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COMPARISON OF SHORT-TERM CHRONIC AND CHRONIC SILVER TOXICITY TO FATHEAD MINNOWS IN UNAMENDED AND SODIUM CHLORIDE–AMENDED WATERS

RAMI B. NADDY,*† ANITA B. REHNER,† GINA R. MCNERNEY,† JOSEPH W. GORSUCH,‡ JAMES R. KRAMER,§ CHRIS M. WOOD,‖ PAUL R. PAQUIN,# and WILLIAM A. STUBBLEFIELD††
†ENSR, 4303 West LaPorte Avenue, Fort Collins, Colorado 80521, USA
‡Gorsuch Environmental Management Services, Webster, New York 14580, USA
§School of Geography and Earth Sciences, McMaster University, Hamilton, Ontario L8S 4K1, Canada
‖Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada
#HydroQual, 1200 MacArthur Boulevard, Mahwah, New Jersey 07430, USA
††Parametrix, 33972 Texas Street South West, Albany, Oregon 97321, USA

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Abstract—The chronic (early life stage [ELS]) and short-term chronic (STC) toxicity of silver (as silver nitrate) to fathead minnows (FHM) was determined concurrently in flow-through exposures (33 volume additions/d). Paired ELS (~30 d) and STC (7 d) studies were conducted with and without the addition of 60 mg/L Cl (as NaCl). The paired studies in unamended water were later repeated using standard flow conditions (9 volume additions/d). The purpose of the paired studies was to determine if short-term chronic endpoints can be used to predict effects in ELS studies. For each experiment, a ''split-chamber'' design (organisms were held in a common exposure chamber) allowed the direct comparison between short-term and chronic exposures. It appeared that the chronic toxicity of silver was mitigated to some extent by NaCl addition. The maximum acceptable toxicant concentration for growth in the ELS study was 0.53 μg dissolved Ag/L under standard flow conditions. Early life stage and STC endpoints in all three studies typically agreed within a factor of two. Whole-body sodium and silver concentrations measured in individual fathead minnows during these studies showed an increase in silver body burdens and a decrease in sodium concentration. These results indicate that the STC study could be used as a surrogate test to estimate chronic toxicity and that the mechanism of chronic silver toxicity may be the same as for acute toxicity.

Keywords—*Pimephales promelas* Early life stage exposure Short-term chronic exposure Sodium chloride

INTRODUCTION

Currently, no nationally recommended chronic freshwater ambient water quality criterion exists for silver [1,2]. The assessment of the toxicity of metals in aquatic systems has been changing in recent years and now incorporates increased consideration of a number of physicochemical factors affecting the bioavailability and thus toxicity of metals. Models such as the biotic ligand model [3] provide a mechanistically based approach to assess the toxicity of metals and are currently being incorporated into criteria/standard derivation procedures in both North America and Europe [4,5]. These approaches, however, typically require increased empirical data to adequately characterize the effects of these factors on metal bioavailability and toxicity. The toxicity of silver has been under investigation over the past decade so that the water quality parameters that impact its acute and chronic toxicity in freshwaters may be better understood [6]. Many of the studies that have been conducted to date have examined the acute effects of silver toxicity under varied water parameter conditions [7–10]. While chronic studies have been conducted in the past, they have been limited to a few species, such as rainbow trout [11,12], or have focused on physiological effects to better understand the mechanisms of silver toxicity [13]. At present, there are few published studies that examined the chronic toxicity of silver to fathead minnows (FHM) [14,15]. LeBlanc et al. [15] conducted their studies using the less bioreactive silver sulfide and silver thiosulfate complexes, while Holcombe et al. [14] used silver nitrate, a more bioreactive silver compound. Vastly different effect concentrations were observed between these studies. LeBlanc et al. [15] reported maximum acceptable toxicant concentrations (MATC) >11,000 μg/L for both silver compounds (silver sulfide and silver thiosulfate), whereas Holcombe et al. [14] reported an MATC of 0.49 μg total Ag/L. Silver nitrate was used in all of our studies in order to examine the chronic toxicity of ionic silver using the most bioreactive (i.e., toxic) form of silver [9].

The objective of the present study was to determine the chronic toxicity of silver to the fathead minnow, *Pimephales promelas*, in early life stage (ELS) studies. In addition, we also wanted to evaluate whether the widely used 7-d short-term chronic (STC) study performed with a sensitive life stage was predictive of the effects observed in more complex ELS studies. To accomplish this, three separate paired ELS–STC studies were conducted. The first two determined effects in laboratory water with and without the addition of sodium chloride (target of 60 mg Cl/L). We later repeated the paired studies in unamended laboratory water under standard flow conditions (15 ml/min, ~9 volume additions/d). These studies are part of a larger testing program intent on generating data that could be used in the development of a chronic biotic ligand model for silver, which requires substantial empirical data over a range of water quality parameters. Standard ELS test methods require a prolonged duration (~32 d for FHM) and are thus

* To whom correspondence may be addressed (rnaddy@ensr.aecom.com).

time consuming and expensive to perform. If the STC test can be shown to be predictive of chronic toxicity, the use of this shorter, more economical test method would make it possible to evaluate the toxicity of silver under a greater number of water conditions (e.g., chloride, hardness). Therefore, to assess this predicted association, an STC study was conducted concurrently with each ELS study mentioned. Previously, Norberg-King [16] reported that STC studies were predictive of ELS studies within a factor of two. However, the STC method used in that research employed a static-renewal design, while the ELS was conducted using a flow-through design. Furthermore, the two tests were conducted in the same laboratory but not at the same time [14]. These differences make direct comparisons between the STC and ELS study more difficult than if they had been conducted concurrently using the same exposure regime.

In addition to examining the effects on classical endpoints (i.e., survival and growth), endpoints of whole-body silver and sodium concentrations were also measured in our studies. Previous research has shown that internal sodium levels can decrease because of exposure to ionic silver and that this decrease is associated with the mechanism of acute silver toxicity [17]. Also, extensive analytical chemistry was conducted with one paired ELS–STC study (under standard flow conditions) to determine silver speciation and demonstrate equilibrium during exposures (J.R. Kramer and R.A. Bell, McMaster University, Hamilton, ON, Canada, unpublished data). An important and confounding finding of these studies was that a significant amount of silver loss can occur during the storage of analytical samples in the low-part-per-billion range. A strategy to address the problems that compromised the accuracy of measured values in the higher-flow studies is presented in the following section.

