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Aarya Venkat

Valdosta State University, avenkat@valdosta.edu


Adriana L. Amerson

Valdosta State University, alamerson@valdosta.edu

Gretchen K. Bielmyer-Fraser

Valdosta State University, gkbielmyer@valdosta.edu

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Cover Page Footnote

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INFLUENCE OF WATER HARDNESS ON SILVER ACCUMULATION AND EFFECTS OF SILVER IN THE GREEN ALGA, *Raphidocelis subcapitata*

Aarya Venkat, Adriana Amerson, and Gretchen K. Bielmyer-Fraser*
Valdosta State University, Valdosta, Georgia
*gkbielmyer@valdosta.edu

ABSTRACT

Metal pollution from anthropogenic sources can pose a threat to aquatic systems. Silver is released into the environment from various industrial processes. In excess, silver can accumulate and cause adverse effects in aquatic organisms, particularly those in lower trophic levels, such as phytoplankton. Water chemistry parameters, such as hardness, have been shown to modify toxicity of metals because divalent cations compete with the metal for binding sites on the biological membrane. The objective of this study was to assess population growth and silver accumulation in the green alga, *Raphidocelis subcapitata*, after silver exposure in waters of varying hardness for 7 d. Throughout the exposure period, a decrease in algal cell density was observed with increasing silver concentrations. Silver accumulation in the algae decreased and cell density increased with increasing water hardness. Additionally, at least some degree of protection was observed against silver toxicity due to increased water hardness.

Keywords: silver, toxicity, hardness, *Raphidocelis subcapitata*

INTRODUCTION

Silver is a nonessential heavy metal that commonly enters aquatic systems through anthropogenic inputs, such as through the photography industry, and through the manufacture of jewelry, silverware, electronic devices (Luoma et al. 1999; GFMS Limited 2011). Additionally, silver nanoparticles are now frequently used in various products such as antibacterials, colloidal silvers, fabric softeners, and water purifiers (Perelshtein et al. 2008). The use of silver from all of these sources may result in contamination in many aquatic systems, particularly those surrounding densely populated areas (Perelshtein et al. 2008).

Once in the aquatic environment, silver may accumulate and potentially cause toxicity in aquatic organisms particularly in lower trophic levels (Ratte et al. 1999; Bielmyer et al. 2002; Bielmyer et al. 2006). Due to its properties as a strong antimicrobial agent, silver strongly inhibits the reproduction of microorganisms like algae and protozoa (Chernousova and Epple 2013). Silver may also affect higher level organisms, such as zooplankton and fish, through silver transfer in aquatic food webs, or by reducing their food sources (Luoma et al. 1999). Silver generally causes toxicity by disrupting ionoregulation in aquatic organisms (Lee et al. 2012). Because the water quality in freshwater ecosystems varies, it is important to understand the influence of water characteristics on silver toxicity to the biota inhabiting those areas. Several studies have shown that water hardness, i.e., the concentration of divalent cations, may reduce metal toxicity to phytoplankton (Deleebeek et al. 2009; Charles et al. 2002). Increased

cation concentration can result in increased competition with silver for binding sites in aquatic biota, thus reducing silver bioavailability and toxicity (Charles et al. 2002). Additionally, increased anion concentrations can change silver speciation, resulting in silver species that are less bioavailable and toxic (Campbell 1996). Generally the silver ion is considered the most bioavailable and toxic silver species (Bielmyer et al. 2002; Campbell 1996). For silver toxicity to occur in aquatic organisms silver must be bioavailable, the silver must interact with the biological membrane of the organism, and the silver must enter the organism, causing some biological effect (Campbell 1996). Several studies have demonstrated the protective effect of water hardness against metal toxicity to algae. Elevated water hardness effectively reduced the toxicity of uranium to *Raphidocelis subcapitata* (Poston et al. 1984). The protective effect was believed to be due to complexation between uranium and carbonate ions (Poston et al. 1984). Heijerick and colleagues demonstrated increased protection against copper toxicity to *R. subcapitata*, with increased Ca^{2+} , Mg^{2+} , and H^+ ions at the cell surface (Heijerick et al. 2005). These cations likely competed with copper for entry into the cell.

