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The Effects of Dietary Silver on Larval Growth in the Echinoderm *Lytechinus variegatus*

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Abstract Previous studies have demonstrated that the euryhaline copepod *Acartia tonsa* is extremely sensitive to dietborne silver (Ag) exposure, with a 20 % inhibition (EC_{20}) of survival occurring when copepods are fed algae with 1.6 μg g^{-1} dry weight (dw) Ag, corresponding to a waterborne Ag concentration of 0.46 μg l^{-1} Ag. In contrast, 43 μg l^{-1} Ag is required to elicit similar effects in copepods exposed to Ag by way of water. In the current study, we investigated whether another planktonic marine organism might also be sensitive to dietary Ag. Specifically, we tested larvae of the echinoderm, *Lytechinus variegatus* in an 18-day study in which larvae were continuously exposed to Ag-laden algae (*Isochrysis galbana*). After 7 days of exposure, no significant effects were observed on larval growth up to the highest concentration tested (10.68 μg g^{-1} dw Ag in algae after exposure to 3.88 μg l^{-1} waterborne Ag). After 18 days, significant effects were observed in all Ag treatments resulting in a lowest–observable effect concentration of 0.68 μg g^{-1} dw Ag in algae and corresponding waterborne Ag concentration of 0.05–0.07 μg l^{-1} Ag (depending on background Ag [see Results]). However, the dose–response relationship was quite flat with a similar level of growth inhibition (approximately 15 %) in all Ag treatments, resulting in an EC_{20} of >10.68 μg g^{-1} dw Ag in algae (>3.88 μg l^{-1} Ag in water). This flat dose–response relationship is characteristic of dietary metal (silver, copper, cadmium, nickel, and zinc) toxicity to copepods as well, although the effect is slightly more robust (approximately 20–30 % inhibition of survival or reproduction). We conclude that echinoderm larvae may be similar to copepods in their sensitivity to dietary Ag, although a better understanding of the mechanisms underlying the apparent flat dose–response relationships is clearly needed.

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During the past 15 years, there has been increasing concern that water-quality criteria (WQC) for the protection of aquatic organisms derived solely on waterborne metal exposures may be underprotective because they do not consider the potentially important dietary-exposure pathway (Meyer et al. 2002). Silver (Ag), which has been a common pollutant in coastal waters near municipal wastewater-treatment plants (Luoma et al. 1995; Hornberger et al. 2000), is probably the best-studied metal in this context. Several studies have investigated the toxicity of dietary Ag to the copepod *Acartia tonsa* and found that the effects at corresponding waterborne Ag concentrations (i.e., the waterborne Ag concentration to which the algal diet was exposed) were more than one order of magnitude lower than effect concentrations observed in water-only exposures (Hook and Fisher 2001a, b, 2002). Most recently in our laboratory, we demonstrated an EC_{20} for *A. tonsa* survival during a continuous 7-day exposure to dietary Ag corresponding to a waterborne Ag concentration of 0.46 μg l^{-1} (1.6 μg g^{-1} Ag in the diet) (Bielmyer et al. 2006).

Although the available data certainly suggest that *A. tonsa* is sensitive to dietary metal exposure at concentrations near or lower than proposed/existing WQC for Ag (0.92 μg l^{-1}) and several other metals (Hook and Fisher 2002; Bielmyer et al. 2006), it is unclear whether this

sensitivity is unique to *A. tonsa* or if it applies to other zooplankton. The objective of the current study was to expand the diversity of marine zooplankton for which the sensitivity to dietary Ag has been examined. In this experiment, we selected larvae of the sea urchin *Lytechinus variegatus*, which is distributed throughout the Caribbean from southern Florida to Venezuela (Hendler et al. 1995).

Echinoderm larvae have been used as a model test organism for >30 years, with the majority of studies focusing on sperm fertilization assays and early larval development to the pluteus stage (Dinnel et al. 1987; United States Environmental Protection Agency [USEPA] 1995; American Society for Testing and Materials 1998). Echinoderm larval development is sensitive to waterborne metal exposures (Nipper et al. 1993; Rumbold and Snedaker 1997; Bielmyer et al. 2005). However, considering that larvae do not begin feeding until they reach the pluteus stage, the standard 48- to 72-h early development protocol typically used in waterborne studies is not appropriate for dietary studies. As a result, we chose to begin exposure at the pluteus stage (16–20 h in *L. variegatus*) and continue through 18 days when the larvae are approaching metamorphosis (George et al. 2004). As end points, we selected whole-body and post-oral arm length as measures of larval growth.

