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VALIDATION STUDY OF THE ACUTE BIOTIC LIGAND MODEL FOR SILVER

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Abstract—An important final step in development of an acute biotic ligand model for silver is to validate predictive capabilities of the biotic ligand model developed for fish and invertebrates. To accomplish this, eight natural waters, collected from across North America, were characterized with respect to ionic composition, pH, dissolved organic carbon, and sulfide. Tests were conducted with the cladoceran *Ceriodaphnia dubia* (48-h static) and the fish *Pimephales promelas* (96-h static renewal) to determine the concentrations causing lethality to 50% of the organisms (LC50s) for silver in each of these waters. Overall, the biotic ligand model adequately predicted silver toxicity to *C. dubia*; however, in some cases, predicted LC50 values exceeded measured values. The accuracy of the biotic ligand model predictions was less convincing for silver toxicity to *P. promelas* with pronounced problems in low–ionic strength waters. Another issue was the use of acclimated organisms in toxicity studies because the biotic ligand model has been developed with the use of a mix of studies with acclimated and nonacclimated test organisms of varying ages and sizes. To evaluate whether effects of acclimation to test waters influence biotic ligand model predictions, a subset of the natural waters were also tested with *P. promelas* that had been acclimated to the natural water for 7 d before testing. These experiments revealed no differences in toxicity between acclimated and nonacclimated *P. promelas*. To determine the influence of organism size, which has been previously correlated to Na^+ turnover and acute silver toxicity across multiple species, Na^+ and Cl^- influx rates were measured in *P. promelas* of different sizes. Our results show that Na^+ and Cl^- influx rates were inversely related to fish mass and positively correlated with silver sensitivity.

Keywords—Biotic ligand model *Pimephales promelas* *Ceriodaphnia* Water chemistry Silver

INTRODUCTION

It is well known that ionic silver (Ag^+) toxicity varies with water chemistry [1–4] and that silver bioavailability is controlled by ligand interactions and competing ions [2,5,6]. The Ag^+ ion is presumed to be the most bioavailable and toxic form of silver in aquatic environments [7]. Some ligands, such as dissolved organic carbon (DOC), chloride, sulfide, or thiosulfate, can form complexes with Ag^+, rendering it generally less available to an organism [6,8–10], whereas ions such as sodium and hydrogen can compete with Ag^+ for potential uptake by the gill, the primary target for most waterborne metals in freshwater [5,11–13]. Silver toxicity occurs in freshwater fishes as a result of Ag^+ disabling the enzymes Na^+/K^+ adenosinetriphosphatase (ATPase) and carbonic anhydrase at the gill, leading to a net loss of Na^+ and Cl^- [13–15].

The biotic ligand model (BLM) was created to help predict the toxicity of metals for waters with defined chemistries. The model has worked reasonably well thus far at predicting the acute toxicity of copper to some organisms such as *Pimephales promelas* and *Ceriodaphnia dubia*. Over the past 10 years, an extensive research program has been undertaken to develop an acute BLM for silver [16,17]. An important final step of this research program was to conduct a validation study to assess the predictive capabilities of the BLM developed for fish and invertebrates. Our first goal was to characterize the toxicity of silver to *P. promelas*, a sensitive fish, and *C. dubia*, a sensitive invertebrate, in waters with varying chemistry and to evaluate the effectiveness of the BLM in predicting acute toxicity values to these organisms by comparing measured toxicity values with those estimated by the BLM.

Past studies have demonstrated that in low–ionic strength waters, the copper BLM underpredicted toxicity to *P. promelas* and varied more than a factor of two from measured toxicity values [18]. Therefore, in this study, several site waters with relatively low ionic strength were collected to determine the effectiveness of the silver BLM at predicting toxicity values.

A potential confounding factor in using the BLM is the issue of acclimating test organisms to ambient water quality conditions. Frequently, organisms less than 24 h old are used for testing because they are generally considered more sensitive to toxicants. However, when water quality parameters (e.g., pH, chloride) in site waters depart significantly from the conditions under which the test organisms were cultured, testing with these early life stages precludes allowing organisms to acclimate to the new conditions. This, in part, could explain discrepancies between measured toxicity values and those predicted by the BLM. The issue of acclimation is potentially important because changes in organism ionoregulatory performance that can result from a change in ambient conditions often take days or even weeks [19,20]. Although this fact is well recognized, studies used to develop the BLM and to derive water quality criteria (WQC) include a mix of experimental conditions and the use of both acclimated and unacclimated organisms. As such, our second objective was to evaluate the importance of acclimation to testing waters. To meet this objective, we compared toxicity tests performed on *P. promelas* acclimated to the test water for 7 d before toxicity testing to tests performed on 7-d-old *P. promelas* transferred directly to the test water at the onset of testing.