Raphidocelis subcapitata (formerly *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*) is a freshwater alga with a widespread distribution that is commonly used as a testing organism to determine the toxicity of various contaminants (Franklin et al. 2007). Due to its photosynthetic capabilities and its fast reproduction rate, it can easily make a tremendous impact on an aquatic environment purely by its presence or absence (dePaiva et al. 2014). *R. subcapitata* has also been shown to be sensitive to metals (Luoma et al. 1999), thus it makes for an ideal testing organism for this study. The objective of this study was to determine the influence of water hardness on silver toxicity to *R. subcapitata*. We hypothesized that algal cell density would decrease with increasing exposure to silver, and that increased water hardness would reduce silver toxicity.

MATERIALS & METHODS

Testing Waters

Reconstituted synthetic very soft, soft, moderately hard, and hard water was made using US EPA recommended guidelines (USEPA, 1989). The following reagent grade salts were added to reverse osmosis deionized water: MgSO_4 , NaHCO_3 , KCl , and CaSO_4 (Fisher Scientific). Each of the testing waters was mixed and aerated in 1 L carboys for 24 h before use. Water quality was measured in each water type using a LaMotte fresh water aquaculture test kit (model AQ-2) to determine pH, hardness (detection limit = 4 ppm), alkalinity (detection limit = 10 ppm), chloride (detection limit = 4 ppm), carbon dioxide (detection limit = 1 ppm), nitrate (detection limit = 0.05 ppm), nitrite (detection limit = 0.05 ppm), and ammonia (detection limit = 0.2 ppm); while dissolved oxygen and temperature were measured using a calibrated YSI meter (YSI® model 85, Yellow Springs, Ohio).

Experimental design

Ten Erlenmeyer flasks were filled with 100 ml of water from each water type. A 1 mg/L AgCl stock solution was then used to make five silver concentrations (control, 0.5, 5, 10, and 25 $\mu\text{g/L}$ AgCl) each with two replicates per treatment in each water type. Additionally, 5 μl of Fritz f/2 Alga Food A and B (Fritz Industries®, Dallas, Texas) was

added to each of the 10 Erlenmeyer flasks. The flasks were covered with aluminum foil and then autoclaved and allowed to cool for at least 24 h.

The flasks were each inoculated with 25 ml of concentrated (1×10^6 cells/ml) *R. subcapitata* (Aquatic Biosystems) and then put in a temperature-controlled growth chamber (20 °C) for 7 d. At 0, 2, and 7 d, algal cell densities for each flask were measured using a hemocytometer. At the beginning of the exposure, water was collected and filtered using a 0.45 μm filter (ThermoScientific Nalgene) and transferred to 15 ml centrifuge tubes for later silver analysis. At the end of the exposure, another water sample containing algae was collected in 50 ml centrifuge tubes, the algal cells were concentrated via centrifugation for 10 min at 3500 RPM, and the supernatant discarded. Algal pellets were pooled and dried in a convection oven at 60 °C for at least 24 h, and then weighed again. The dried algae were digested via addition of concentrated trace metal grade nitric acid (1 ml) and diluted with 18 m Ω Milli-Q water. Water samples and digested algae were measured for silver using a Perkin Elmer atomic absorption spectrometer (AAAnalyst 800).

Using the program Sigma plot, a one-way analysis of variance was conducted along with a Tukey's test (multiple pairwise comparison) to determine significant differences between treatments. A t-test was also performed between the control and each silver treatment ($\alpha = 0.05$). Data were analyzed for normal distribution and equality of variance using a Shapiro-Wilk's test and a Bartlett test, respectively.

RESULTS

Generally, pH, alkalinity, hardness, chloride, and carbon dioxide increased from very soft water to the hard water with only two exceptions (alkalinity in soft water and chloride in hard water; Table I). Additionally, the measured hardness of each water type was slightly below that classified by the US EPA. For example, the moderately hard water we used in this experiment had a hardness value of 72.5 mg CaCO₃/L; however the US EPA classifies moderately hard water as ranging from 80-100 mg CaCO₃/L. The measured silver concentrations in the highest two silver treatments were slightly below nominal values and similar across water types (Table II). The measured silver concentrations in the lower two silver treatments were more variable across water types and in some cases varied more from the nominal values.

