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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^+:2Cl^- 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.

Keywords: Chloride; Ionoregulation; Ag; Aquatic toxicity; Freshwater teleosts

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^+:2Cl^- 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

in all tests were collected at 0, 48, and 96 h and immediately acidified (nitric acid, trace metal grade, 1% v/v, Fisher Scientific, Pittsburgh, PA, USA) for subsequent analysis as described below.

2.3. Na^+ and Cl^- flux experiments

All flux experiments were conducted generally following the methods of Boisen et al. (2003). *P. promelas* (65-d-old; 97.9 ± 40.9 mg) were placed in 1-L plastic beakers filled with (900 ml) soft or moderately hard de-chlorinated tap water. Fish (10 fish/beaker) were allowed to settle for at least 30 min. Isotope (2 μCi ^{22}Na or ^{36}Cl) was added to the designated beakers and water samples were taken at the start (~5 min after isotope addition) and end of the exposure. At the end of the flux period, the fish were rinsed twice with 100 mM NaCl to displace loosely bound isotope, euthanized by overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA), blotted dry, and weighed. Radioactivity of individual fish and water samples were measured on a gamma counter for ^{22}Na and a beta counter for ^{36}Cl. In addition, water samples were analyzed for Na and Cl concentrations as described below. In separate experiments, silver (10 or 30 μg/L nominal Ag) was added to half the treatments in both soft and moderately hard water, allowed to equilibrate and then fish were added and measured for Cl^- and Na^+ influx, respectively.

In addition, separate experiments employing the drugs, ethoxzolamide (10^{-4} M in DMSO), DIDS (10^{-3} M in DMSO), bumetanide (10^{-4} M in DMSO), and a combination of DIDS (10^{-3} M in DMSO) and bumetanide (10^{-4} M in DMSO) were added to half the treatments and the other half were used as controls (0.1% DMSO addition only). These three drugs were administered in a series of experiments attempting to inhibit carbonic anhydrase, the Cl^-/HCO_3^- exchanger, the NKCC co-transporter, and both the Cl^-/HCO_3^- exchanger and the NKCC co-transporter in combination, respectively.

2.4. Branchial short-term silver accumulation

A branchial silver binding experiment was conducted in soft water with both soft water- and tap-water-acclimated *P. promelas* (69.8 ± 21.8 mg). Fish were exposed to dissolved silver concentrations of 0.2–12 μg/L for 3 h in 1-L plastic beakers filled with 900 mL of water and constantly aerated. There were approximately 10 fish per beaker. Fish were then rinsed with de-ionized water and sacrificed by an overdose of MS-222. Gill baskets were obtained by dissection, then weighed, and digested in 1 N HNO_3 (trace metal grade, Fisher Scientific, Pittsburgh, PA, USA) for 24–48 h.

2.5. Analytical chemistry

Immediately upon collection, water samples were passed through a 0.45 μm filter (Pall Life Sciences, East Hills, NY, U.S.A.) and acidified. At the end of each experiment, all samples and tissue digests were analyzed for silver using graphite furnace atomic absorption spectrophotometry (AAS; Varian 220Z Mulgrave, Victoria, Australia). Each test water was analyzed for cations and anions by flame AAS (Varian 220FS Mulgrave, Victoria, Australia) and ion chromatography (DX-120; Dionex Corp., Sunnyvale, CA, U.S.A.), respectively. The detection limit for both chloride and sulfate was 1 μM. Total CO_2 was measured using a total CO_2 analyzer (965 Corning Limited, Halstead, Essex, England), and pH using a combination glass electrode (pHC3005-8 Radiometer, Villeurbanne Cedex, France) coupled to a meter (PHM220 MeterLab^{TM}, Radiometer, Copenhagen).

2.6. Statistical analysis

Probit and Spearman Karber analysis were used to estimate 96-h concentrations which caused lethality to 50% of the organisms (LC50s). Analysis of variance, followed by Dunnett's test were used to determine significant differences between treatments ($\alpha = 0.05$).

3. Results and discussion

3.1. Protection of Cl^- against Ag toxicity

F. heteroclitus were not protected against silver toxicity in waters with increased ambient Cl^- concentration. The 96-h LC50s were 3.8, 1.2, and 3.2 μg/L Ag for *F. heteroclitus* exposed to silver in soft water, 1 mM KCl and 0.5 mM $MgCl_2$, respectively, and not statistically different ($p > 0.05$) between treatments (Fig. 1). Considering only physiology, the lack of protection from ambient Cl^- was not surprising since *F. heteroclitus* do not actively take up Cl^- at the gill (Patrick et al., 1997).