Another confounding factor in the BLM is the influence of

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Table 1. Composition of test waters from eight different sites and dechlorinated reference waters from the University of Miami (Florida, USA).[a] Asterisks represent filtered (60-μm) samples. Water chemistry parameters measured include pH, hardness (Hard), ion concentrations, dissolved organic carbon (DOC), and reactive sulfide (CRS). ND = nondetectable

Water	pH	Hard (mg/L)	Total CO_2 (μM)	Cl^- (μM)	NO_3^- (μM)	SO_4^{2+} (μM)	Na^+ (μM)	Ca^{2+} (μM)	Mg^{2+} (μM)	K^+ (μM)	DOC (μM)	CRS (nM)
DES	8.10	225	2,050	4,358	1,067	625	4,473	1,485	761	294	472	49
dRef	6.17	6	40	173	33	14	123	40	23	6	202	6.0
EG*	8.04	197	3,940	796	ND	52	752	1,719	250	8	960	1.2
GCS	8.14	131	1,300	210	ND	520	257	682	628	8	148	3.0
HT	7.75	28	320	138	56	50	184	166	117	7	321	1.5
MC*	7.84	129	2,950	105	ND	24	520	600	693	11	362	2.0
Ref[b]	7.74	76	888	1,352	66	121	1,076	512	251	105	370	<1.0
Ref[c]	8.07	63	730	1,189	59	130	928	451	175	100	379	<0.7
RW*	7.20	39	500	594	22	83	456	284	109	6	513	5.0
SLB[b]	7.79	85	1,400	187	ND	78	208	508	338	32	1,037	4.0
SLB[c]	7.82	80	1,340	204	7	94	226	499	298	44	1,000	27
TL	6.84	12	100	183	4	48	138	79	35	27	418	1.0

[a] In alphabetical order: DES = Desjardin Canal, ON, Canada; dRef = diluted (10%) reference; EG = northern Everglades National Park, FL, USA; GCS = Green Cove Springs, FL, USA; HT = Horsetooth (Cache la Poudre River) Reservoir, CO, USA; MC = Miller Creek, Seattle, WA, USA; Ref = reference; RW = Ringwood River, Passaic County, NJ, USA; SLB = unnamed canal, upper St. Louis Bay, MN, USA; TL = Trout Lake, ON, Canada.

[b] The waters were used for *Pimephales promelas* only.

[c] The waters were used for *Ceriodaphnia dubia* only.

age and size of freshwater organisms on silver toxicity, with smaller invertebrate species being the most sensitive [21]. This has traditionally been dealt with by BLM calibration (i.e., fitting the model to experimental values [17]), which generally yields successful model predictions. The underlying assumption for model calibration in this case is that the mode of action of the metal in question is similar among vertebrates and invertebrates, which appears to have some experimental support [22]. Sodium turnover rate, which correlates with body mass, was recently proposed to be important for determining sensitivity to acute silver exposure [21,23]. Consequently our third objective was to measure Na^+ and Cl^- turnover rates and acute silver sensitivity at various life stages of *P. promelas* ranging from 48 h posthatch to fully adult fish.

MATERIALS AND METHODS

Collection of natural waters

At each site, surface waters (<60 cm depth) were collected in plastic (low-density polyethylene) cubitainers, packed in ice, and shipped overnight to the University of Miami (Florida, USA), where the samples were stored at 4°C until use. The selected site waters spanned a wide range of DOC, chloride, hydrogen ion, and sulfide concentrations. Water samples were collected at the following locations: Desjardin Canal (Ontario, Canada); northern Everglades National Park (Florida, USA); Green Cove Springs (Florida, USA); Horsetooth (Cache la Poudre River) Reservoir (Colorado, USA); Miller Creek (Seattle, WA, USA); an unnamed canal, Ringwood River, Passaic County (New Jersey, USA); upper St. Louis Bay (Minnesota, USA); and Trout Lake (Ontario, Canada).