Another objective of this research was to help generate chronic aquatic toxicity data that could be used for derivation of a freshwater chronic criterion. The toxicity studies were conducted following U.S. Environmental Protection Agency (U.S. EPA) good laboratory practices [18] with modifications noted in the following section.

METHODS

Culture conditions/control water

Culture methods for fathead minnow (*P. promelas*) at the ENSR Fort Collins Environmental Toxicology Laboratory (Fort Collins, CO, USA) in-house cultures have been previously reported [19].

The dilution/control water used in the present study can be characterized as a very soft to soft water, with both hardness and alkalinity ranging from 20 to 35 mg/L as $CaCO_3$. The culture and testing waters for FHMs in our laboratory were obtained from Horsetooth Reservoir (Fort Collins, CO, USA). The water was passed through a sand filter, cartridge (10 and 1 μm) filters, and ultraviolet light sterilization and heated to 25°C prior to use. Water for unamended studies was used without further treatment; water chemistry parameters measured during the course of these studies are summarized in Table 1. For the sodium chloride study (HT-high NaCl [higher flow conditions in Horsetooth water amended with NaCl]), a sodium chloride (American Chemical Society [ACS] reagent grade, 99.9% NaCl; Fisher Scientific, Fair Lawn, NJ, USA) stock was prepared in Milli-Q® (Millipore, Billerica, MA, USA) water and added to Horsetooth water to achieve a nominal chloride concentration of 60 mg/L (1.7 mM), a considerable elevation relative to background levels of 1.9 mg/L (0.05 mM). Similarly, the background sodium level in our laboratory water increased in the NaCl studies from 3.5 to approximately 38 mg/L.

Table 1. Water quality parameters measured in unamended Horsetooth Reservoir water (Fort Collins, CO, USA) at the Fort Collins Environmental Toxicology Laboratory (Fort Collins, CO, USA) from 2000 to 2004. Unless specified does not included data from the studies

Water quality parameter	Average	Range
Calcium (mg/L)	9.95	8.3–12.8
Magnesium (mg/L)	1.85	1.4–2.4
Sodium (mg/L)[ab]	3.5	2.7–5.5
Potassium (mg/L)[a]	<1	—
Sulfate (mg/L)	5.6	3.4–10
Chloride (mg/L)[ab]	1.9	0.5–3.6
Alkalinity (mg/L)[c]	28.8	25–33
DOC[d] (mg/L)	2.4	2.1–2.9
Chromium-reducible sulfide (nM)[a]	1.14	—

[a] Measured during the short-term chronic and early life stage studies conducted under standard flow conditions.
[b] Not including measurements taken during the NaCl study.
[c] Represents data measured during all studies.
[d] Dissolved organic carbon.

Test material

Silver nitrate (ACS reagent grade $AgNO_3$; ≥99.7%, CAS 7761-88-8) (Sigma, St. Louis, MO, USA) stock solutions were prepared in Milli-Q water for toxicity tests. The stock solution was metered into the test water as described below to deliver nominal silver concentrations specific for the individual study. The test substance and dilution water delivery rates to the diluter were checked twice daily, and adjustments were made as necessary. Nominal test concentrations are provided elsewhere in the document.

Test methods

Early life stage tests were conducted following American Society of Testing and Materials (ASTM) guidance, either Method E1241-92 [20] or Method E1241-98 [21], depending on when the studies were conducted with slight modification (i.e., chamber design). The STC studies were conducted following U.S. EPA Method 1000.0 [22,23], as appropriate, depending on when they were conducted. The split-chamber design used to conduct both the ELS and the STC study simultaneously was a modification to these procedures (see below for details). All studies were conducted following good laboratory practices [18], except for measurements of aqueous silver and fish silver and sodium analysis. Nomenclature used to refer to the three pairs of ELS and STC studies was as follows: HT (normal flow conditions in unamended Horsetooth water), HT-high (higher flow conditions in unamended Horsetooth water), and HT-high NaCl. The study type, either ELS or STC, is also included in the name to differentiate between test types.

A continuous-flow serial diluter [24] with a dilution factor of 0.5 was used for the tests. Diluter testing methods followed during these studies were similar to those previously reported [25] for brown trout. The dilution system delivered five silver concentrations, a sodium chloride control (sodium chloride studies only [HT-high NaCl]), and a dilution water control to the test chambers. The test chamber positions (four replicates per treatment) were randomly assigned within the water bath.