In contrast to *F. heteroclitus*, *D. rerio* have high affinity transporters for Cl^- uptake, which is sensitive to inhibited carbonic anhydrase activity, similar to that of rainbow trout (Boisen et

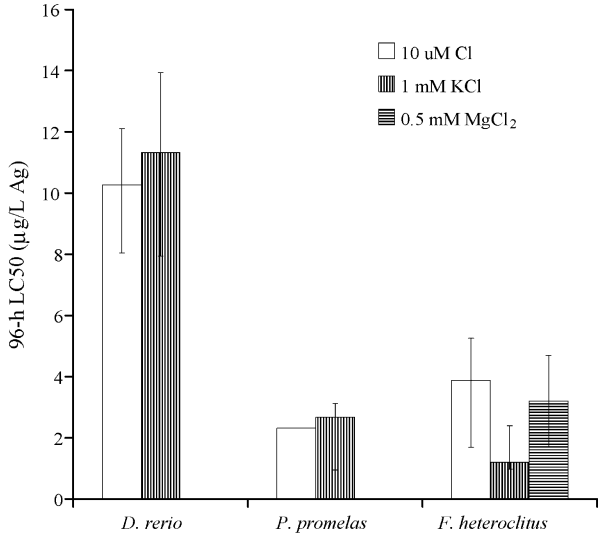

Fig. 1. Estimated concentrations causing lethality in 50% of the organisms (LC50s) in 96 h for *Danio rerio, Pimephales promelas,* and *Fundulus heteroclitus* exposed to silver in soft water with 10 μM Cl^-, 1 mM KCl, and 0.5 mM $MgCl_2$. Error bars represent 95% confidence intervals.

Table 1
Existing literature on the protective effect of Cl^- against Ag^+ toxicity to fish

Species	Age or weight	Cl^- protection	Cl^- (μM)	Endpoint	Reference
Oncorhynchus mykiss	5.17 g	Yes	50–5000	7-d LC50	Galvez and Wood (1997)
*Oncorhynchus mykiss**	5.17 g	Yes	50–5000	7-d LC50	Galvez and Wood (1997)
*Oncorhynchus mykiss**	2.2 g	Yes	50–1500	96-h LC50	Bury et al. (1999)
Oncorhynchus mykiss	20-d-old	Yes	84.6–1128	96-h LC50	Karen et al. (1999)
Oncorhynchus mykiss	25 g	Yes	10–1200	Na^+, Cl^- Flux	Grosell et al. (2000)
*Pimephales promelas**	30-d-old	Yes	200	96-h LC50	Erickson et al. (1998)
Pimephales promelas	4-d-old	No	84.6–1692	96-h LC50	Karen et al. (1999)
*Pimephales promelas**	0.23 g	No	50–1500	96-h LC50	Bury et al. (1999)
Anguilla anguilla	60 g	No	10–1200	Na^+, Cl^- Flux	Grosell et al. (2000)
Fundulus heteroclitus	7-d-old	No	10–1000	96-h LC50	Present study
Pimephales promelas	4-d-old	No	1000	96-h LC50	Present study
Danio rerio	Adult	No	1000	96-h LC50	Present study

Asterisks indicate the addition of Cl^- as NaCl, otherwise added as KCl.

al., 2003; Morgan et al., 2004). Therefore, we predicted that like rainbow trout, *D. rerio* would be protected by ambient Cl^- against silver toxicity. However, the 96-h LC50s of *D. rerio* exposed to silver in soft water and 1 mM KCl were 10.3 and 11.3 μg/L silver and not significantly different. This result was surprising because the Cl^- uptake mechanisms appear to be similar in rainbow trout and *D. rerio*. The lack of effect of ambient Cl^- might suggest that the Cl^- uptake pathways in *D. rerio* are less sensitive to silver than those in rainbow trout. One possible explanation for this would be species differences in the sensitivity of carbonic anhydrase to inhibition by silver. For example, Skaggs and Henry (2002) demonstrated a three order of magnitude difference in carbonic anhydrase sensitivity to silver in the closely related euryhaline crabs *Callinectes sapidus* and *Carcinus maenas*. It is possible that such a species difference in carbonic anhydrase sensitivity to silver occurs in fish as well. However, more research is needed to evaluate this hypothesis. Additionally, in at least one study reporting the protective effects of Cl^- against silver toxicity in rainbow trout, Cl^- was added as NaCl and protection may have been the result of increased ambient Na^+ and/or Cl^- (Bury et al., 1999).

