μg/L Ag) is lower than the current criterion for seawater systems (2.3 μg/L = 0.021 μM [36]), it is still 50 times greater than the highest Ag concentrations measured in North American coastal waters (0.0001–0.032 μg/L Ag in estuaries [73]).

To summarize so far, our current view of the sequence of events leading to acute Ag toxicity to marine teleost fish is as follows. Silver enters the fish both via the gills and intestinal tract. Water uptake in the intestine is impaired by the inhibition of drinking and by blockage of ion and water transport across the intestinal epithelium. The net loss of water results in increased plasma osmolality, which pulls water out from the tissues. Thus, although details are lacking, tissue dehydration is likely the direct cause of death in marine teleost fish.

Elasmobranch fish

Recent evidence indicates that elasmobranch fish may be more sensitive to Ag than are teleost fish. Exposure of the spiny dogfish (*Squalus acanthias*) to concentrations of Ag as low as 0.28 μM resulted in acute mortalities (G. DeBoeck et al., unpublished results). In another study, the spiny dogfish and the long-nose skate (*Raja rhina*) both accumulated much more Ag on the gills—yet none in the intestines—than did several teleost species when exposed to the same Ag concentration [67].

The elasmobranch gill resembles a freshwater teleost gill, and overall the strategy to regulate water and ions is very

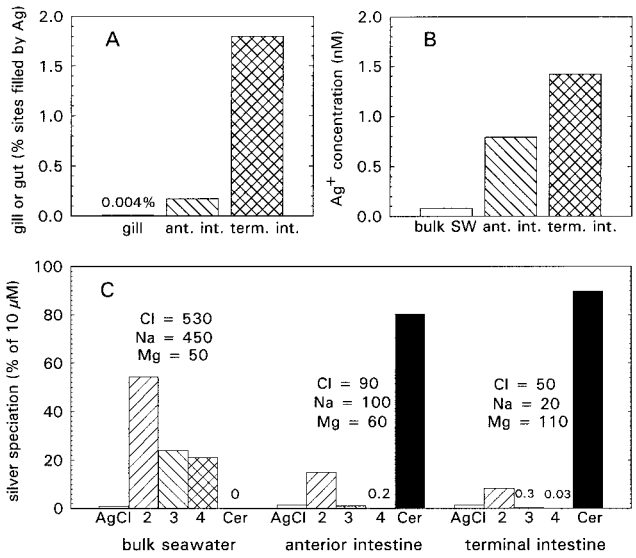

Fig. 8. A modeling exercise of silver (Ag) binding to the gills and gut of rainbow trout in 33 ppt seawater. The gill-Ag model [12] was used to calculate the fate of 10 μM Ag (as $AgNO_3$) using the ion concentrations reported in [76] for bulk seawater (SW), the anterior intestine (ant. int.), and the terminal intestine (term. intest.). In **C**, 2, 3, 4, and Cer represent $AgCl_2^-$, $AgCl_3^{2-}$,$AgCl_4^{3-}$ and cerargyrite, respectively. See text for details.

different from that of marine teleost fish. Elasmobranchs are slightly hyperosmotic to their environment, although the blood Na^+ and Cl^- concentrations are only 40 to 50% of those in seawater [16,64]. This feat is accomplished by supplementing inorganic osmolytes with high concentrations of urea and trimethylamine oxide.

Because elasmobranchs are hyperosmotic relative to seawater, water enters passively across the gills and there is no need to drink, which is the situation in freshwater fish. The elasmobranch gill is very permeable to water, but rather impermeable to ions and urea. When spiny dogfish were exposed to acutely toxic concentrations of Ag, the branchial permeabilities for Na^+, Cl^-, and urea increased drastically so that urea was lost and Na^+ and Cl^- rapidly entered the shark (G. DeBoeck et al., unpublished results). Thus, the similarity of elasmobranch gills to freshwater teleost gills seems to result in the increased sensitivity of elasmobranchs to Ag in seawater.