Table I. Water quality parameters in very soft, soft, moderately hard, and hard synthetic water. Abbreviation: ppm = parts per million.

<u>Water Type</u>	<u>Very Soft</u>	<u>Soft</u>	<u>Moderately Hard</u>	<u>Hard</u>
pH	6.5±0.0	7.0±0.0	7.5±0.0	7.5±0.0
Alkalinity (ppm)	6.5±0.7	36.0±0.7	17.0±0.0	22.0±2.8
Hardness (ppm)	45.0±4.2	58.0±3.5	73.0±0.7	96.0±19.8
Chloride (ppm)	5.0±1.4	15.0±0.0	21.0±1.4	12.0±0.0
Ammonia (ppm)	0.2±0.0	0.4±0.0	0.2±0.0	0.2±0.0
Nitrate (ppm)	<0.05±0.0	<0.05±0.0	<0.05±0.0	<0.05±0.0
Nitrite (ppm)	<0.05±0.0	<0.05±0.0	0.05±0.0	0.05±0.0
CO ₂ (ppm)	3.0±0.0	<1.0±0.0	<1.0±0.0	16.5±4.9

Table II. Measured silver concentrations (mean \pm standard error in micrograms per liter) in the experimental media at the start of the experiment (prior to addition of the algae).

Nominal Ag values	Water Type			
	Very Soft	Soft	Moderately Hard	Hard
Control (0)	0.0	0.0	0.0	0.0
0.5	0.5 \pm 0.0	1.5 \pm 1.5	0.5 \pm 0.0	0.2 \pm 0.8
5.0	5.8 \pm 0.8	3.3 \pm 0.0	6.8 \pm 0.4	7.7 \pm 0.2
10.0	7.8 \pm 0.1	7.8 \pm 0.2	8.1 \pm 0.0	8.1 \pm 0.0
25.0	18.1 \pm 0.0	18.1 \pm 0.0	18.1 \pm 0.0	18.2 \pm 0.0

In all synthetic freshwaters, a concentration-dependent increase in silver accumulation in the algae was observed with increasing silver exposure (Figure 1). Water hardness generally reduced the magnitude of silver accumulation in the algae with one exception (very soft water) (Figure 1). Silver accumulated to a much lower extent in very soft water and hard water.