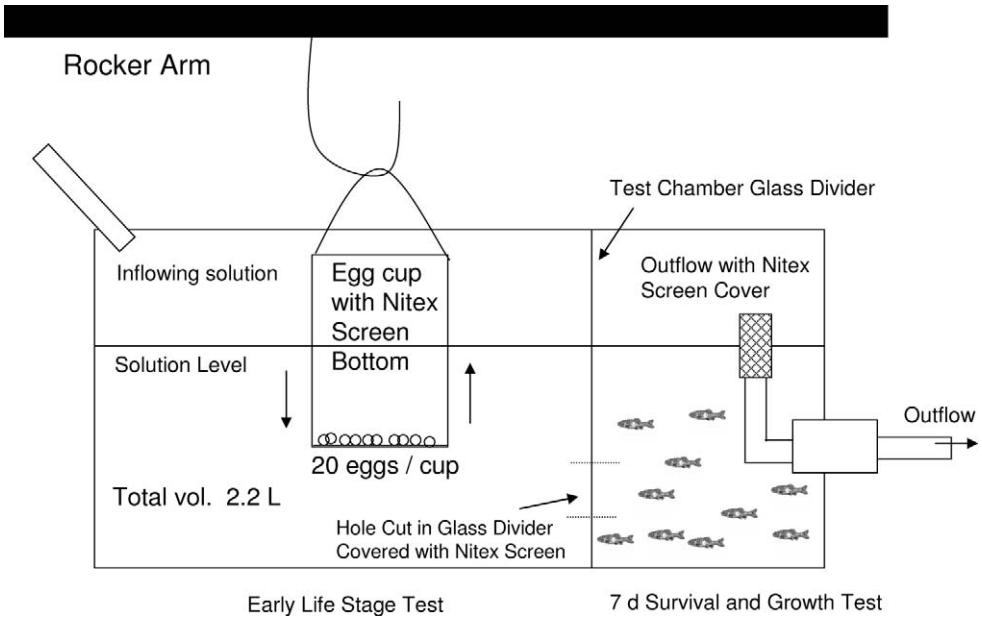

Fig. 1. Split-chamber test design for the concurrent fathead minnow (FHM) short-term chronic (7-d survival and growth) and early life stage studies.

Flow rate was adjusted to deliver a target rate of 50 ml/min (HT-high and HT-high NaCl) or 15 ml/min (HT) to each exposure chamber. Flow rates into individual chambers were measured at test initiation and test termination and at least weekly.

For ELS studies, individual spawning tiles were isolated on the days of test initiation to obtain freshly fertilized eggs. Twenty fertilized eggs were assigned to each replicate egg cup in each treatment of an individual test using a randomized block design. Egg cups hung from a rocker arm (2 rpm) apparatus installed above each of the two rows of test chambers (Fig. 1). The total volume of water in each chamber was approximately 2.2. L. Egg cups were constructed of glass tubing (approximately 4 cm outside diameter and 7 cm length) with a Nitex® (Wildlife Supply Company, Buffalo, NY, USA) screen bottom. Cups were gently oscillated in the test water until all 20 eggs had either hatched or been noted as dead. Hatched larvae were removed by pipette to the surrounding test chamber on a daily basis. The cups and rocker arm were removed after all living eggs had hatched. Feeding of hatched *P. promelas* was started at a ration of 0.5 ml (per test chamber) of a concentrated suspension (~800 nauplii/0.1 ml) of newly hatched brine shrimp (Argent Chemical Laboratories, Redmond, WA, USA) three times daily (i.e., morning, noon, evening). The volume of food was increased if little or no food was left in chambers 1 h after feeding. Food volume was halved for individual replicates if mortality exceeded 50% for the HT studies following U.S. EPA guidance [23]. This decrease in feeding rate did not occur in either of the other two studies (HT-high or HT-high NaCl) as these were conducted in 2000 and followed previous U.S. EPA [22] guidance. Approximate feeding rates by the end of the test were 2.0 to 2.5 ml of concentrated brine shrimp nauplii per feeding. Fish were not fed within 24 h of test termination.

Organisms less than 24 h old were used to initiate all STC testing. Between hatching and use in testing, larvae were fed a concentrated suspension of newly hatched brine shrimp nauplii (*Artemia* sp. [23]) ad libitum. During STC testing, *P. promelas* were fed 0.1 ml (per test chamber) of a concentrated suspension of newly hatched brine shrimp three times daily (i.e., morning, noon, evening). As with the HT-high and HT-high NaCl ELS studies, feeding rates for the HT-high and HT-high NaCl STC studies were not decreased when mortality exceeded 50% [22]. Organisms were not fed within 24 h of test termination.

Paired STC and ELS studies were conducted concurrently in the same test chambers (Fig. 1) for each study (i.e. HT, HT-high), except for the HT-high NaCl studies. A STC study was originally initiated during the ELS study; however, this study had to be repeated just after termination of the ELS study. Test chambers (2.5 L) were divided to accommodate organisms for each test (Fig. 1). Exposure vessels, constructed of plate glass and silicone adhesive, were covered with a glass plate to minimize possible contamination, evaporation, and/or loss of silver from the test chambers. Each test chamber drain consisted of a piece of 5-mm (inner diameter) glass tubing inserted through a silicone stopper that was pressed into a small hole drilled in the side of the aquarium. The test solution volume was maintained at the level of the top of the drain. Spent test solution overflowed into glass standpipes and was discharged directly to a waste conduit. During testing, the drain openings were covered with a small piece of nylon mesh to prevent test organisms from being lost through the drain. For the HT studies, each test vessel received a flow rate of 15 ml/min that resulted in approximately nine volume additions per day or approximately two turnovers (99% molecular replacement) per day. The HT-high and HT-high NaCl studies were conducted at a flow rate of 50 ml/min, which resulted in approximately 33 volume additions per day or approximately six turnovers per day [26]. Exposure containers and flow rates were sufficient to maintain a loading rate of ≤5 g of fish per liter of water in each test chamber in a 24-h period. The exposure aquaria were housed in a temperature-controlled water bath that maintained the water temperature at 25 ± 1°C. Lighting was controlled to provide a 16:8-h light:dark photoperiod; light intensity was low ambient (538 lux).

During the STC tests, live organisms were counted and recorded on a daily basis for each test chamber. During the ELS, live eggs, number of hatched larvae, partial hatches, and potential deformities were counted daily. Dead eggs or larvae were removed on a daily basis at counting or when chambers were cleaned to remove excess feces and food buildup. Any organisms that were not found during the test were considered dead. Fish were considered dead if no gill movement or visible response was observed in response to gentle prodding. For STC studies, the exposure continued for 7 d ± 1 h. Early life stage studies were terminated after 28 d posthatch of the control organisms. Test durations for the ELS studies were 32 (HT), 33 (HT-high), and 34 d (HT-high NaCl).

At test termination, all surviving fish were counted and sacrificed via immersion in isopropyl alcohol. The fish were then rinsed with deionized (Milli-Q) water and blotted dry. Fish from each replicate were transferred to a tared weighing boat and dried at 100°C for at least 96 h prior to weighing. Ten individual fish per treatment (less if limited by mortality) were sent to the McMaster University Department of Biology to be analyzed for whole-body concentrations of sodium and silver.