Influences on silver toxicity

Silver is much less toxic to marine fish than to freshwater fish (seawater 96-h LC50 values: 1.7 to 11.1 μM or 183 to 1,200 μg/L [5–7]; freshwater 96-h LC50 values: 0.05 to 0.65 μM or 5 to 70 μg/L [5]). The lower toxicity of Ag in seawater is primarily because ionic Ag^+ is virtually nonexistent in Cl^--rich seawater. Indeed, even $AgCl^0$ is no more than a few percent of the total Ag. Instead, anionic Ag chlorides, $AgCl_n^{n-1}$, and possibly organothiol-bound Ag complexes dominate Ag speciation in marine surface waters [5,8,53] (see also Fig. 8B and C). Closer to the sea bottom, Ag sulfides and especially Ag thiols may become more important, at least when the total Ag concentration is not higher than a few nanograms per liter (e.g., in most marine environments [53]).

Within the seawater range, the uptake and toxicity of Ag generally decrease with increased salinity [6,7,67] (Fig. 1). Perhaps the most important contributor to this effect is the progressive reduction of the small $AgCl^0$ fraction (see below). There appear to be two major exceptions to the general rule of lower Ag toxicity as salinity increases, one of them being anadromous euryhaline species that are primarily freshwater fish but that spend part of their life cycle at sea. These fish may not always be as well adapted to hypoosmoregulation (osmoregulation in seawater) as fish that are native to seawater. For such species (e.g., rainbow trout), the seawater itself imposes an ionoregulatory stress on the fish, which can result in greater Ag toxicity at higher salinities [8] (''ion stress'' in Fig. 1).

The other exception to the rule of lower Ag toxicity at higher salinity relates back to the geochemistry of Ag in seawater. Formation of the insoluble Ag chloride complex cerargyrite ($AgCl_{(s)}$) is salinity and concentration dependent so that cerargyrite forms at lower Ag concentrations at lower salinities [8] (see also Fig. 8C). For practical purposes, cerargyrite can be regarded as nontoxic, at least on an acute scale. In several acute experiments, it was found that, if mortality was not observed at Ag concentrations lower than the threshold for cerargyrite precipitation, toxicity would not occur within 168 h [6–8]. In fact, it is possible to *rescue* a fish in an otherwise lethal concentration of Ag by further increasing the Ag concentration above the threshold for cerargyrite precipitation (J.R. Shaw et al., unpublished results). Cerargyrite precipitation is not a normal phenomenon in seawater because of the unrealistically high Ag concentrations required (>4.6 μM, >500 μg/L), but it is an important consideration in laboratory tests.

In addition to salinity, ammonia can significantly modulate Ag toxicity in seawater. Shaw et al. [6] found that Ag toxicity was enhanced and mortality was hastened (i.e., decreased LC50 values and decreased time to reach 50% mortality [LT50] values) when Ag was tested in combination with ammonia (Fig. 1). Ammonia is an important pollutant from domestic, agricultural, and industrial sources, and its additional effects on Ag toxicity, especially in coastal areas, warrants more attention [5]. Similarly, increased concentrations of other toxicants such as Cu, which has a mode of action similar to Ag [74], would be expected to increase the apparent toxicity of Ag in seawater (Fig. 1).

Organothiols are effective in reducing acute Ag toxicity if they are present in high enough concentrations. The addition of 3-mercaptoproprionic acid (3-MPA) in equimolar concentrations to Ag almost completely eliminated mortalities in various species of fish at Ag concentrations at or above their 96-h LC50 values for Ag alone [7]. Organothiols such as 3-MPA can be present at micromolar concentrations in reducing environments [53] and would need to be present in at least these concentrations to reduce acute Ag toxicity to marine fish [7]. However, little is known about organothiol concentrations in oxic marine environments where fish and most other eukaryotic organisms reside. It should also be noted that, although 3-MPA was effective in preventing acute Ag toxicity when administered in equimolar concentrations to Ag, nothing is known about the effects of organothiols on Ag uptake and on possible chronic toxicity from long-term Ag accumulation.

Influences on silver uptake

Waterborne Ag readily enters seawater fish and appears to accumulate primarily in the liver [1,6,67]. As with toxicity, our current understanding of the mechanisms involved in uptake and distribution of Ag in marine fish is poor. Either the gills, the intestinal tract, or most likely both are the routes of

entry for Ag. However, as noted above, there are substantial differences among fish species in the amounts of Ag accumulating in these tissues during sublethal exposures.