Methods and Materials

Experimental Animals

Reproductively ripe adult *L. variegatus* were obtained from Biscayne Bay, FL, and transported to the University of Miami, where spawning was induced the same day by injecting 1 ml 0.5 M KCl into the haemocoel. Gametes from individual echinoderms were inspected using a microscope, and only properly developed gastrula were used for testing (USEPA 1995).

Algal Diet

The algae *Isochrysis galbana* was used as algal diet in this experiment. Algae were cultured in natural seawater from Bear Cut, FL (salinity 34 psu, pH 8.1, dissolved organic carbon (DOC) 110 μM). Seawater was filtered (0.2 μm), autoclaved, and spiked with F/2 micronutrients (except ethylene diamine tetraacetic acid [EDTA]) (Guillard 1975). Cultures were maintained at 21 °C in 1-l flasks under continuous light and aeration. Six 1-l cultures of algae (one for each treatment) were continuously maintained during the course of the experiment. Seven days before test initiation, each culture was spiked with Ag (as $AgNO_3$) from either a 612 μg l^{-1} or 25.5 mg l^{-1} Ag stock solution, depending on concentration. Each stock solution had ^{110}Ag (Eckert and Ziegler, Valencia, CA) added as a radiotracer to facilitate measurement of test concentrations. For the 612 μg l^{-1} Ag stock solution, 0.19 μCi ml^{-1} ^{110}Ag was added yielding a specific activity of 395 cpm ng^{-1} Ag, whereas 6.3 μCi ml^{-1} ^{110}Ag was added to the 25.5 mg l^{-1} Ag stock solution resulting in a specific activity of 322 cpm ng^{-1} Ag.

Nominal waterborne Ag concentrations of 0, 0.1, 0.25, 0.5, 1, and 5 μg l^{-1} were targeted. Waterborne Ag concentrations in each culture were measured daily by centrifuging 1 ml of each culture at 16,000×*g* for 5 minutes and then assaying the supernatant for radioactivity using a gamma counter. Additional Ag was then added as necessary to maintain approximately constant waterborne Ag concentrations.

Algae were cultured in the presence of Ag for 7 days before initiation of the echinoderm exposure. Ag concentrations in the different algal cultures were determined daily for the 7-day pre-exposure period and then on each day algae were fed to echinoderm larvae. To determine algal Ag concentrations, the cell density of each culture was determined spectrophotometrically. A 0.5-ml aliquot of algae was centrifuged at 16,000×*g* for 1 minute, the supernatant decanted, and the algal pellet rinsed with autoclaved seawater. This process was repeated two additional times, and the algal pellet was assayed for radioactivity.

The algal cultures were renewed every 3 to 5 days whenever algae moved out of log-growth phase and cell density began to decrease. Culture renewal was accomplished by decanting 70 % of the culture and renewing with fresh autoclaved seawater, F/2 nutrients, and Ag as appropriate.

Echinoderm Dietary Exposures

Echinoderm larvae at the gastrula stage (approximately 6 h) were transferred into a single 2-l polycarbonate bottle with 0.2 μm filter-sterilized seawater at a density of 500 larvae l^{-1} and fed 1 × 10^5 cells ml^{-1} of Ag-laden *I. galbana*. One bottle was used per Ag treatment, and bottles were given gentle aeration to maintain larvae in suspension. Larvae were maintained at 23–25 °C under a 16:8–h light-to-dark cycle for the study duration. Larvae begin feeding when they reach the pluteus stage (approximately 16–20 h), and after 1 day, all larvae had reached this developmental stage. At this time, larvae were split into three replicate 1.2-l polycarbonate bottles per Ag treatment at a density of 0.3 larvae ml^{-1}. Larvae were fed 2 × 10^4 cells/ml of the appropriate Ag-laden *I. galbana*, and test bottles were sealed and placed on a mechanical roller table at approximately 1 revolution min^{-1} to maintain larvae in suspension. On days 1, 4, 7, 11, and 14, water changes were

performed by gently pouring the contents of each test bottle through a container with an 80-μm filter in the bottom to retain the larvae. The filter was submerged in seawater to prevent damage to larval arms. Retained larvae were then gently rinsed back into the test container with fresh-filter sterilized seawater, and fresh Ag-laden algae was added.