Control mortality was within the specified acceptability limits for the STC and ELS studies (<20% and <30%, respectively) for all experiments. Reference toxicant tests (positive control test with sodium chloride) were conducted in moderately hard water with fathead minnows using an STC study on a monthly basis. Organism responses from these STC studies were within historical control limits, which represent ±2 standard deviations from the historical laboratory mean growth endpoints (i.e., IC25 values [inhibition concentration 25%, or the concentration estimated to cause a 25% reduction in organism response compared with the control]).

Water quality

On the day of test initiation, temperature, pH, and dissolved oxygen (DO) concentrations were measured and recorded for each test chamber. These parameters were also measured and recorded for each test chamber with surviving test organisms on the day of test termination and in alternating replicates from each treatment with surviving test organisms on a daily basis during the test. Hardness, alkalinity (both as mg/L $CaCO_3$), conductivity (μS/cm), and total ammonia (mg/L as nitrogen [N]) were measured in the control and highest treatments at test initiation and in the control and the highest treatment containing surviving organisms at test end. Calcium, magnesium, potassium, sodium, chloride, and sulfate concentrations (mg/L) were measured during the studies (i.e., for the HT studies) or taken from annual measurements from 2000 to 2004. Dissolved organic carbon (DOC) and total organic carbon concentrations were measured in all studies.

Water quality parameters (e.g., hardness, alkalinity, pH, DO, and conductivity) were performed following standard laboratory operating procedures developed from standard methods [27]. Chloride analyses were conducted following U.S. EPA Method 325.3, and DOC was analyzed following U.S. EPA Method 415.1 [27]. Determinations of waterborne sodium, calcium, potassium, and magnesium concentrations were made using inductively coupled plasma/atomic emission spectroscopy, SW-846 (Paragon Analytics, Fort Collins, CO, USA).

Chemical analyses

Aqueous samples for ''total'' and dissolved (0.45 μm; GHP Acrodisc Syringe Filters, Pall Gelman Scientific, Ann Arbor, MI, USA) silver analysis were collected using clean metal techniques from all test chambers at test initiation (STC and ELS) and termination where surviving fish were present (ELS only). In addition, samples were collected from one alternating or randomly chosen replicate from each treatment on days 3 (or 4) and 7 for the STC studies and weekly for the ELS studies where surviving fish were present. Sampling equipment was rinsed three times with the water to be sampled prior to collection. In addition, filters were cleaned prior to use, and samples were preserved with one drop (per 11-ml sample) of Ultrex® II ultrapure reagent nitric acid (J.T. Baker, Phillipsburg, NJ, USA) after sampling. Samples from the HT studies were analyzed on the day of collection using a PerkinElmer 1100B atomic absorption spectrophotometer with a HGA 700 graphite furnace (PerkinElmer, Waltham, MA, USA) (U.S. EPA Method 200.9) [27]. Total silver samples were analyzed as collected and were not digested prior to analysis. Aqueous silver analyses were not conducted following good laboratory practice methods [18]. Matrix spikes, duplicates, and analysis of reference samples were included as quality control samples.

Samples from the HT-high and HT-high NaCl studies were collected and sent to McMaster University for analysis using a PerkinElmer Elan 6000 inductively coupled plasma mass spectrometry (U.S. EPA Method 200.8) [27]. However, it was unknown at the time that, despite the use of standard sample preservation methods, even a short holding period would lead to significant reductions in silver concentrations in the low-μg/L range compared with analysis conducted at the time of collection. Although an analytical technique was developed and used to recover the silver from the storage vessels (J.R. Kramer, unpublished data), the results from this analysis were low compared to the analytical results measured in-house within 24 h of sampling in subsamples or from analytical measurements conducted during later studies under similar conditions (Table 2). Because of the lower silver concentrations measured following extended storage of the primary samples, these data were judged to be unreliable and were not used because they underrepresented exposure concentrations under these conditions. Analytical results from the subset of samples measured shortly after sampling and those from later studies at similar test conditions were comparable and more representative of expected exposure concentrations. Therefore, we used total-to-nominal and dissolved-to-nominal ratios in the latter studies to estimate total and dissolved silver concentrations in the HT-high and HT-high NaCl ELS and STC studies.

Dried fathead minnow larvae to be analyzed for whole-body sodium and silver concentrations were ground with a Teflon® tissue grinder and digested individually in situ in 50 volumes (50 μl/mg sample) of 1 N trace metal–grade nitric acid (Fisher Scientific) at 60°C until all solid material had disappeared. This digest was then diluted with 200 μl (fivefold) of 1% HNO_3. Silver was measured on a portion of the supernatant from this step. The remaining supernatant (150 μl) was then diluted with 1.5 ml (11-fold) of 1% HNO_3 and used for sodium analyses. Silver was measured using a Varian 1475 graphite furnace atomic absorption unit equipped with a GTA-95 graphite furnace and sodium by flame atomic absorption on a Varian 1475 atomic absorption unit (Varian, Palo Alto, CA, USA). Analyses were optimized according to the manufacturer's instructions, and addition/recovery tests were run routinely. Certified standards (Fisher Scientific, Burlington, ON, Canada) in an appropriate tissue matrix were used throughout.

Table 2. Comparison of total and dissolved silver concentrations measured in high-flow studies: from limited samples measured in-house during the studies (in-house samples), from samples collected during the studies but measured after extended storage using an extraction technique (stored samples), and from surrogate studies conducted later at similar conditions measured on the day of collection (surrogate samples). Some studies were conducted in higher-flow conditions in unamended Horsetooth water (HT-high), while other studies were conducted in laboratory water amended with NaCl (target of 60 mg Cl/L) at higher-than-normal flow conditions (HT-high NaCl)