Most striking of all is the observation that elasmobranchs accumulate up to 20 times more Ag in their livers than do teleosts during extended exposures to sublethal concentrations of Ag [67]. This finding is in accord with earlier work of Pentreath [75], who used the radiotracer ^{110m}Ag to record Ag turnover rates in fish. Elasmobranchs do not drink and do not accumulate Ag in intestinal tissue but accumulate much larger amounts of Ag in the gill tissue than do marine teleosts. This observation, together with the fact that teleosts generally take up less Ag at higher salinities (see below) where drinking rates are higher [64,76] could suggest that the gills are a more important route than the intestine for Ag uptake in some marine fish. However, this supposition must be qualified by the different nature of the gills in elasmobranchs versus teleosts and the effects of salinity on silver speciation.

It is apparent that salinity itself dramatically alters Ag uptake rates in marine teleosts. In euryhaline tidepool sculpins, whole-body Ag burdens were much lower at 32 ppt than 25 ppt salinity during acute Ag exposures [6], and Ag burdens in liver and other tissues were much lower at 30 ppt than 18 ppt salinity during chronic exposures [67]. Based on the evidence from freshwater studies that $AgCl^0$ readily penetrates across gill membranes, we attribute at least part of this effect to the decline and disappearance of the small $AgCl^0$ fraction at higher salinities. However, both the physiology of the fish [67] and the contributions of the various anionic chloride complexes ($AgCl_n^{n-1}$) to total Ag are also altered at higher salinity [8] (Fig. 8B and C), so the reduced proportion of $AgCl^0$ at higher salinities may not be the complete explanation. Disturbances of liver enzymes [77], ammonia production, and metabolic rate [67] have been seen in marine fish chronically exposed to waterborne Ag, so whether internalized Ag causes any toxicity is an important topic for future research.

Can the gill modeling approach be applied to marine fish?

The gill modeling approach was developed for metals that have their primary, acute toxic action at the gills of freshwater fish [10,12,41,42,46] and clearly cannot be applied directly to the marine situation where the intestine is an important site of toxicity. Another crucial consideration is that, whereas Ag taken up as ionic Ag^+ is clearly the main cause of acute Ag toxicity in freshwater fish, the uncharged $AgCl^0$ complex must be considered a possible toxic Ag species to marine fish.

The reason for considering $AgCl^0$ as a toxic Ag species relates back to the target site for toxicity. In freshwater fish, there is good reason to believe that ionic Ag^+ is taken up specifically by gill cells involved in ion transport (ionocytes) that constitute no more than 5 to 10% of the gill surface area. Thus, Ag is likely to become much more concentrated in these cells than in the rest of the gill tissue. $AgCl^0$ probably permeates the gill membrane almost as well as ionic Ag^+, but its entry is likely nonspecific and spread over most of the gill surface area, thus ''diluting'' its toxicity. In addition, in freshwater the Ag-sensitive chloride cells have only a tiny fraction of their apical surface exposed to the water [39], which would further limit nonspecific effects of $AgCl^0$ accumulation. In the intestine of marine fish, the situation is different because in sections of the intestine virtually all epithelial cells are engaged in ion transport and are presumably equally sensitive to Ag, and there is much more Cl^- to bind Ag than in freshwater. Thus, the dilution effect that may be important in the gills of freshwater fish is less likely to occur in the intestine of marine fish.

An additional factor to consider is that the physiology of the drinking process changes seawater chemistry greatly in favor of the formation of cerargyrite, slightly more $AgCl^0$, and more trace ionic Ag^+ as imbibed seawater enters the intestinal tract. As seawater passes down the esophagus, it is rapidly desalinated by passive or active Na^+ and Cl^- uptake in the esophagus, probably augmented by dilution from an osmotic backflux of water into the stomach lumen from the blood plasma [63]. Concentrations of Na^+ and Cl^- fall below plasma levels, whereas divalent ions (Mg^{2+}, SO_4^{2-}, Ca^{2+}, and CO_3^{2-}) and HCO_3^- rise well above their concentrations in plasma [72,76,78] (C. Hogstrand and C.M. Wood, unpublished data).