On day 7, larvae (approximately 30–40) were subsampled from each test container and preserved in 10 % buffered formalin for later size measurements. The test was terminated on day 18 before metamorphosis, and additional larval samples were collected and preserved from each test container.

Larval growth was determined by measuring both body and post-oral arm length in 10 animals from each replicate at the two sampling time points (days 7 and 18). Length was determined using a dissecting microscope and calibrated ocular micrometer.

Analytical Chemistry

Dissolved Ag concentration in the stock solutions was determined after passing samples through a 0.45-μm cellulose nitrate syringe filter (Acro-Disc; Pall Life Sciences, MI) and acidifying by addition of HNO_3 (Fisher Scientific, trace-metal grade) to a final concentration of 1 %. Ag concentrations in stock solutions were analyzed by graphite furnace atomic absorption spectrophotometry (Varian 220Z; Varian, Walnut Creek, CA). The background dissolved Ag concentration in seawater was measured by multi collector-inductively coupled plasma–mass spectrometry using a standard bracketing technique with a detection limit of 200 pg l^{-1}. Considering that seawater was diluted by a factor of 100 before the analysis, an upper limit of 20 ng l^{-1} was determined for the Ag concentration in seawater. A gamma counter (Tm Analytic) was used to measure ^{110}Ag radioactivity using specific detection windows as outlined previously (Hansen et al. 2002). Total Ag concentration in each treatment was determined from sample activity and measured specific activity of the Ag stock solution, thus allowing for a method detection limit of 0.01 μg l^{-1} Ag. To calculate Ag concentrations in the algal diets, cell-density measurements were converted to dry-weight mass. Algal dry weights were determined using the methods of Xu and Wang (2004). Briefly, algal cells were filtered onto a preweighed filter, rinsed with 0.5 M ammonium formate, and dried at 80 °C for 1 day. Sample radioactivity in the algal pellets was then used to determine algal Ag concentrations.

Data Analysis

All analyses were performed on measured test concentrations. Data are presented as mean ± SEM and $n = 3$ in all cases. Significant echinoderm growth effects were assessed using analysis of variance followed by *post hoc* Dunnett's test to determine no–observable effect concentration (NOEC) and lowest–observable effect concentration (LOEC) values. In addition, linear interpolation was used to estimate the EC_{20} (USEPA 2002).

Results

Ag Concentrations in Water and Algae

The background Ag concentration in seawater did not exceed 0.02 μg l^{-1}. Mean measured waterborne Ag concentrations (using the radiotracer method) in water from algal cultures were 50–78 % of nominal (Table 1). However, given that background Ag may be as high 0.02 μg l^{-1} (it is most likely much lower), these concentrations should be treated with caution, particularly at the low end of the range. In the remainder of this article, we report concentrations assuming background Ag was negligible. Waterborne Ag, as measured by radioisotope tracer, was not detected at any point in the echinoderm exposure chambers (<0.01 μg l^{-1} added Ag), indicating that leaching of Ag from the algal diet to the water was minimal.

The corresponding silver concentrations in algae were relatively stable in all test concentrations except the highest treatment (3.88 μg l^{-1} Ag). In this treatment, algal Ag was significantly greater during the pretest equilibration phase of the exposure, with an initial concentration of 23.8 μg g^{-1} dw and then a gradual decrease throughout the rest of the experiment (Fig. 1). The Ag concentration in this treatment when first fed to echinoderms (day 0 in Fig. 1), was 16.6 μg g^{-1} dw and decreased to 8.2 μg g^{-1} dw on the last day larvae were provided fresh algae (day 14). The exact mechanism for this decrease in concentration over time is unknown, but the data suggest that the observed effects might simply be the result of growth dilution because the

Table 1 Measured waterborne and algal Ag in the algal cultures (mean ± SEM)[a]

Nominal Ag (μg l^{-1})	Waterborne Ag (μg l^{-1})	Dietary Ag (μg g^{-1} dw)
0	≤0.02	<0.03
0.1	0.05 ± 0.01	0.68 ± 0.06
0.25	0.16 ± 0.02	1.14 ± 0.12
0.5	0.32 ± 0.03	1.90 ± 0.27
1	0.64 ± 0.08	3.57 ± 0.62
5	3.88 ± 0.27	10.69 ± 1.41