	In-house samples		Stored samples[a]		Surrogate samples[b]	
Nominal treatment (μg Ag/L)	Total (μg Ag/L)	Dissolved (μg Ag/L)	Total (μg Ag/L)	Dissolved (μg Ag/L)	Total (μg Ag/L)	Dissolved (μg Ag/L)
HT-high						
Control	—	—	0.025	0.011	<0.04	<0.04
0.125	0.08 (n = 1)	—	0.082	0.025	0.09	<0.04
0.25	0.17 (n = 1)	—	0.152	0.037	0.20	0.10
0.50	0.48 (n = 4)	—	0.274	0.061	0.41	0.23
1.0	0.81 (n = 1)	—	0.425	0.091	0.85	0.58
2.0	1.67 (n = 2)	—	0.967	0.355	1.75	1.33
HT-high NaCl						
Control	—	—	0.026	0.021	<0.04	<0.04
NaCl control	—	—	0.037	0.025	<0.04	<0.04
0.19	—	—	0.191	0.070	0.14	0.05
0.38	—	—	0.320	0.098	0.32	0.23
0.75	0.54 (n = 3)	—	0.536	0.243	0.69	0.54
1.5	—	—	1.050	0.492	1.48	1.27
3.0	2.42 (n = 3)	—	2.044	1.039	3.02	2.78

[a] Analytical results from 7-d short-term chronic (STC) studies; values are time-weighted averages (TWA) of samples measured on days 0, 3 (or 4), and 7 of the respective studies.

[b] Analytical results for HT-high study from a subsequent 7-d STC study (n = 3 for each treatment; TWA of days 0, 3, and 7) and analytical results associated with HT-high NaCl study determined from a subsequent study to replicate measured results in the original study.

The chromium-reducible sulfide concentration was measured in duplicate according to Bowles et al. [28], except that the purge gas was ultrapure nitrogen and all gas-delivery tubing was made from either Teflon or medical-grade Tygon® (Saint-Gobain Performance Plastics, Akron, OH, USA) (containing no plasticizers). Two Milli-Q water blanks were included during the analysis.

Statistical analysis

Data analyses (for hypothesis testing) was performed with Toxstat software Version 3.5 [29] to determine whether organism performance in the experimental treatments was similar to that observed in the control. Normality and homogeneity assumptions for these data was evaluated by the Shapiro–Wilk's test and Bartlett's test, respectively ($p \leq 0.01$). Biological data that met these assumptions or that met these assumptions through (log) transformations were tested by analysis of variance, followed by Dunnett's procedure ($p \leq 0.05$). For data that did not meet the assumptions of normality and homogeneity, the Steel's many-one rank test ($p \leq 0.05$) was used.

Survival data was entered as proportions and was transformed by arcsine square root prior to hypothesis testing. Treatments with no surviving organisms were excluded from hypothesis testing of survival data. Only treatments that did not show a significant reduction in survival relative to the control were included in hypothesis testing of sublethal endpoints. For hypothesis testing, growth in each replicate was determined by the dry weight (dry wt) per original fish. An IC20 value (inhibition concentration 20%, or the concentration estimated to cause a 20% reduction in mean dry weight per original number of organisms compared with the control) was also calculated [30]. Hypothesis testing was also conducted on whole-body silver and sodium data. Error bars for IC20 values represent 95% confidence intervals. Significant differences in effects between STC and ELS studies were presumed to be indicated by nonoverlapping 95% confidence intervals. Unless otherwise stated, data have been expressed as means ± 1 standard deviation.

RESULTS

Water quality characteristics

Water quality parameters measured during the paired ELS and STC studies were within expected values. Water hardness averaged 30.5 mg/L as $CaCO_3$, alkalinity averaged 28.8 mg/L as $CaCO_3$, pH ranged from 7.3 to 8.2, DO was >4.9 mg/L (72% saturation at 1,585 m), and time-weighted average temperature was 25 ± 1°C. The chloride concentration in unmodified HT water ranged from 0.5 to 3.6 mg/L with an average of 1.9 mg/L. In contrast, chloride levels in the HT-high NaCl studies averaged 58.2 mg/L for both the ELS and the STC study. Corresponding sodium concentration in unamended Horsetooth water averaged 3.5 mg/L and was approximately 38 mg/L in the HT-high NaCl studies. Dissolved organic carbon values in each set of tests averaged 2.53 (HT), 4.02 (HT-high), and 3.55 (HT-high NaCl) mg/L. Chromium-reducible sulfide measured in our HT laboratory water averaged 1.14 nM in the HT ELS and STC studies. Other water quality parameters of interest measured in unmodified Horsetooth Reservoir water from 2000 to 2004 as part of our annual monitoring program or measured during the studies are also reported (Table 1).

The confounding issues associated with silver concentrations measured during the HT-high and HT-NaCl high studies were previously discussed. On the basis of the analysis described and presented in Table 2 (i.e., a few original measurements at the time of sampling for total silver, plus later measurements of both total and dissolved silver from subsequent similar tests), we estimate that total silver concentrations in the HT-high studies averaged 81% nominal concentrations, while dissolved silver treatments averaged 53% of nominal

Table 3. Primary endpoints (average ± standard deviation) for early life stage (ELS) and short-term chronic (STC) studies examining the toxicity of silver in unamended Horsetooth water under normal flow conditions (HT). Analytical samples (time-weighted averages) were taken on days 0, 3, and 7 for the STC study and on days 0, 7, 14, 21, 25, 28, and 32 for the ELS study