Toxicity testing with P. promelas

Pimephales promelas (<24 h old) were obtained from Aquatic Biosystems (Fort Collins, CO, USA) and held in University of Miami dechlorinated tap water for 7 d before testing. Natural waters visibly containing suspended solids were filtered through a mesh (60 μm) before use and are indicated in Table 1. Each natural water, as well as reference water (University of Miami dechlorinated tap water), was spiked with varying concentrations of $AgNO_3$ (reagent grade, Assurance, Spex, Certiprep, Metuchen, NJ, USA) and equilibrated for 24 h before testing. Ten fish (7 d old) were added to each replicate 1,000-ml plastic beaker containing 500 ml of testing solution for 96 h at a temperature of 26°C [24]. Each treatment was replicated three times, with six to seven treatments per test. *Pimephales promelas* were fed *Artemia* nauplii before solution renewal at 48 h. Samples for total and dissolved (that which passes through a 0.45-μm filter) silver analysis were collected at 0, 48, and 96 h and were acidified immediately. Samples were analyzed immediately after each test. Mortality was assessed at 48 and 96 h. Prior acclimation to testing waters before use in toxicity tests might affect the results of the tests. As such, three experiments were conducted in which *P. promelas* were acclimated to the test water (Horsetooth, Trout Lake, diluted reference water) for 7 d before testing. The same methods (as described above) were used in testing. Because the effect of acclimation to test water is expected to be greatest for water with extreme chemical composition, particularly low–ionic strength waters, the three test sites with the most divergent water chemistry and lowest ionic strength were selected for these studies.

Additional toxicity testing was conducted with *P. promelas* of different ages (1, 4, 7, 27, and 41 d old) in dechlorinated tap water by the same methods as described above.

Toxicity testing with C. dubia

Ceriodaphnia dubia were obtained from an in-house culture originally from Aquatic Biosystems (Fort Collins, CO, USA) and maintained in U.S. Environmental Protection Agency (U.S. EPA) moderately hard water (80–100 mg $CaCO_3$) at 26°C [24]. Natural water solutions were spiked with varying concentrations of $AgNO_3$ and equilibrated for 24 h. Five *C. dubia* (<24 h old) were added to each replicate 30-ml plastic cup containing 20 ml of testing solution for a 48-h exposure following standard methods [24]. Each treatment was replicated three times. Samples for total and dissolved silver analysis were collected at 0 and 48 h and acidified immediately. Samples were analyzed immediately after each test. Mortality, as determined by nonresponsiveness under gentle prodding, was assessed at 48 h.

Na^+ and Cl^- uptake experiments with P. promelas

Pimephales promelas (10 fish/beaker) were placed in either 400-ml beakers filled with 200 ml of dechlorinated tap water (42- and 150-d-old fish) or 10-ml beakers filled with 5 ml of dechlorinated tap water (1-, 4-, and 7-d-old fish). The fish were allowed at least 30 min to recover from being handled, after which 2 μCi of ^{22}Na or ^{36}Cl (Amersham, Princeton, NJ, USA) was added to the beakers. Water samples were taken at the start (~5 min after isotope addition) and at the end of the exposure. Flux times were 3 h for the 42- and 150-d-old fish and 12 min for the 1-, 4-, and 7-d-old fish, with continuous aeration in all beakers. The short flux time for the smaller fish was necessary because of their higher ion turnover rates, which would have resulted in significant isotope backflux at longer exposure times [21]. At the end of the flux time, the fish were rinsed twice with 100 mM NaCl to displace any loosely bound radioisotope, euthanatized by overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA), blotted dry, and weighed to the nearest 10 μg with a Mettler Toledo AX105 analytical balance (Columbus, OH, USA). Weights of the 42- and 150-d-old fish were recorded individually. Weights of the 1-, 4-, and 7-d-old fish were recorded as pooled values (of 10 fish each). Radioactivities of the fish and water samples were measured on a gamma counter (Tm Analytic, Elk Grove Village, IL, USA) for ^{22}Na and a beta counter (Tm Analytic) for ^{36}Cl. In addition, water samples were measured for Na and Cl content by flame atomic absorption spectrophotometry ([AAS]; Varian 220FS, Mulgrave, VIC, Australia) or ion chromatography (DX-120; Dionex, Sunnyvale, CA, USA), respectively.