[a] Ag concentrations reported using ^{110}Ag radiotracer. Background Ag measured as <0.02 μg l^{-1} such that waterborne Ag may be ≤0.02 μg l^{-1} greater than reported for each treatment

decrease in algal Ag concentrations approximately corresponded with the time period when algal specific growth rates were high and Ag concentrations stabilized when specific growth rates were low (Fig. 2). It is possible that growth rates, and therefore Ag accumulation, were sufficiently high within the 24-h period between solution renewals to cause decreases in waterborne Ag. Alternative explanations, such as adaptation to increased Ag leading to decreased metal uptake, are also possible.

Based on mean measured Ag concentrations in algae, bioconcentration factors (BCFs) ranged from 13,600 in the lowest treatment to 2,755 in the highest Ag treatment. As has been observed for other metals in numerous studies (McGeer et al. 2003), a log-linear relationship was observed between BCF and exposure concentration (Fig. 3).

Effect of Dietary Ag on Echinoderm Growth

Body length and post-oral arm length were measured in a subsample of larvae from each treatment replicate on day 7 and at test termination on day 18. On day 7, no significant effects were observed in any of the Ag treatments tested, with a waterborne NOEC of 3.88 μg l^{-1} and corresponding dietborne NOEC of 10.68 μg g^{-1} dw Ag (Fig. 4). In contrast, on day 18, a statistically significant decrease in both body and post-oral arm length was observed in all treatments tested relative to the control, resulting in a NOEC of <0.05 μg l^{-1} Ag (<0.68 μg g^{-1} dw in the diet) and LOEC of 0.05 μg l^{-1} Ag (0.68 μg g^{-1} dw in the diet). Although this LOEC suggests that echinoderms are quite sensitive to dietary Ag exposure, decreases in both body length and post-oral arm length were relatively modest (approximately

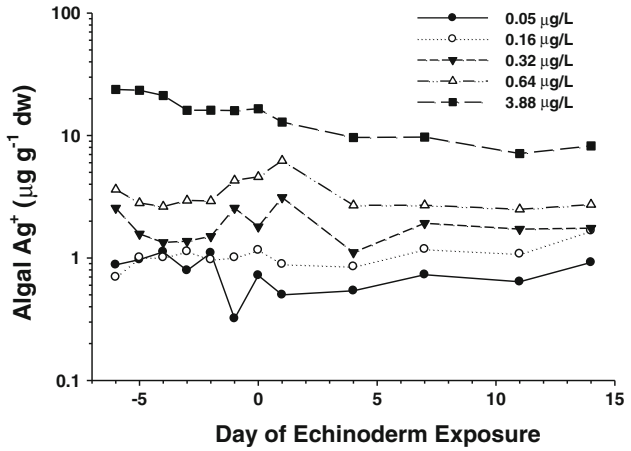

Fig. 1 Silver concentrations in algal diets (μg g^{-1} dw) over time at different waterborne Ag concentrations. Day 0 indicates the first day algae were fed to echinoderm larvae