Increased ambient Cl^- was also not protective against silver toxicity to *P. promelas*, which is consistent with the majority of existing literature (Table 1; Bury et al., 1999; Karen et al., 1999). The 96-h LC50s were 2.3 and 2.7 μg/L Ag for *P. promelas* exposed to silver in soft water and 1 mM KCl, respectively (Fig. 1). *P. promelas* and *F. heteroclitus* were similar in age and had similar 96-h LC50s despite the fact that *P. promelas* weighed more than an order of magnitude less than *F. heteroclitus*. The *D. rerio* used in the toxicity tests were adults and weighed substantially more than both *P. promelas* and *F. heteroclitus* and as such had the highest 96-h LC50s after Ag exposure.

3.2. Effects of Ag^+ on Cl^- and Na^+ uptake in *P. promelas*

Because *P. promelas* rely on branchial uptake of Cl^- (average control uptake rate for 103 mg fish = 3000 ± 862 nmol/g/h; Bielmyer et al., 2007), we next investigated whether Cl^- uptake in these fish is sensitive to Ag^+. Chloride uptake was not significantly affected by a 2 h exposure to 10 μg/L dissolved silver in soft or moderately hard water, as compared to control values (Fig. 2). The silver concentration used in our experiments was more than twice the concentration (4.3 μg/L silver) reported to cause a significant decrease in Cl^- uptake and carbonic anhydrase activity in rainbow trout during the first two hours of exposure to silver (Morgan et al., 2004). To verify that *P. promelas* respond to Ag^+ in the same manner as has been reported for other fish species, Na^+ uptake was also measured. The average control Na^+ uptake rate (480 ± 285 nmol/g/h) was similar to other reported values (Bury et al., 1999). *P. promelas* exposed to 30 μg/L silver for 30 min had a significant decrease in Na^+ uptake (Fig. 3), in accordance with the previously characterized mechanism of silver induced Na^+/K^+ ATPase inhibition and subsequent Na^+ depletion reported for other fishes (Wood et al., 1996; Morgan et al., 1997, 2004).

3.3. Effects of acclimation on Ag^+ uptake in soft and moderately hard water

Gill ion transport physiology is sensitive to acclimation conditions (Boisen et al., 2003) and differences in ion transport physiology likely translate to differential sensitivity to silver

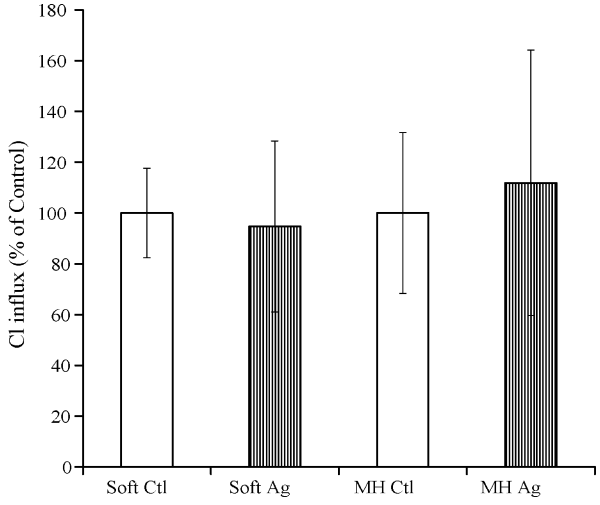

Fig. 2. Chloride influx (as percent of control) in *Pimephales promelas* exposed to silver (Ag) in soft or moderately hard water (MH). Error bars represent 95% confidence intervals.

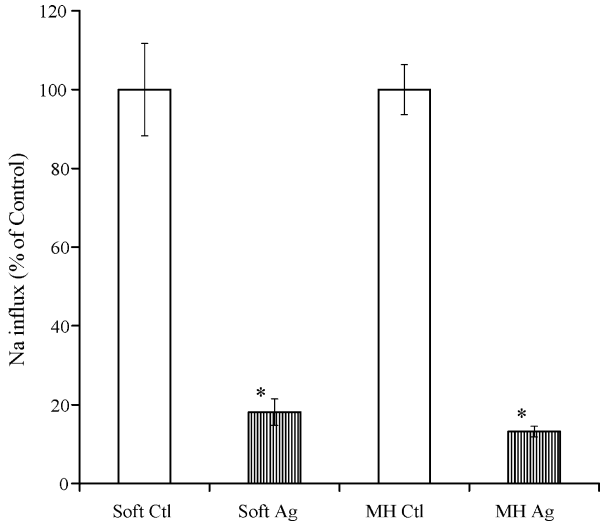

Fig. 3. Sodium influx in *Pimephales promelas* (as percent of control) exposed to silver (Ag) in soft or moderately hard water (MH). Error bars represent standard error. Asterisks denotes significant difference from corresponding control ($P < 0.05$).