Water chemistry

All samples were analyzed for silver with the use of graphite furnace AAS (detection limit = 1.0 μg/L). For silver concentrations less than 1 μg/L, a series of multiple injections were used to concentrate analyte mass and achieve a detection limit of 0.25 μg/L. Samples were analyzed in duplicate with appropriate quality control methods. Each water was characterized for ions via flame AAS (Na^+, K^+, Mg^{2+}, Ca^{2+}) or ion chromatography (DX-120; Cl^-, SO_4^{2-}), pH with a combination glass electrode (pHC3005-8 Radiometer, Villeurbanne Cedex, France) coupled to a meter (PHM220 MeterLab™, Radiometer, Copenhagen, Denmark), reactive sulfides (CRS) via column chromatography [25], total CO_2 via a total CO_2 analyzer (965 Corning Limited, Halstead, Essex, UK), and DOC as described previously [26].

Data analyses

Trimmed Spearman Karber analysis was used to estimate 48- and 96-h concentrations causing lethality to 50% of the organisms (LC50s). Water effect ratios (WERs) were calculated by dividing the LC50 value of the site water by the LC50 value of the University of Miami reference water (dechlorinated tap water) [27]. Traditionally, site water LC50 values are normalized for hardness to the laboratory water LC50 value. However, at least for rainbow trout, silver toxicity is predominantly controlled by chloride concentration and not water hardness [2,8]. Therefore, hardness corrections were not made in our WER calculations and they were only used for comparison to the reference laboratory water to assess the relative change in toxicity. Water chemistry parameters for individual waters were entered into the BLM (Ver. 2.1.2, Mahwah, NJ, USA) where humic acid was assumed to be 10%. All other parameters used in the model were measured values. The appropriate organism was selected (*P. promelas* or *C. dubia*), and predicted LC50 values were generated. All silver measurements are reported as the dissolved silver fraction.

Table 2. *Ceriodaphnia dubia* toxicity values (μg/L dissolved silver), 95% confidence intervals (CI), and water effect ratios (WER).[a] Refer to Table 1 for water locations

Water	LC50	95% CI	WER
Desjardin Canal	3.24	1.98–5.28	4.26
Everglades	1.45	1.19–1.77	1.91
Green Cove Springs	0.34	0.19–0.61	0.45
Horsetooth Reservoir	1.28	0.63–2.60	1.68
Miller Creek	4.24	3.36–5.34	5.58
Reference	0.76	0.53–1.09	NA
Ringwood	1.15	0.58–2.31	1.51
St. Louis Bay	9.52	7.7–11.77	12.5
Trout Lake	1.69	1.30–2.20	2.22

[a] LC50 = lethal concentration for 50% of the organisms; NA = not applicable.

Whole-body influx rates of Na^+ and Cl^- were calculated from the appearance of radioactivity in the tissues as follows: whole body influx = $(q/\text{wt})/t/\text{SA}$, where q represents the count per minute (cpm) for whole body, t is flux time (h), wt is fish wet weight (g), and SA is the specific radioactivity of the water. The $\text{SA} = \{(\text{cpm}_i/[\text{ion}]_i) + (\text{cpm}_f/[\text{ion}]_f)\}/2$, where cpm_i represents the initial count in the water (cpm/ml), cpm_f represents the final count in the water (cpm/ml), and $[\text{ion}]_i$ and $[\text{ion}]_f$ represent the initial and final Na and Cl concentration of the water, respectively.