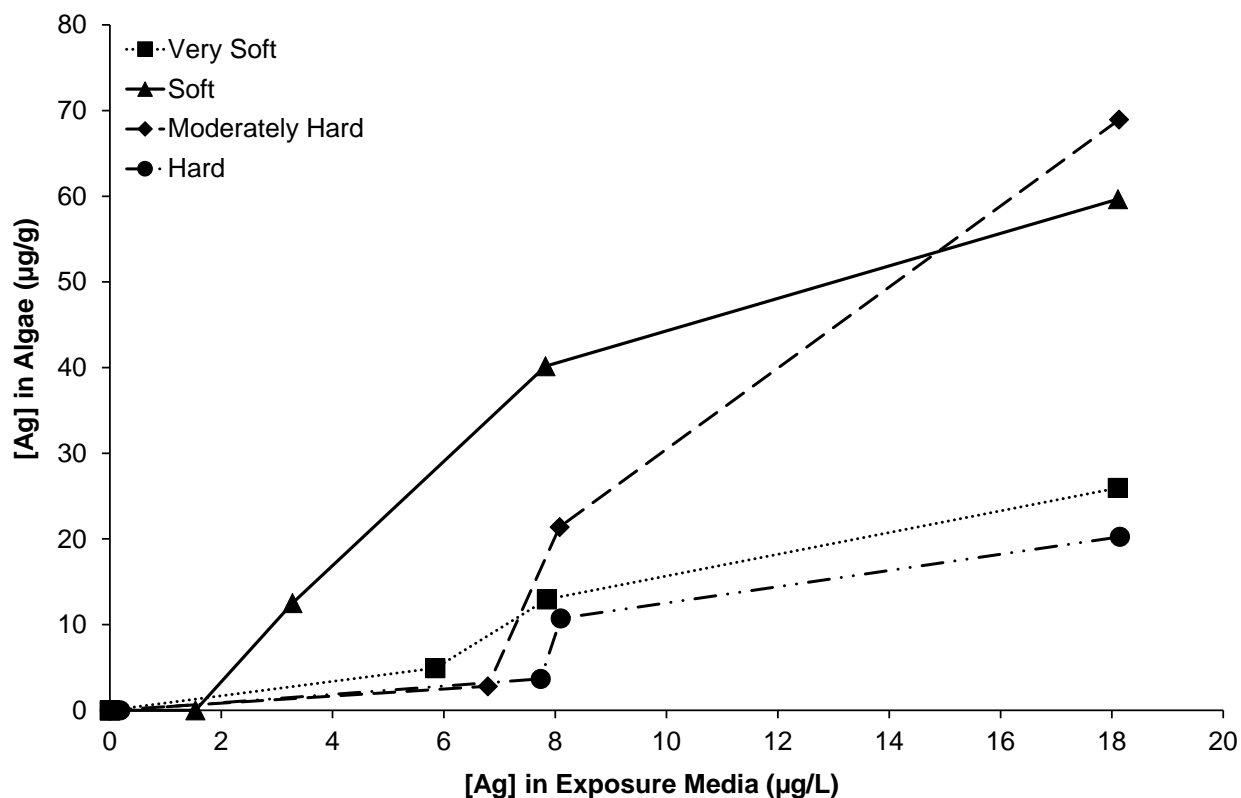


Figure 1. Silver accumulation (micrograms per gram dry weight) in *R. subcapitata* after silver exposure for 7 d in waters of varying hardness.

Increasing water hardness generally reduced silver toxicity to *R. subcapitata* (Figure 2). *R. subcapitata* cell density was significantly reduced after 7 d of silver exposure in every silver treatment, as compared to the control in very soft water (Figure 2A). However, no significant differences were observed between treatments in any other freshwaters, except in hard water at the highest silver concentration of 25 µg/L.