(and other metals), therefore, silver binding affinity at the gills of fish previously acclimated to either soft or moderately hard water was assessed. Results demonstrated that fish previously acclimated to soft water accumulated silver at a higher rate than those previously acclimated to moderately hard water (Fig. 4) even when exposed under identical conditions. Bury et al. (1999) reported silver accumulation of 329 ± 71 μg/kg on the gills of *P. promelas* exposed to 8 μg/L Ag^+, for 4 h in soft water, after previous acclimation to moderately hard water. Similarly, after 10 μg/L Ag^+ exposure for 3 h, 245 μg/kg silver accumulated on the gills of *P. promelas*, previously acclimated to moderately hard water in this study. Due to the differences in silver binding affinity and rate of silver accumulation at the gill of fish acclimated to different water types, all flux experiments were conducted in both soft and moderately hard water.

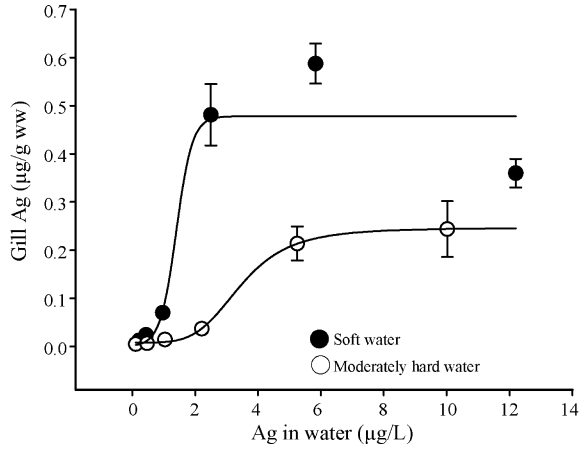

Fig. 4. Silver (Ag) accumulation on the gill (wet weight; ww) of *Pimephales promelas* (previously acclimated to soft water or moderately hard water) during 1-h Ag exposure in soft water. Error bars represent standard error.

3.4. Investigation of Cl^- uptake pathways in *P. promelas*

The inhibition of Cl^- uptake was first investigated by individually blocking the NKCC co-transporter, the Cl^-/HCO_3^- exchanger, and carbonic anhydrase. The concentration of each pharmacological agent, intended to inhibit different Cl^- uptake pathways in *P. promelas* was high and at or above concentrations reported in other experiments to inhibit Cl^- transport (Boisen et al., 2003). Chloride uptake is reported as percent of control in all the figures to enable comparisons. The average control group Cl^- influx values and standard errors for all experiments were 2519 ± 471.3 and 6398 ± 1065 nmol/g/h, in soft water and moderately hard water, respectively. Unlike the carbonic anhydrase inhibitor, ethoxzolamide, which caused no statistically significant effect on Cl^- uptake in soft or moderately hard water (Fig. 5A), both the anion exchange inhibitor (Fig. 5B) and the NKCC inhibitor (Fig. 6A) showed a trend (although not statistically significant) of reducing Cl^- uptake. This is in contrast to rainbow trout experiments showing substantial Cl^- inhibition following exposure to an anion exchange inhibitor solely (Perry et al., 1981). Sodium influx in *P. promelas* was unaffected after exposure to bumetanide (Fig. 6B) which is not surprising given