RESULTS AND DISCUSSION

A relatively diverse range of waters were tested with pH ranging from 6.17 to 8.28, hardness from 11 to 197 mg $CaCO_3$/L, Cl^- from 105 to 4,358 μM, and Na^+ from 123 to 2,747 μM (Table 1). Reactive sulfide values ranged from 0.7 to 49 nM, and DOC values ranged from 148 to 1,037 μM. Most of the waters used to calibrate the BLM had hardness >50 mg/L $CaCO_3$ and generally higher ionic strength than many of the waters used in this study. *Ceriodaphnia dubia* were more sensitive to silver than *P. promelas*, and toxicity was dependent on the water composition. These results agree with previous studies reporting that *Daphnia magna* were more sensitive to silver than *P. promelas* [1,9]. In general, both *C. dubia* and *P. promelas* were less sensitive (higher LC50s) in waters with a combination of higher hardness and DOC, with a few exceptions for *C. dubia* (Tables 2 and 3). For two of the waters tested (Everglades and Ringwood), it appeared that different parameters or interactions between parameters were controlling the bioavailability and toxicity of silver for the two species, as shown by the differences in WERs (Tables 2 and 3). For example, the WER for the Everglades water was 13.1 for *P. promelas* and only 1.91 for *C. dubia*. No single factor effectively predicted the toxicity of Ag^+ from the data generated in this study.

The measured 48-h LC50 values for *C. dubia* exposed to silver and the 48-h LC50 values predicted by the BLM correlated fairly well (Fig. 1A) and were within the range of values used to calibrate the model. However, for three outliers (Horsetooth, Trout Lake, St. Louis Bay), the model was conservative and overpredicted toxicity. Horsetooth River and Trout Lake had the lowest ionic strength and hardness values of all the

Table 3. *Pimephales promelas* toxicity values (μg/L dissolved silver), 95% confidence intervals (CI), and water effect ratios (WER).[a] Refer to Table 1 for water locations

Water	LC50	95% CI	WER
Desjardin Canal	20.9	19.2–22.7	6.20
Diluted reference	1.99	1.7–2.34	0.59
Diluted reference (Acc)	2.18	1.69–2.81	0.65
Everglades	44	42.5–45.5	13.1
Green Cove Springs	6.02	3.63–10.0	1.79
Horsetooth (Acc)	2.44	1.21–4.89	0.72
Horsetooth Reservoir	5.23	4.40–6.23	1.55
Miller Creek	16	12.6–20.4	4.75
Reference	3.37	3.02–3.75	NA
Ringwood	20.2	18.3–21.9	5.99
St. Louis Bay	44.1	UC	13.1
Trout Lake	2.49	1.88–3.30	0.74
Trout Lake (Acc)	2.96	2.41–3.64	0.88

[a] Acc = fish acclimated to testing waters before testing; UC = the value was unable to be calculated; LC50 = lethal concentration for 50% of the organisms.

site waters tested. The CRS concentration in the St. Louis Bay water sample might have been underestimated by the BLM for both *C. dubia* and *P. promelas* because the sample was stored for three months before analysis.

The 96-h LC50 values for *P. promelas* exposed to silver correlated fairly well with predicted values in higher ionic strength waters, but for waters with lower ionic strength in particular, the BLM tended to underpredict toxicity (Fig. 1B). Van Genderen and colleagues [18] reported similar findings with copper, indicating that factors unaccounted for in the current BLM might be important in predicting toxicity in low–ionic strength waters. One such factor concurrently explored in our laboratory is the possibility of changes in binding affinity of Ag^+ for the gill in waters of different ionic strength.

In the acclimation studies, we expected the acclimated *P. promelas* to have higher LC50 values (less susceptible to silver) than nonacclimated organisms, as has been previously demonstrated for copper and *C. dubia* [28]. However, no differences in silver toxicity were detected between *P. promelas*, which were acclimated to the test waters, and those held in laboratory water before testing (Table 3). The acclimation studies were conducted with lower ionic strength waters, and the composition of these waters might not have been optimal for *P. promelas* or 7 d might have not been a long enough time for full acclimation. Alternatively, a more likely explanation is that *P. promelas* acclimated to lower ionic strength water had a more efficient uptake of Na^+ and Cl^-, thereby enhancing Ag^+ uptake and accumulation at the gill. Recent results demonstrated that *P. promelas* previously acclimated to soft water accumulated twice the amount of branchial silver and at a higher rate than those previously acclimated to moderately hard water, even when exposed under identical conditions in soft water (Bielmyer et al., unpublished data).