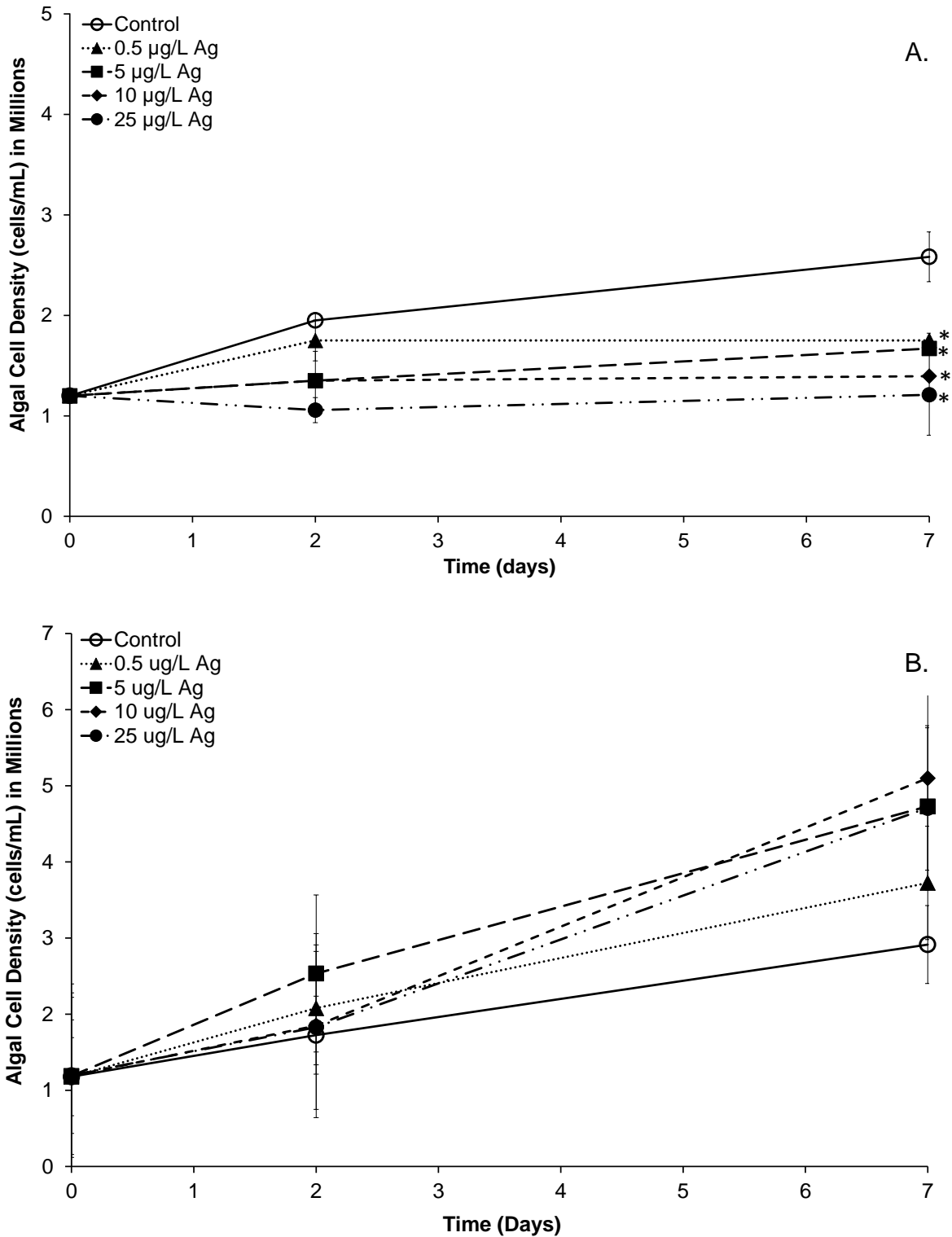


Figure 2A and B. *R. subcapitata* cell density (mean±standard deviation; n = 2) after exposure to silver in A. very soft and B. soft water. Asterisks denote significant differences from the control (p < 0.05).

