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# Is $Cl^-$ protection against silver toxicity due to chemical speciation?

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**Abstract**

In freshwater teleosts, the primary mechanism of acute silver toxicity is inhibition of $Na^+/K^+$ ATPase and carbonic anhydrase at the gill, leading to net $Na^+$ and $Cl^-$ loss due to the continued diffusion of these ions into the hypoosmotic external environment. External $Cl^-$ has been shown to protect rainbow trout (*Oncorhychus mykiss*) against silver toxicity presumably by complexation to form AgCl. However, $Cl^-$ does not appear to greatly influence silver toxicity to at least two other species, the European eel (*Anguilla Anguilla*) and the fathead minnow (*Pimephales promelas*). We hypothesized that differences in protective effects of $Cl^-$ at the gill were due to differing requirements or mechanisms for $Cl^-$ uptake among fish species. To test this hypothesis, we exposed *Fundulus heteroclitus*, which does not take up $Cl^-$ across the gills, and *Danio rerio* and *P. promelas*, which do rely on $Cl^-$ uptake across the gills, to $Ag^+$ in waters of varying $Cl^-$ concentration. The 96-h LC50s of *F. heteroclitus* exposed to $Ag^+$ in soft water with 10 μM $Cl^-$, 1 mM KCl, and 0.5 mM $MgCl_2$ were 3.88, 1.20, and 3.20 μg/L, respectively, and not significantly different. The 96-h LC50s for *D. rerio* exposed to $Ag^+$ in soft water with 10 μM $Cl^-$ and 1 mM KCl were 10.3 and 11.3 μg/L, respectively and *P. promelas* exposed under the same conditions were 2.32 and 2.67 μg/L, respectively. Based on these results, increasing external $Cl^-$ concentration by as much as 1 mM (35.5 mg/L) did not offer protection against $Ag^+$ toxicity to any fish species tested. Although previous results in our laboratory have demonstrated that *P. promelas* do take up $Cl^-$ at the gill, a mechanism of uptake has not been identified. Additional experiments, investigating the mechanisms of $Na^+$ and $Cl^-$ influx at the gill of *P. promelas* and the influence of silver, demonstrated that $Cl^-$ uptake in *P. promelas* acclimated to soft water occurs through both a $Na^+$:$K^+$:2$Cl^-$ co-transporter and a $Cl^-/HCO_3^-$ exchanger, but is not dependent on carbonic anhydrase. Further, acclimation water chemistry was found to greatly influence subsequent branchial silver accumulation, but $Cl^-$ uptake was not sensitive to 10 μg/L $Ag^+$. Published by Elsevier B.V.

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## 1. Introduction

Silver nitrate, which readily dissociates to yield the free silver ion ($Ag^+$), is known to be very toxic to freshwater organisms (Ratte, 1999). Silver exerts toxicity on rainbow trout (*Oncorhychus mykiss*) at the gill by initially inhibiting carbonic anhydrase activity followed by inhibition of $Na^+/K^+$ ATPase leading to a net loss of $Na^+$ and $Cl^-$ (Morgan et al., 2004). This effect of silver on ion transport at the gill is detrimental to freshwater fish which rely on active uptake of $Na^+$ and, for some species, also $Cl^-$ to maintain normal salt balance (Wood, 2001; Marshall and Grosell, 2005). External $Cl^-$ has been shown to protect *O. mykiss* against silver toxicity presumably by complexation to form AgCl, thereby reducing the concentration of $Ag^+$, a more bioavailable form of silver (Galvez and Wood, 1997; Bury et al., 1999; Grosell et al., 2000). Circumneutral AgCl, however, may passively enter and accumulate within *O. mykiss* and $Cl^-$ appears to protect against Ag toxicity but not accumulation at least in rainbow trout (Hogstrand et al., 1996; Wood et al., 2002).

In efforts to evaluate the bioavailability and acute toxicity of waterborne metals, a biotic ligand model (BLM) has been developed as a predictive tool that will allow site-specific water quality standards to be generated when the water chemistry of the site is known (McGeer et al., 2000; Di Toro et al., 2001). This model takes into account a variety of water chemistry parameters, including $Cl^-$ concentration, to predict the amount of free metal ion available to bind to the fish gill. The gill is considered the proximate site of toxic action and a surrogate for the actual biotic ligand, which is understood to be one or more sensitive enzyme systems (carbonic anhydrase and/or $Na^+/K^+$-ATPase). The concentration of metal bound to the gill is then related to

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acute toxicity to provide an estimate of the site-specific toxicity of a metal. Many of the toxicity studies used to calibrate the Ag BLM were conducted with *O. mykiss*.

The question of the broader application for the BLM to predict toxicity for species other than the few standard test organisms investigated to date remains to be thoroughly addressed. Of particular interest to us was the question of whether protection against silver toxicity arising from ambient $Cl^-$ is a general phenomenon, not just specific to rainbow trout. Because not only silver speciation but also gill transport physiology vary with water chemistry (Boisen et al., 2003), we also questioned whether gill silver accumulation reflects the ambient water chemistry to which the organism is acclimated.