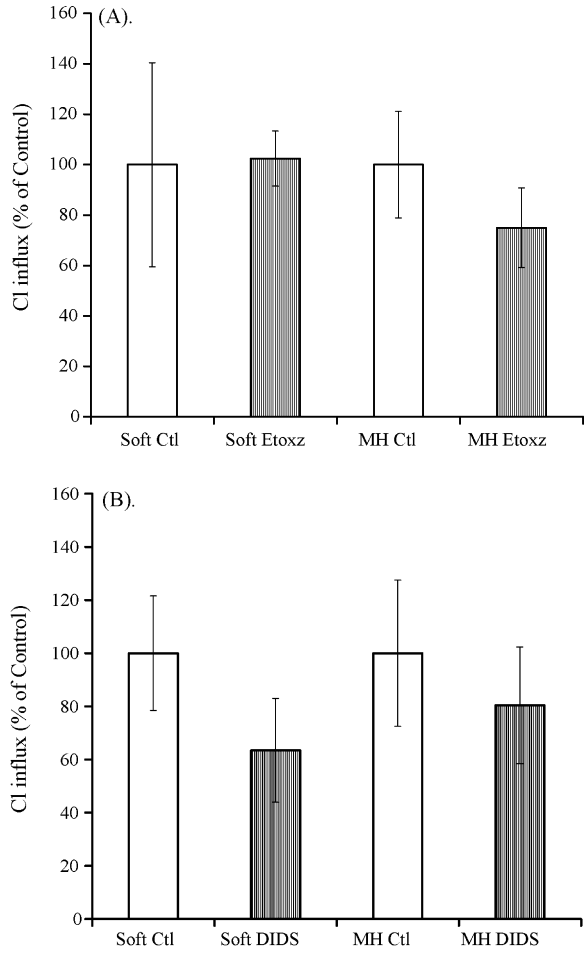

Fig. 5. Chloride influx (as percent of control) in *Pimephales promelas* after (A) exposure to ethoxzolamide (Etoxz) and (B) exposure to DIDS, in both soft and moderately hard (MH) water. Error bars represent standard error.

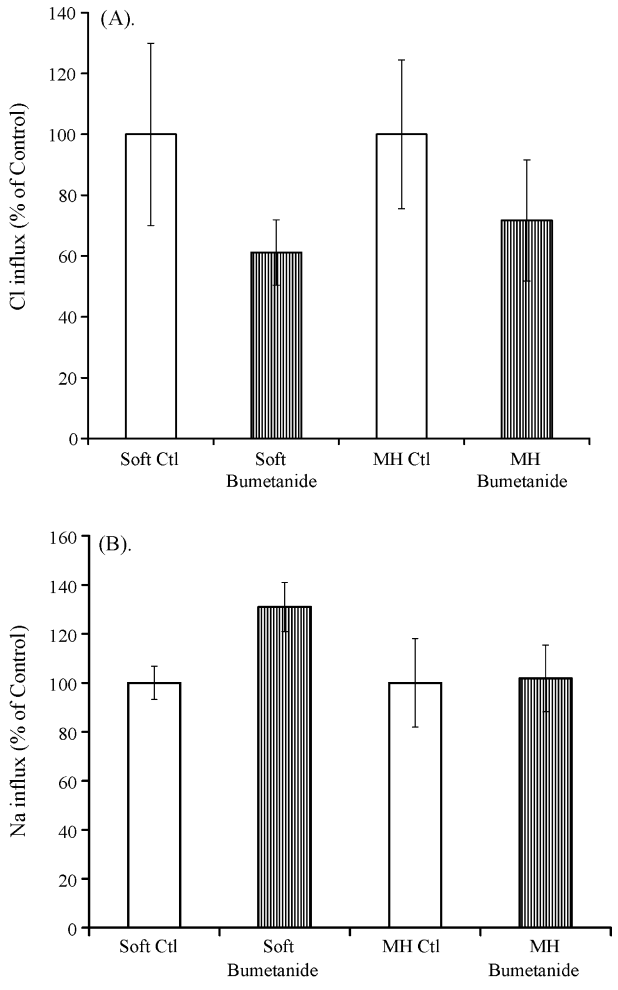

Fig. 6. (A) Chloride influx (as percent of control) and (B) sodium influx (as percent of control) in *Pimephales promelas* after exposure to Bumetanide in both soft and moderately hard (MH) water. Error bars represent standard error.