Silver toxicity also varied with the age and size of *P. promelas* (Table 4). As fish aged and mass increased, the 96-h LC50 values increased (organism sensitivity decreased). This observation is consistent with the findings of other researchers [21,23]. Furthermore, Na^+ and Cl^- uptake rates also decreased with increasing mass of *P. promelas* (Fig. 2A and B), resulting in a strong correlation between turnover of both Na^+ and Cl^- and toxicity. Grosell and colleagues [21] reported higher Na^+ turnover in smaller animals compared with larger animals in freshwater. Furthermore, they demonstrated that smaller ani-

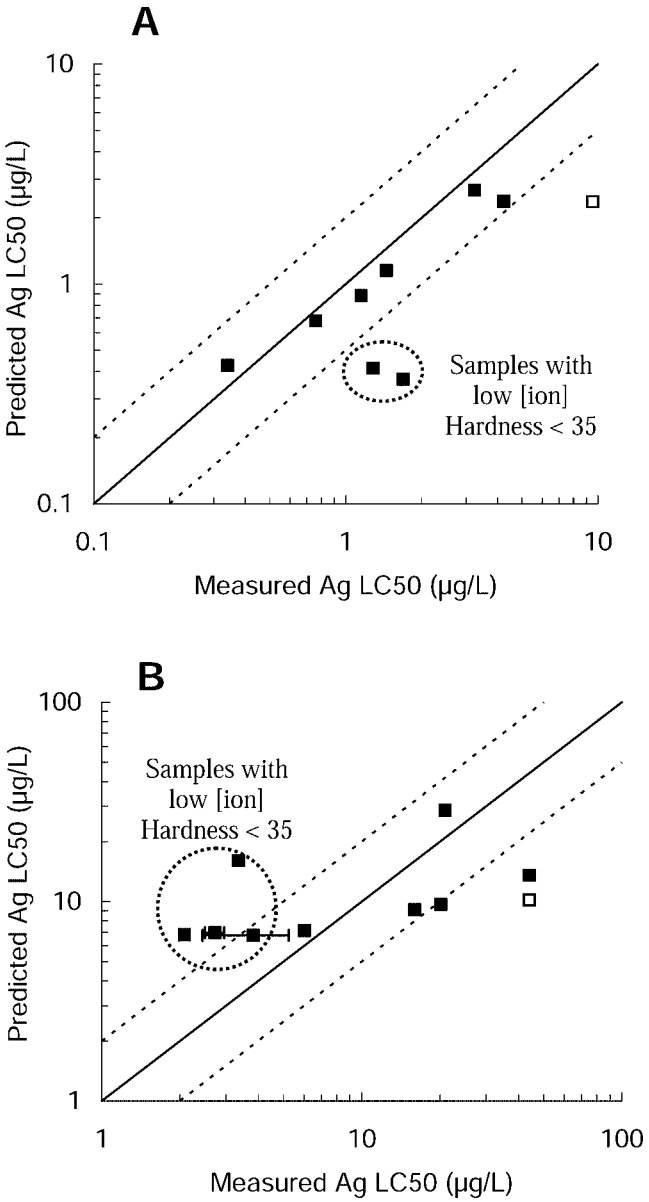

Fig. 1. The solid boxes represent concentrations causing lethality to 50% of the organisms (LC50s; analytically measured, or predicted by the biotic ligand model) for *Ceriodaphnia dubia* (**A**) or *Pimephales promelas* (**B**) exposed to dissolved silver. (×) Error bars represent a range of values from acclimation experiments. Open boxes represent waters, possibly with artificially low reactive sulfide values. Points within the circle indicate water samples with low ion concentration (hardness < 35 mg $CaCO_3$/L). Dashed lines represent a factor of two from the measured values.

Table 4. Ninety-six–hour concentrations causing lethality to 50% of the organisms (LC50s) and confidence intervals (CI) for *Pimephales promelas* exposed to Ag^+ at different ages

Fish age (d)	Fish weight (mg)	96-h LC50 (μg/L dissolved Ag)	95% CI
1	0.16	1.20	0.72–2.07
4	0.50	1.20	0.74–1.84
7	0.86	3.37	3.02–3.75
27	192	5.90	4.99–7.18
41	880	10.4	8.66–12.6