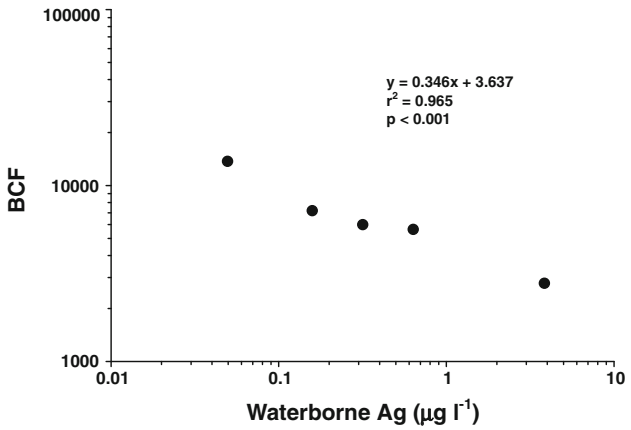

Fig. 3 Relationship between mean waterborne Ag concentration and BCF for algae

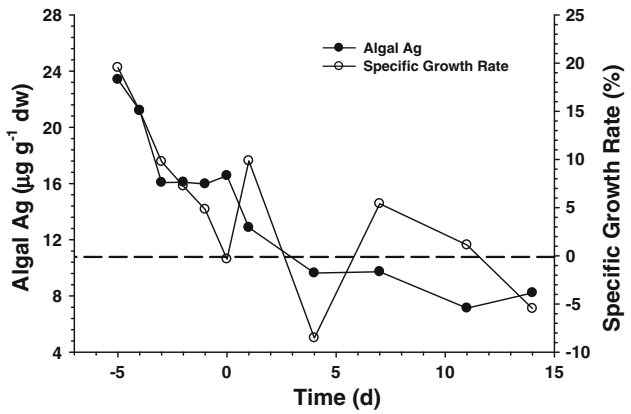

Fig. 2 Silver concentrations in algal diets (μg g^{-1} dw) over time in the highest waterborne Ag exposure and corresponding specific growth rates in the same treatment. *Dashed line* indicates 0 % specific growth

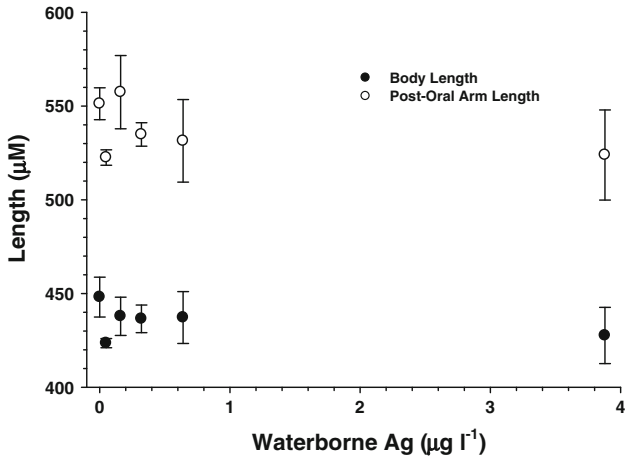

Fig. 4 Effects of dietary Ag on day 7 echinoderm whole-body and post-oral arm length. Exposure is expressed as the waterborne Ag concentration to which the algae were exposed (μg l^{-1}). (mean ± SEM; $n = 3$)

15 %), and the observed dose–response relationships were flat for both end points with no significant difference in effects between the lowest and highest Ag treatments (Fig. 5). This observation is supported by regression-based analysis of the data, which lead to estimated EC_{20} values of >3.88 μg l^{-1} Ag (>10.68 μg g^{-1} dw Ag in the diet) for both end points.

Discussion

The toxicity of dietary Ag to marine planktivores has previously been characterized in the copepod *A. tonsa*. Hook and Fisher (2001b) were the first to conduct this type of experiment, and they showed that dietary Ag inhibited copepod egg hatching success at 4.2 μg g^{-1} dw Ag, corresponding to a waterborne Ag concentration of 0.22 μg l^{-1} Ag. The experimental design for this study was somewhat unusual in that the copepods were only pulse-exposed to dietary Ag. Bielmyer et al. (2006) repeated this study using a continuous-exposure experimental design and observed effects on both survival and reproduction with survival being the most sensitive end point. They estimated an EC_{20} for copepod survival of 1.6 μg g^{-1} Ag in the diet, corresponding to 0.46 μg l^{-1} waterborne Ag.

Results from the current study suggests that echinoderm larvae are also sensitive to dietary Ag exposure with significant effects on growth observed at the lowest concentration tested (0.05 μg l^{-1} waterborne Ag [0.68 μg g^{-1} dw dietary Ag]). However, the observed flat dose response leads to an estimated EC_{20} greater than the highest concentration tested (3.88 μg l^{-1} waterborne Ag [10.68 μg g^{-1} dw dietary Ag]).