Nominal exposure concn. (μg Ag/L)	Avg. total silver (μg/L)	Avg. dissolved silver (μg/L)	Total hatched (%)	Total % survival	Growth (dry wt/orig. [mg])	Whole-body Ag (μg/g dry wt)	Whole-body Na[a]
ELS study							
Control	<0.044	<0.044	83.8 ± 14	76.2 ± 6.3	13.3 ± 1.2	0.51 ± 0.03	248 ± 16
0.19	0.100	0.038	82.5 ± 19	73.8 ± 18	12.8 ± 0.73	0.54 ± 0.13	235 ± 18
0.38	0.195	0.079	87.5 ± 10	77.5 ± 8.7	13.1 ± 0.69	0.99 ± 0.21*	235 ± 20
0.75	0.455	0.147	83.8 ± 2.5	77.0 ± 13	13.2 ± 0.77	4.59 ± 1.6*	237 ± 39
1.5	0.899	0.351	91.2 ± 7.5	82.5 ± 8.7	13.3 ± 1.1	19.7 ± 2.6*	252 ± 7.7
3.0	1.972	0.795	80.0 ± 11	25.0 ± 31*	2.08 ± 3.0*	33.2 ± 3.2*	158 ± 27*
STC study							
Control	<0.04	<0.04	—	100 ± 0.0	0.414 ± 0.02	0.658 ± 0.08	145 ± 32
0.19	0.104	0.032[b]	—	95 ± 5.8	0.391 ± 0.06	0.718 ± 0.05	137 ± 5.6
0.38	0.198	0.070	—	100 ± 0.0	0.438 ± 0.05	1.23 ± 0.37*	148 ± 40
0.75	0.431	0.117	—	100 ± 0.0	0.456 ± 0.01	2.57 ± 0.72*	162 ± 41
1.5	0.862	0.290	—	100 ± 0.0	0.411 ± 0.04	10.2 ± 3.1*	137 ± 35
3.0	1.84	0.606	—	82.5 ± 35	0.368 ± 0.22	10.6 ± 2.1*	115 ± 70

[a] Units for whole-body Na are concentration for ELS studies (mmol/kg dry wt) or content for STC studies (nmol).
[b] Average was determined to be below the detection limit.
* Significantly different from the control ($p \leq 0.05$).

values. In addition, we estimated that total silver concentrations in the HT-high NaCl studies averaged 90% nominal concentrations, while dissolved silver treatments averaged 67% of nominal values.

In HT normal flow studies, silver analyses were not confounded by storage. Here the total silver concentrations averaged 60% of nominal concentrations, while dissolved silver treatments averaged 20% of nominal values (Table 3). The percentage of dissolved to total silver concentrations for these studies ranged from 27 to 40%. The amount of silver measured in solution increased in the studies at higher flow rates and in the presence of chloride compared to silver concentrations measured under normal flow conditions or without chloride.

HT studies

The percentage of eggs hatched in the HT ELS study was not negatively impacted in any of the silver treatments (Table 3). Overall survival was affected only in the highest silver treatment (3.0 μg/L), which had 25% survival. Survival in all other treatments was ≥73.8%, meeting the ASTM criterion of ≥70% for control survival [21]. Growth (dry wt per original number of organisms) and whole-body sodium levels were significantly lower than control values at the 3.0 μg/L treatment. On the other hand, whole-body silver accumulation was significantly increased compared to the control organisms in all but the lowest silver treatment, 0.19 μg nominal Ag/L (Table 3).

Endpoints in the STC HT study were, typically, unaffected by exposure up to 3.0 μg/L treatment (Table 3). The only endpoint that had a significant impact from silver exposure was whole-body silver concentration, which was significantly increased from the control at all but the lowest silver treatment (0.19 μg nominal Ag/L). The reduced flow rates in these studies accounted for the lower dissolved silver concentrations (Table 3) and the lack of significant effects in this study compared to the HT-high STC study.

Three endpoints in the ELS study—survival, growth, and whole-body Na—were lower than those in the STC study by one treatment level (Table 3). However, the Ag concentration (i.e., 0.38 μg nominal Ag/L) that resulted in a significant increase in whole-body Ag body burden was similar between the STC and the ELS study.

HT-high studies

For the ELS study, the 1.0 and 2.0 μg nominal Ag/L treatments had significantly lower total hatches than the control (Table 4). Both of these treatments had ≤30% overall survival, which was significantly less than in the control treatment. All other treatments in the ELS study had ≥82.5% survival. Growth was not significantly lower than the control at silver concentrations below 1.0 μg/L (Table 4). Other sublethal endpoints of whole-body silver (μg/g dry wt) and sodium (mmol/kg dry wt) residues were significantly different from the control at the 0.125- and 0.5-μg/L treatments but not at the 0.25-μg/L treatment.

In the STC study, survival was significantly reduced only at 2.0 μg/L, and the other endpoints (growth per original number of organisms and whole-body sodium) did not show a consistent negative effect at concentrations lower than 2.0 μg/L (Table 4). Whole-body silver residues were significantly increased in the ≥0.5-μg/L treatments, demonstrating that silver accumulation was directly related to exposure concentration.

In general, the endpoints for the STC in the HT-high study were less sensitive than the ELS study (Table 4). Whole-body silver accumulation was the most sensitive endpoint for both ELS and STC studies, although the whole-body sodium endpoint was just as sensitive in the ELS study. Both endpoints were affected at the lowest Ag concentration tested in the ELS study, 0.125 μg nominal Ag/L.

HT-high NaCl studies

The total percentage of FHMs hatched was not significantly reduced up to the highest tested nominal silver concentration of 3.0 μg nominal Ag/L (Table 5). However, survival, whole-body Ag, and Na were significantly different from the control at the highest concentration tested. Growth (dry wt/original) was affected only at the two highest treatments, 1.5 and 3.0 μg/L (Table 5).

Survival of FHMs in the STC was not significantly reduced

Table 4. Primary endpoints (average ± standard deviation) for early life stage (ELS) and short-term chronic (STC) studies examining the toxicity of silver in unamended Horsetooth water under high-flow conditions (HT-high)

Nominal exposure concn. (μg Ag/L)	Total hatched (%)	Total % survival	Growth (dry wt/orig. [mg])	Whole-body Ag (μg/g dry wt)	Whole-body Na[a]
ELS study					
Control	88.8 ± 8.5	83.8 ± 4.8	22.4 ± 1.7	0.28 ± 0.02	193 ± 5.2
0.125	92.5 ± 5.0	83.8 ± 4.8	22.2 ± 0.73	0.33 ± 0.03*	165 ± 4.3*
0.25	93.8 ± 2.5	82.5 ± 6.4	21.1 ± 0.51	0.52 ± 0.26	179 ± 2.5
0.5	91.2 ± 11	86.2 ± 12	21.2 ± 0.94	1.46 ± 1.6*	172 ± 12*
1.0	72.5 ± 8.7*	30.0 ± 25*	5.3 ± 4.3*	3.63 ± 2.9*	127 ± 14*
2.0	40.0 ± 10*	0*	0*	—	—
STC study					
Control	—	95 ± 5.8	0.837 ± 0.07	1.07 ± 0.26	180 ± 74
0.125	—	92.5 ± 9.6	0.691 ± 0.10	0.921 ± 0.08	118 ± 26
0.25	—	90 ± 8.2	0.682 ± 0.12	0.901 ± 0.22	113 ± 30
0.5	—	95 ± 5.8	0.713 ± 0.07*	1.80 ± 0.19*	115 ± 26
1.0	—	72.5 ± 25	0.595 ± 0.41	2.82 ± 1.3*	114 ± 72
2.0	—	0*	0*	—	—