Other complicating factors in modeling Ag uptake and toxicity in marine teleosts are that fish continually change the volume as well as the composition of their intestinal fluid along the gut [63,72,76], and ingested water is mixed with food that will alter Ag speciation in ways that may be difficult to predict. For example, a diet of fish will produce many Ag binding sites in the form of thiol-containing amino acids, whereas a diet consisting of *Ophiura* sp. (a brittlestar) will add large amounts of calcium carbonate to the intestinal fluid. By analogy to gastrointestinal absorption of Cu in mammals, it is possible that Ag can be taken up while bound to amino acids [79,80]. It is not known whether Ag taken up as Ag bound to amino acids, as $AgCl^0$, or as some other complex can cause harmful effects in marine fish. In principle, any toxicity from forms of absorbed Ag other than ionic Ag^+ would limit the applicability of the gill binding model for predicting Ag toxicity in marine teleost fish. To solve this issue, research should focus on the mechanisms of Ag uptake and toxicity in the gut of marine fish.

Modeling silver uptake in marine fish

If, however, ionic Ag^+ is the sole absorbed Ag species causing toxicity in marine fish, then modeling would use the same principles of metal complexation and cationic competition [12], although in a much more complicated system. Our conceptual model of Ag-gut binding is similar to the freshwater, Ag-gill model summarized in Figure 3. The concentration of Cl^- is important in the model, but DOM and particulate matter are much less important in seawater than in freshwater (DOM = 0.4–2.5 mg C/L, suspended particulate matter = 20–50 μg/L for seawater, vs 2–100 mg C/L and 0.08–38 g/L for freshwater, respectively [51]). In our hypothetical gut model, the particulate part of the gill model would therefore be replaced by organothiols and a food component, but we have no data to allow us to include this component at present. Cationic competition at Ag uptake sites at the intestinal epithelium would likely be strong because of the relatively high concentrations of cations in the intestines of seawater fish.

Marine fish drink about 3 ml/kg/h, whereas ventilation rate is about 18,000 ml/kg/h, so the exposure and hence potential dose of Ag at the two possible routes of Ag toxicity are different. As yet, we do not know how to weight these two components for Ag dose. In addition, changes in Ag speciation in the gut as Na^+, Cl^-, and water are removed must substantially alter the availability of Ag to the fish, as follows.

Shehadeh and Gordon [76] measured intestinal ion composition as 11, 17, and 33 ppt seawater passed through the guts of salinity-acclimated rainbow trout. When 33 ppt sea-

water containing (in millimolar concentrations) 530 Cl^-, 450 Na^+, 50 Mg^{2+}, 30 SO_4^{2-}, 9 Ca^{2+}, and 10 K^+ at pH 8.1 was imbibed by rainbow trout, by the time the water reached the anterior intestine it contained 90 Cl^-, 100 Na^+, 60 Mg^{2+}, 40 SO_4^{2-}, 5 Ca^{2+}, and 6 K^+. At the terminal intestine it contained 50 Cl^-, 20 Na^+, 110 Mg^{2+}, 110 SO_4^{2-}, 2 Ca^{2+}, and 0.8 K^+ at pH 8.1 [76]. The removal of Na^+, Cl^-, and water (the latter causing the increased concentrations of other ions such as Mg^{2+} and SO_4^{2-}) has large effects on the speciation of Ag in imbibed seawater.

The complexity of Ag uptake and toxicity in marine fish is well illustrated in our attempt at modeling Ag binding at the gills and intestine. We modeled both the gill and gut systems in seawater fish using the gill-Ag model [12] inserted into MINEQL$^+$, assuming 10 μM total Ag (added as $AgNO_3$), water chemistry from [76], and ignoring the complications of DOM, particulate matter, food, and fluid volume changes in the intestine. In the absence of any direct measurements in seawater fish, for modeling purposes we used freshwater trout gill values, with the same concentration of binding sites and the same strength of Ag binding for seawater trout gill and gut tissue. Activities of Na^+,K^+-ATPase are broadly similar in gill and intestinal tissue of marine teleosts [67] and are only slightly higher than in the gills of freshwater teleosts [58–61], so we feel these assumptions are reasonable. In essence, Figure 3 can be turned into the Ag-gut model simply by substituting ''gut'' for ''gill.''