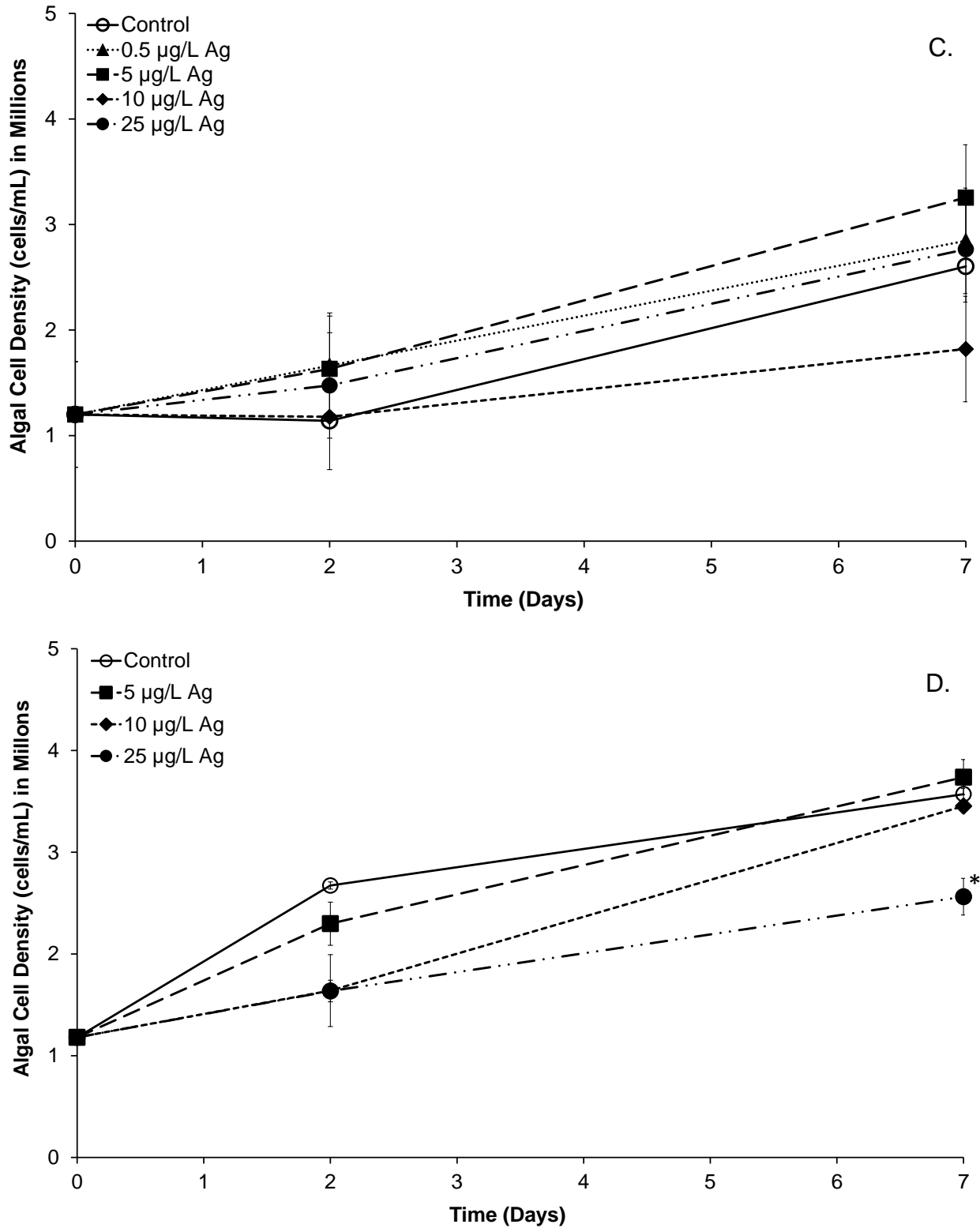


Figure 2C and D. *R. subcapitata* cell density (mean±standard deviation; n = 2) after exposure to silver in C. moderately hard and D. hard water. Asterisks denote significant differences from the control (p < 0.05).

The lowest silver treatment (0.5 µg/L) in hard water was contaminated; therefore, the results were not presented.

DISCUSSION

Silver accumulation in algae as a consequence of increased silver exposure has been reported in other studies (Luoma et al. 1999; Bielmyer 2000). With the exception of very soft water, silver accumulation in *R. subcapitata* was reduced with increasing water hardness in a concentration-dependent manner. The effects of hardness on silver accumulation were particularly evident at the lower silver exposure concentrations. This decreased silver bioavailability to the algae was likely due to increased cation concentration and competition with silver for uptake into the algae in waters of higher hardness (Campbell 1996). Additionally, increased anion concentration in harder waters could have resulted in silver complexation, thereby rendering it less bioavailable to the algae (O'Shea and Mancy 1978). Bielmyer et al. (2008) reported a higher rate of silver accumulation in the fish, *Pimephales promelas*, when the fish was previously acclimated to soft water, as compared to moderately hard water.

By 7 d of silver exposure, silver accumulation in the algae occurred to a lesser extent in very soft water, as compared to the accumulation observed in soft and moderately hard water. Likewise, in very soft water cell density did not significantly increase in any silver treatment. The lack of silver accumulation in the very soft water at 7 d could have been a consequence of the severe silver toxicity that occurred over the exposure period. Exposure of the more bioavailable silver in very soft water could have, over time, resulted in the algae inhibiting silver uptake by down regulating transport proteins or by increasing silver excretion. These actions could have caused the lower silver accumulation in the algae observed at 7 d.

Silver toxicity was greatly reduced as water hardness increased above very soft water; however, at higher levels of water hardness, no additional protection against silver toxicity was observed. The initial protection against silver toxicity in the algae was likely due to changes in silver speciation. In soft, moderately hard, and hard water, the proportion of silver in the ionic fraction, which is considered the most bioavailable, was likely reduced below that causing a toxic effect.

It is important to recognize that these experiments were performed with synthetic waters with low organic matter concentrations and thus may not completely reflect the effects of water hardness in natural waters (McLaughlin and Bonzongo 2012). McLaughlin and Bonzongo (2012) reported that natural waters with high dissolved organic carbon and low ionic strength, such as very soft water, are not comparable with their synthetic counterparts in toxicity testing. With that caveat, this study clearly demonstrates decreased silver accumulation and toxicity in the alga, *R. subcapitata*, with increasing water hardness. However, more research is needed to determine mechanism, e.g., cation competition or anion complexation, leading to the protection provided.

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