[a] Units for whole-body Na are concentration for ELS studies (mmol/kg dry wt) or content for STC studies (nmol).
* Significantly different from the control ($p \leq 0.05$).

at any of the silver treatments; however, growth (per original number of organisms) and whole-body sodium were affected at both the 1.5- and the 3.0-μg/L treatment (Table 5). Whole-body silver was increased over the control treatment in the ≥0.75-μg nominal Ag/L treatments.

Survival in the ELS study was more sensitive than survival in the STC study; however, the remaining STC endpoints were either as sensitive as (growth) or more sensitive (whole-body Ag and Na) than ELS endpoints (Table 5).

DISCUSSION

The chronic and subchronic toxicity levels of silver to fathead minnows were determined in three separate paired experiments to determine the chronic toxicity of silver to the fathead minnow, *P. promelas*, and to determine if the STC study could be used to estimate the toxicity of Ag determined by the ELS studies. Our intention was to use the more economically feasible STC study method in further silver testing (R. Naddy, unpublished data) to cost effectively evaluate other water quality effects (e.g., chloride, hardness, DOC). Two of the experiments were conducted in our base laboratory dilution water, HT, using two different flow rates (standard and high), while the third was conducted in Horsetooth water amended with NaCl at a high flow rate. The results of these comparative studies indicate that while the ELS endpoints were typically more sensitive, STC endpoints were generally within a factor of two of the ELS endpoints. For the NaCl studies, the endpoints in the STC test proved to be more sensitive or equally sensitive to those in the ELS study. Growth endpoints (IC20 values) were similar between the STC and ELS studies for two of the three tests we conducted (Fig. 2). We could not fully evaluate the relationship between the STC and ELS IC20 values in the HT study because no significant effect was observed in the HT STC study at the highest concentration tested, 3.0 μg nominal Ag/L (0.61 μg dissolved Ag/L). Norberg-King [16] and Holcombe et al. [14] reported a twofold agreement

Table 5. Primary endpoints (average ± standard deviation) for early life stage (ELS) and short-term chronic (STC) studies examining the toxicity of silver in Horsetooth water amended with NaCl (target of 60 mg Cl/L) under high-flow conditions (HT-high NaCl)

Nominal exposure concn. (μg Ag/L)	Total hatched (%)	Total % survival	Growth (dry wt/orig. [mg])	Whole-body Ag (μg/g dry wt)	Whole-body Na[a]
ELS study					
Control	95.0 ± 7.1	91.2 ± 4.8	31.2 ± 1.3	0.60 ± 0.15	163 ± 8.1
NaCl control	86.4 ± 4.2	77.5 ± 2.3	31.6 ± 1.6	0.69 ± 0.16	161 ± 20
0.19	95.0 ± 4.1	92.2 ± 9.2	31.8 ± 2.7	0.55 ± 0.11	166 ± 8.2
0.38	93.8 ± 7.5	87.5 ± 9.6	32.1 ± 0.4	0.69 ± 0.03	164 ± 13
0.75	89.0 ± 10	82.8 ± 9.3	31.4 ± 2.1	0.70 ± 0.08	174 ± 23
1.5	93.8 ± 6.3	72.5 ± 9.6	21.0 ± 1.2*	0.57 ± 0.09	150 ± 19
3.0	86.2 ± 8.5	1.2 ± 2.5[b]*	0.01*	3.22*	84.0*
STC study					
Control	—	100 ± 0.0	0.684 ± 0.14	0.65 ± 0.07	167 ± 16
NaCl control	—	95 ± 10	0.694 ± 0.10	0.62 ± 0.12	156 ± 26
0.19	—	100 ± 0.0	0.774 ± 0.04	0.76 ± 0.14	147 ± 28
0.38	—	97.5 ± 5.0	0.732 ± 0.11	0.90 ± 0.30	136 ± 33
0.75	—	97.5 ± 5.0	0.740 ± 0.12	1.68 ± 1.1*	138 ± 46
1.5	—	85 ± 10	0.465 ± 0.09*	2.68 ± 0.93*	98.8 ± 21*
3.0	—	85 ± 13	0.226 ± 0.04*	6.25 ± 2.3*	30.5 ± 5.4*

[a] Units for whole-body Na are concentration for ELS studies (mmol/kg dry wt) or content for STC studies (nmol).
[b] Only one organism survived.
* Significantly different from the concurrent NaCl control ($p \leq 0.05$).

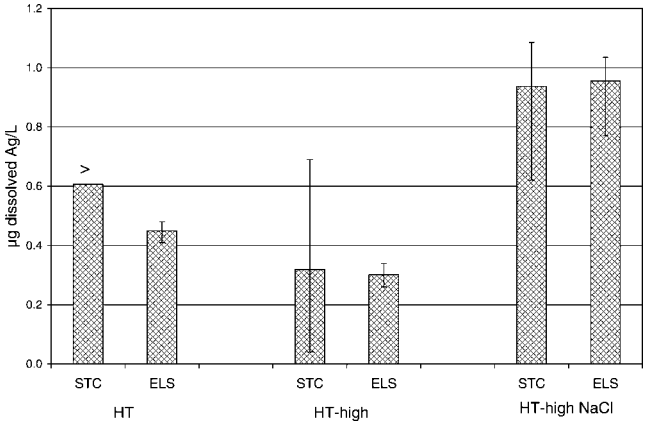

Fig. 2. The silver concentration estimated to cause a 20% reduction in fathead minnow growth per original number of organisms (dry wt) compared to the control response (i.e., IC20) are presented for the studies in unamended laboratory water under normal flow conditions (HT), in unamended laboratory water under high-flow conditions (HT-high), and in laboratory water amended with NaCl under high-flow conditions (HT-high NaCl). Short-term chronic (STC) and early life stage (ELS) studies were conducted under all three flow/water conditions. Silver concentrations for HT-high and HT-high NaCl studies were estimated from other studies under similar conditions. Error bars represent 95% confidence intervals. The IC20 was >0.606 µg dissolved Ag/L (highest concentration tested) for the HT STC study.

between STC and ELS studies, similar to our results. Demonstrating the relationship between the STC study and the ELS studies was important so we could use the more economical STC study design to evaluate the effect of various water quality parameters on silver toxicity in later studies.