Virtually no Ag is modeled to bind to the gills of marine fish (0.004% of 1.8 nM gill sites filled by Ag; Fig. 8A) because the high concentration of Cl^- in seawater effectively binds virtually all the Ag, leaving just 0.08 nM ionic Ag^+ (Fig. 8B). The rest of the 10 μM Ag is bound as $AgCl^0$ and especially as $AgCl_2^-$, $AgCl_3^{2-}$, and $AgCl_4^{3-}$ (Fig. 8C; log K_{AgCln} = 3.3, 5.4, 5.3, and 5.5, respectively). In addition, competition by Na^+ and by Ca^{2+} helps keep Ag off the gills; if cationic competition is removed from the model, about 36% of the gill sites are calculated to be filled by Ag, in spite of the low concentration of ionic Ag^+ in the bulk seawater, because Ag^+ is ''stripped off'' the weaker Ag–chloride complexes by the strong binding of Ag^+ to the now unoccupied binding sites on the gills (log $K_{Ag\text{-}gill}$ = 10.0 vs log K_{AgCln} = 3.3–5.5).

The anterior intestine and especially the terminal intestine of marine fish are modeled to accumulate more Ag than the gills, with 0.17 and 1.8% of the 1.8 nM gut sites filled by Ag (Fig. 8A). More Ag would theoretically bind to the intestines than to the gills because the complexation of Ag by Cl^-, and competition by Na^+ and Ca^{2+}, is reduced as these ions plus water are removed. Much less $AgCl_2^-$, $AgCl_3^{2-}$, and $AgCl_4^{3-}$ are calculated to form, and about 80% of the 10 μM total Ag is calculated to form cerargyrite (''Cer'' in Fig. 8C). The passage of water through the marine fish gut is slow enough (probably 24–48 h) that there is plenty of time for cerargyrite to form (i.e., there are no kinetic constraints in the equilibrium model). The concentration of ionic Ag^+ is only 0.08 nM in bulk seawater but is calculated to increase considerably in both the anterior and terminal intestine (Fig. 8B). However, as a percentage of the 10 μM Ag used in the simulation, even in the terminal intestine ionic Ag^+ amounts to only about 0.014% of the total Ag (cf. with Fig. 8C).

Does this modeling exercise help us interpret the trends of Ag toxicity in seawater first illustrated in Fig. 1? In marine fish where Ag is not very toxic, little silver is calculated to accumulate on either the gills or gut, with only ≤2% of the binding sites filled by Ag (Fig. 8A). In contrast, in freshwater fish where Ag toxicity is greatest, about 94% of gill sites were calculated to be filled by Ag in an exposure to just 0.1 μM Ag (Fig. 6).

The upward trend in Ag toxicity as fish move from brackish water into seawater (Fig. 1) must be related to Ag's action on the intestinal tract as fish begin to drink Ag-laden water. Indeed, going from 11 to 17 to 33 ppt seawater, progressively *less* Ag is calculated to bind to the gills (0.058, 0.024, and 0.004% of binding sites filled by Ag) because of Cl^- complexation of ionic Ag^+ and because of competition by Na^+ and Ca^{2+} for Ag binding sites at the gills. In contrast, the accumulation of Ag by the anterior intestine is modeled as approximately constant when going from 11 to 17 to 33 ppt salinity (0.1 to 0.2% of sites filled), and the accumulation of Ag by the terminal intestine should actually increase as salinity increases (0.3, 0.6, and 1.8% of sites filled by Ag). These patterns indicate that the increase in Ag toxicity as a fish moves from brackish water to about 25 ppt seawater could be related to the need to drink and the accompanying toxic effects of Ag at the gut, and the decrease in Ag toxicity when going from 25 ppt to full-strength seawater could reflect reduced Ag binding at the gills.

Although we can model the gill and gut systems in a speculative manner to yield insight into changes in Ag speciation as seawater chemistry changes, and can suggest the effects of these speciation changes on Ag toxicity to marine fish, we currently understand little of the physiological mechanisms involved in Ag toxicity in seawater and do not know the specific sites of Ag interaction with the fish. We also do not yet know how to weight the relative contributions of Ag–gill and Ag–gut interactions in Ag toxicity to marine fish. It is probable that the gill and intestinal uptake of Ag in marine fish can be modeled in future, but we need to learn much more about the interactions among Ag, intestinal fluid components, and the intestinal epithelium before such a model will meaningfully reflect reality. Finally, to evaluate Ag effects in the environment, more chronic studies need to be conducted, and laboratory experience needs to be taken into the field.

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