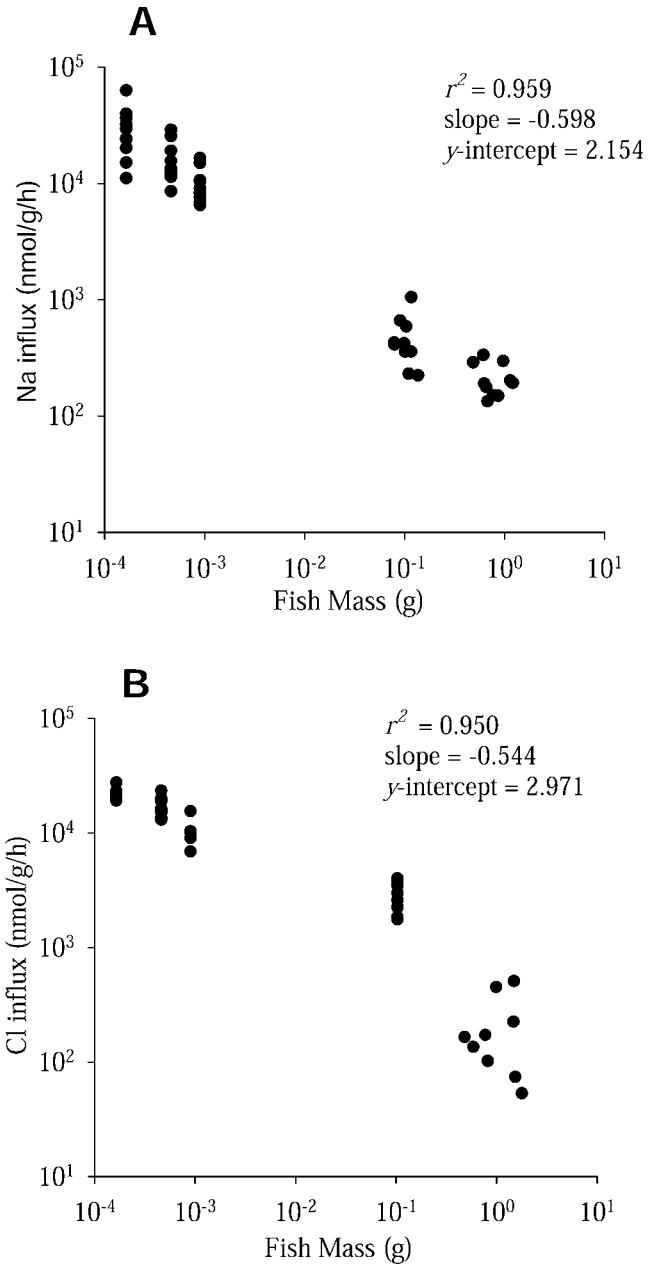

Fig. 2. Na^+ (**A**) and Cl^- (**B**) influx in *Pimephales promelas* of different sizes.

mals therefore are likely more sensitive to silver toxicity because inhibition of Na^+ uptake results in faster depletion of internal Na^+ than in larger animals [21]. Considering our measurements for Cl^- turnover in *P. promelas* of different sizes, a similar argument could be made for Cl^- because silver exposure inhibits Cl^- uptake at least in rainbow trout [15]. The higher silver sensitivity of *C. dubia* compared with *P. promelas* in our study agrees with this theory. The ion influx measurements provide a physiological explanation for differences in sensitivity that have been observed in results in which different age, and thereby size, of fish have been tested as well as a plausible rationale for adjusting the BLM to account for fish size/age.

CONCLUSION

The BLM made reasonable (within a factor of two of measured values) predictions of silver toxicity to *C. dubia* with three exceptions, including the two waters with the lowest ionic strength and hardness <35 mg $CaCO_3$/L. For these waters, the model overpredicted silver toxicity. The BLM predictions were less accurate (50% within a factor of two measured values) for *P. promelas*. The BLM underpredicted silver toxicity in low–ionic strength waters to *P. promelas*, in contrast to the overpredictions for *C. dubia*. Differences in silver toxicity were not detected between *P. promelas* acclimated to test waters for 7 d before testing and those that were not acclimated, suggesting that poor BLM predictability in low–ionic strength waters is not an acclimation issue. Physiological differences, such as Ag^+ binding affinity to the gill, might exist between the organisms, especially in low–ionic strength waters. Additionally, decreasing silver toxicity as a function of increasing size is likely due to rates of Na^+ and Cl^- turnover in these organisms. These physiological explanations could be useful in predicting silver toxicity and in further refining the BLM.

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