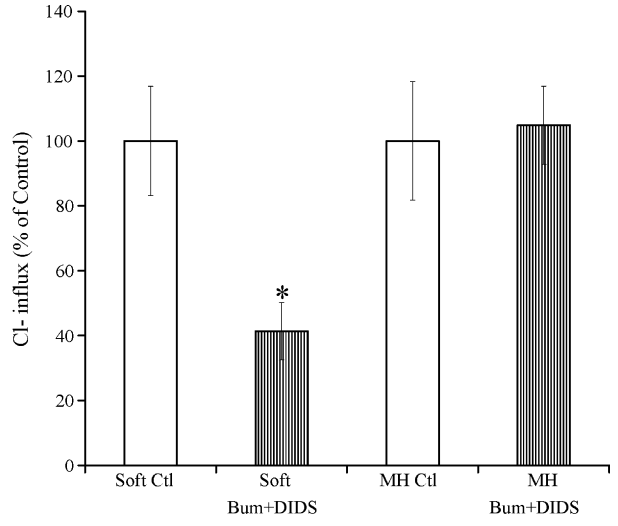

Fig. 7. Chloride influx (as percent of control) in *Pimephales promelas* after combined exposure to the pharmacological agents, Bumetanide (Bum) and DIDS, in both soft and moderately hard (MH) water. Error bars represent standard error. Asterisk denotes significant difference from corresponding control ($P < 0.05$).

the stoichiometry of this transporter (2 Cl^- ions transported for every Na^+ ion). Sodium is likely taken up by other pathways.

Due to the observed trend in Cl^- reduction after blocking individual Cl^- uptake pathways, we conducted an additional experiment where the pharmacological agents, DIDS and bumetanide were both employed simultaneously. In soft water, a statistically significant decrease in Cl^- uptake was observed after both the anion exchanger and the NKCC co-transporter were blocked (Fig. 7). Contrary to the observations in soft water, no statistically significant difference was detected in moderately hard water indicating that additional Cl^- uptake pathways may exist under such conditions. Although no statistical differences were detected, there appeared to be a slight reduction in Cl^- uptake from moderately hard water in *P. promelas,* after exposure to ethoxzolamide (Fig. 5A).

4. Conclusions and implications

Clearly, Cl^- protection is not simply due to water chemistry and silver speciation. Of the fishes tested to date, only *O. mykiss* conclusively displays Cl^--induced protection against Ag^+ toxicity and at least four other fish species do not get protection from ambient Cl^- alone, at the concentrations tested (Bury et al., 1999; Karen et al., 1999; Grosell et al., 2000—summarized in Table 1).

We hypothesized that varying Cl^- requirements and/or mechanisms of Cl^- uptake at the gill might explain this discrepancy between fish species. The lack of Cl^- protection against Ag^+ toxicity in *F. heteroclitus* is consistent with lack of Cl^- uptake in these fish. It is important to realize that lack of Cl^- uptake at the gill likely also explains the lack of protection from Cl^- for the European eel (Grosell et al., 2000) and that multiple other freshwater fish species lack gill Cl^- uptake (Tomasso and Grosell, 2005). Additionally, *P. promelas* in these studies did not benefit from Cl^- protection and their Cl^- uptake pathways differ from those of *O. mykiss* and apparently are not inhibited by silver exposure. *P. promelas* are dependent on multiple Cl^- uptake pathways, including the NKCC co-transporter and the anion exchanger, independent of carbonic anhydrase, in soft water. However, the primary mechanism for Cl^- uptake in moderately hard water is still unknown.

Unlike *A. anguilla*, *F. heteroclitus* and *P. promelas*, *D. rerio* relies heavily on carbonic anhydrase and Cl^-/HCO_3^- exchange like *O. mykiss* but was not protected against Ag^+ toxicity by Cl^-, again perhaps because Cl^- uptake and possibly carbonic anhydrase in this species is insensitive to silver. Protective effects of external Cl^- against Ag^+ toxicity in fishes so far appear to be unique to *O. mykiss* and further research is needed to characterize the mechanism of Cl^- protection. The results of this study demonstrate that *Fundulus heteroclitus* without requirements for branchial Cl^- uptake and *Pimephales promelas* in which branchial Cl^- uptake is not influenced by silver exposure achieve no protection against silver toxicity from elevated ambient Cl^-. It is unknown why *Danio rerio* is not protected by elevated Cl^- but lack of sensitivity of branchial Cl^- uptake to silver exposure, as in *P. promelas*, seems a likely explanation. It should be acknowledged that different Cl^- uptake pathways

and requirements perhaps are not the only explanations for the observed differences among species. For example, it is puzzling that the enzyme Na^+/K^+-ATPase apparently receives protection from ambient Cl^- against silver inhibition (McGeer et al., 2000), an observation which may be explained by differences in cellular silver speciation.

Currently the BLM includes Cl^- protection largely based on toxicity data from *O. mykiss*. Given the lack of Cl^- protection against silver toxicity demonstrated for other fish species, Cl^- protection should only be applied on a species-specific basis rather than to all species used in deriving BLM-based AWQC.

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