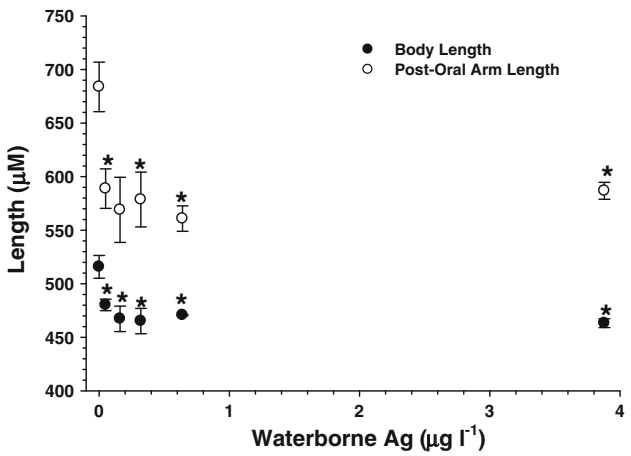

Fig. 5 Effects of dietary Ag on day 18 echinoderm whole-body and post-oral arm length. Exposure is expressed as the waterborne Ag concentration to which the algae were exposed (μg l^{-1}). (Mean ± SEM; $n = 3$). *Significantly ($p < 0.05$) different from the control

There are several key points of comparison with the previously described copepod studies. First, the daily respiking of algal cultures with Ag to achieve a constant waterborne Ag concentration, combined with the extended equilibration of algae with waterborne Ag in the current study, represents a significant departure from previous methodologies. Hook and Fisher (2001b) only exposed algae to waterborne Ag for 4 days, whereas Bielmyer et al. (2006) exposed algae to waterborne Ag for 7 days before harvesting and feeding to copepods. In both studies, waterborne Ag exposure was accomplished by a single spike of Ag at the beginning of the 4–7 days of exposure, and waterborne Ag decreased rapidly during the first 48 h of exposure. We hypothesized that this methodology might lead to a substantial underestimate of the bioconcentration potential of algae exposed to waterborne Ag and that if a constant waterborne exposure was maintained, dietary Ag concentrations would be significantly greater than previously observed.

This hypothesis was shown to be valid at low waterborne Ag concentrations but not at high concentrations. In the current study, we observed an inverse relationship between waterborne Ag exposure and BCF for algae, with BCFs ranging from 13,600 in the lowest treatment to 2,755 in the highest treatment (Fig. 3). In contrast, Bielmyer et al. (2006) observed the opposite, with the lowest BCF (approximately 1,000) in the lowest treatment and the highest BCF in the highest treatment (approximately 22,000). The net result of the differences is that despite approximately comparable waterborne Ag exposures, dietary Ag was an order of magnitude greater in the current study for the lowest treatment and an order of magnitude greater in the study Bielmyer et al. (2006) for the highest treatment. Clearly, the manner in which algae are dosed has a dramatic impact on corresponding dietary metal exposures, and we suggest using the method employed in the current study because continuous exposure is more likely to reflect environmentally realistic scenarios.

Another important observation from the current study is that similar to both the reproductive effects observed by Hook and Fisher (2001b) and the survival effects observed by Bielmyer et al. (2006), the dose–response relationship in the echinoderm study was quite flat with a approximately 15 % inhibition of growth observed at both the lowest and highest Ag concentration tested (factor of 16 difference based on Ag in the algal diets). Hook and Fisher observed a constant approximately 30 % decrease in egg production over more than nearly a factor of 10 in dietary Ag exposure concentrations, and Bielmyer et al. (2006) observed a constant approximately 25 % decrease in copepod survival on exposure to dietary Ag ranging from 1 to 24 μg g^{-1} dw. A further increase in effects with a 65 % decrease in survival was only observed after exposure to very high (120 μg g^{-1} dw) dietary Ag. Hence, the statistically

significant but flat dose–response relationship appears characteristic of dietary Ag exposure and, in fact, several other metals. For example, similar flat dose–response relationships have been observed for Cd, Cu, Ni, and Zn in copepods (Hook and Fisher 2001a, 2002; Bielmyer et al. 2006) and for Zn in daphnids (De Schamphelaere et al. 2004). The mechanism(s) underlying this atypical dose–response relationship remains to be determined.

Conclusion

Overall, results from the current study suggest that copepods are not the only marine zooplankton group sensitive to dietary Ag exposure and that existing/proposed WQC may be underprotective of this exposure pathway for at least two major groups of zooplankton. This conclusion is tempered by the relatively modest effects (15 % inhibition) that were observed over a relatively wide range of dietary Ag exposure concentrations. The ecological implications of such modest effects should be given careful consideration when evaluating these data for use in setting WQC. Given the current results, further research into the diversity of zooplankton that might be affected by dietary Ag and other metals, as well as the mechanisms underlying the observed flat dose–response relationships, seems warranted.

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