The MATC for the HT ELS study was 0.53 µg dissolved Ag/L (1.33 µg total Ag/L) for growth with a calculated IC20 of 0.45 (0.41–0.48) µg dissolved Ag/L. Under high-flow conditions (i.e., HT high), the MATC was 0.36 µg dissolved Ag/L (0.59 µg total Ag/L), while the IC20 was 0.30 (0.26–0.34) µg dissolved Ag/L. Holcombe et al. [14] conducted an ELS study in soft water using normal replacement conditions (~7 volume additions/d) and reported no observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) values of 0.37 and 0.65 µg total Ag/L, respectively, which results in a calculated MATC of 0.49 µg total Ag/L. Although dissolved silver values were not reported, their total silver effect concentration was similar to the results in our HT-high study on a total basis (i.e., MATC of 0.49 and 0.59 µg total Ag/L, respectively). The HT STC study LOEC value was greater than the highest test concentration tested, >0.606 µg dissolved Ag/L (>1.84 µg total Ag/L); however, the MATC value for the HT-high STC study was 0.88 µg dissolved Ag/L (1.22 µg total Ag/L) with an IC20 value of 0.32 (0.04–0.69) µg dissolved Ag/L. Our endpoints in the STC studies were comparable to the value reported by Norberg-King [16], 1.01 µg total Ag/L.

The MATC and IC20 values increased with the addition of 60 mg Cl/L (as NaCl). The MATC for both ELS and STC studies was 0.83 µg dissolved Ag/L, while the respective IC20 values were 0.96 (0.77–1.04) and 0.94 (0.62–1.09) µg dissolved Ag/L, respectively. The addition of NaCl appeared to have a threefold protective effect, based on IC20 values, in the ELS and STC studies (Fig. 2) conducted at high-flow conditions. While we observed a degree of protection from Ag toxicity using NaCl, others have reported little or no appreciable mitigation of silver toxicity due to the presence of sodium chloride in acute exposures with FHMs [9]. However, to our knowledge, there has been no other research with FHM evaluating the mitigating ability of NaCl to Ag under chronic conditions. Sodium chloride (60 mg/L or 1.7 mM Cl) also appeared to provide a level of protection from silver accumulation in the ELS study but not in the STC study (Table 5). While NaCl inhibited silver accumulation in FHMs in the ELS study, the longer exposure period did not necessarily translate to increased protection from silver toxicity because the growth IC20 was similar between short-term and chronic exposures (Fig. 2). The results of these studies suggest a protective chloride and/or sodium effect in short-term and chronic exposures from ionic Ag (Ag^+, the toxic moiety) using FHMs.

The most sensitive endpoint in the Holcombe et al. [14] study was survival, while survival and growth were equally sensitive in our HT and HT-high studies. For the HT and HT-high STC and ELS studies and the STC HT-high NaCl study, the most sensitive endpoint was whole-body silver concentration. Whole-body sodium concentration was equally sensitive in the HT-high ELS study; however, growth was the most sensitive endpoint for the HT-high NaCl ELS study. While whole-body silver accumulation was one of the more sensitive endpoints, caution should be used when identifying these endpoints as threshold effect levels. While threshold effect levels (e.g., NOEC, LOEC, and MATC values) typically imply biologically significant effect (i.e., fitness) levels for survival and growth data, this may not necessarily be the case for whole-body residue endpoints. An accumulation in whole-body silver does not necessarily imply biological impact (i.e., decrease in fitness). However, during acute exposures, it has been clearly demonstrated that an organism's loss of whole-body sodium can be linked to fitness effects, such as a decrease in survival [17,31,32]. The primary mechanism of action provides support for this idea. Ionic silver disrupts ionoregulation in fish by decreasing active sodium (Na^+) and chloride (Cl^-) ion uptake at the gill by the inhibition of Na^+, K^+-ATPase activity and carbonic anhydrase activity [31,33]. This results in osmoregulatory failure because of decreased Na^+ and Cl^- levels in the organism. In fact, we were able to observe a decrease in whole-body sodium concentration (ELS) or content (STC) for FHMs, which appeared to correlate well with other adverse effect endpoints, such as decreased survival or growth. Therefore, it appears that whole-body sodium levels were predictive of effect levels and could be potentially used for threshold effect levels. Interestingly, the whole-body Na data of the present study suggest that the same toxic mechanism observed in acute exposures is responsible for the chronic toxicity of silver in early life stages. However, we cannot eliminate the possibility that other effects may be involved that are more adverse than the loss of Na. If the chronic mode of action is similar to acute exposures, this would be an important finding alleviating one objection to the extension of biotic ligand modeling or acute-to-chronic ratio approaches to the prediction of chronic toxicity, as discussed by Wood et al. [32].

Our research demonstrated that the STC study design could be used as a surrogate study design instead of ELS studies. The use of the STC study, instead of the ELS study, would enable us to conduct more studies investigating the potential impacts of different water quality parameters on Ag toxicity to the fathead minnow, *P. promelas*. Obtaining more chronic data that takes into account various water quality parameters would be helpful in determining a new chronic ambient water quality criterion. It would also be important in the development

of a chronic biotic ligand model for Ag and would minimize or eliminate the need to use acute data with a safety factor to determine a freshwater chronic criterion for Ag.

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