While external $Cl^-$ has been demonstrated to reduce silver toxicity to *O. mykiss*, discrepancies in the literature exist, because the formation of AgCl does not appear to substantially influence silver sensitivity for any other fish species tested (Erickson et al., 1998; Bury et al., 1999; Karen et al., 1999; Grosell et al., 2000). Additionally, Wood et al. (2002) demonstrated that unlike silver toxicity, branchial silver accumulation was not reduced in rainbow trout exposed to AgCl as compared to those exposed to the silver ion. If the protective effect of ambient $Cl^-$ was simply related to chemical speciation, equal protection against silver toxicity from ambient $Cl^-$ would be expected for all species. Because varying protection from ambient $Cl^-$ is reported for different species, we hypothesized that differences in protective effects of $Cl^-$ are due to differing requirements or mechanisms for $Cl^-$ uptake among fish species. Researchers have shown that *O. mykiss*, which has high branchial $Na^+$ and $Cl^-$ uptake, also has a high sensitivity to silver (Morgan et al., 2004) whereas, the European eel, *Anguilla anguilla*, which has low ion uptake rates is relatively resistant to silver (Grosell et al., 2000). By increasing external $Cl^-$ concentration, branchial $Cl^-$ uptake would be less unfavorable for fish which depend on $Cl^-$ uptake at the gill. Fish, which are not dependent on branchial $Cl^-$ uptake, perhaps obtaining $Cl^-$ through the diet, may not be sensitive to silver-induced inhibition of active $Cl^-$ uptake at the gill and ambient $Cl^-$ would likely not offer protection against silver.

To test this hypothesis, we exposed the killifish, *Fundulus heteroclitus*, which does not take up $Cl^-$ (Patrick et al., 1997), the zebra fish, *Danio rerio*, which relies on $Cl^-$ uptake across the gills (Boisen et al., 2003), and the fathead minnow, *Pimephales promelas*, which has just recently been shown to take up $Cl^-$ (Bielmyer et al., 2007), to silver in waters of varying $Cl^-$ concentration. We proposed that sensitivity to silver may differ with changing external $Cl^-$ concentration and will correspond to the rate of branchial $Cl^-$ uptake in these fish.

Thus, one objective of this research was to determine if external $Cl^-$ is protective against silver toxicity in three different fish species with differing requirements or different $Cl^-$ uptake mechanisms. Secondly, we investigated the mechanisms of $Na^+$ and $Cl^-$ influx at the gill of *P. promelas*, a commonly used toxicity test organism for which very little is known about ion uptake at the gill. We hypothesized that *P. promelas* take up $Cl^-$ via the traditional pathway of the $Cl^-/HCO_3^-$ exchanger and possibly the $Na^+$:$K^+$:2$Cl^-$ co-transporter (NKCC), which has been shown to be important in at least two fish species, Mozambique tilapia and goldfish (Hiroi et al., 2005; Preest et al., 2005).

Finally, the influence of acclimation water chemistry on subsequent short-term silver accumulation was examined using *P. promelas* in an attempt to discern the dynamics of silver binding at the gill in low ionic strength waters. By comparing gill silver binding in fish acclimated to low and intermediate ionic strength fresh waters we attempted to address recently identified limitations of the BLM in predicting toxicity at low ionic strength (Bielmyer et al., 2007).

## 2. Methods

### 2.1. Testing waters

For all silver toxicity tests with *F. heteroclitus*, *D. rerio*, and *P. promelas*, low chloride, soft water was made by adding reagent grade salts to deionized water at the following concentrations: 0.05 mM $NaHCO_3^-$, 0.06 mM $CaSO_4{\cdot}2H_2O$, 0.02 mM $MgSO_4{\cdot}2H_2O$, and 0.01 mM KCl. The high $Cl^-$ water was made exactly the same as the low chloride, soft water with the addition of 1 mM KCl or 0.5 mM $MgCl_2$. Both KCl and $MgCl_2$ were used in the experiment with *F. heteroclitus* to determine if $K^+$ or $Mg^{2+}$ would confound the effects of $Cl^-$. All subsequent tests were conducted with KCl only.

For all other experiments with *P. promelas*, moderately hard (MH), de-chlorinated tap water was obtained from the University of Miami and had the following ion concentrations: 1 mM $Na^+$, 0.15 mM $Mg^{2+}$, 0.5 mM $Ca^{2+}$, 0.1 mM $K^+$, 1.2 mM $Cl^-$, and 0.73 mM total $CO_2$, and also contained approximately 200 μM (2.4 mg/L) of dissolved organic carbon (DOC). The MH water was diluted approximately 10-fold to formulate the soft water used in these experiments. The pH of the soft and MH waters were 7.3 and 7.7, respectively. All of the waters were aerated for 24-h prior to use.

### 2.2. Toxicity testing

*F. heteroclitus* (7-d-old; 24.6 ± 5.8 mg), *D. rerio* (adult; 314 ± 35.0 mg), and *P. promelas* (4-d-old; 0.50 ± 0.15 mg), obtained from our laboratory culture, Pet Supermarket (Miami, FL), and Aquatic Biosystems (Fort Collins, CO), respectively, were held in soft water with or without 1 mM $Cl^-$ for at least 48 h prior to testing. Varying concentrations of silver, as $AgNO_3$ (Sigma Aldrich) were added from a 10 mg/L stock solution (1% nitric acid in de-ionized water) to the testing waters and equilibrated for 24 h in 1-L plastic beakers filled to volume.

Fish were exposed to control water (silver-free low $Cl^-$ or silver-free high $Cl^-$ water) or one of five silver solutions in either a low or high $Cl^-$ water for 96 h according to standard methods (U.S.E.P.A., 1993). Nominal silver concentrations ranged from 0.5 to 8 μg/L in tests with *P. promelas* and 2–48 μg/L in tests with *F. heteroclitus* and *D. rerio*. There were eight fish per replicate and three replicates per treatment in each test. At 48 h, *P. promelas* and *F. heteroclitus* were fed for 1 h after which 80% of the test water was renewed. Solutions were continuously aerated throughout testing with *D. rerio*. Samples for silver analysis