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Physiological effects of chronic silver exposure in *Daphnia magna*☆

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Abstract

Daphnia magna were exposed to a total concentration of 5.0 ± 0.04 μg Ag/l, added as $AgNO_3$ (dissolved concentration, as defined by 0.45 μm filtration $= 2.0 \pm 0.01$ μg Ag/l) in moderately hard synthetic water under static conditions (total organic carbon $= 4.80 \pm 1.32$ mg/l) with daily feeding and water renewal, for 21 days. There was no mortality in control daphnids and 20% mortality in silver-exposed animals. Silver exposure caused a small but significant reduction of reproductive performance manifested as a 13.7% decrease in the number of neonates produced per adult per reproduction day over the 21-day exposure. However, silver exposed daphnids also exhibited a much more marked ionoregulatory disturbance, which was characterized by a 65% decrease in whole body Na^+ concentration, and an 81% inhibition of unidirectional whole body Na^+ uptake. Previous work on the acute toxicity of Ag^+ to daphnids has shown that Na^+ uptake inhibition is directly related to inhibition of Na^+,K^+-ATPase activity. Therefore, we suggest that the Na^+ uptake inhibition seen in response to chronic silver exposure was explained by an inhibition of the Na^+ channels at the apical 'gill' membrane, since a 60% increase in whole body Na^+,K^+-ATPase activity was observed in the chronically silver-exposed daphnids. Our findings demonstrate that, in broad view, the key mechanism involved in chronic silver toxicity in *D. magna*, the most acutely sensitive freshwater organism tested up to now, resembles that described for acute toxicity—i.e. ionoregulatory disturbance associated with inhibition of active Na^+ uptake, though the fine details may differ. Our results provide encouragement for future extension of the current acute version of the Biotic Ligand Model (BLM) to one that predicts chronic silver toxicity for environmental regulation and risk assessment. The results strongly suggest that Na^+ uptake inhibition is the best endpoint to determine sensitivity to both acute and chronic toxicity in the scope of future versions of the BLM for silver.

Keywords: Biotic Ligand Model; Chronic toxicity; *Daphnia magna*; Ionoregulation; Na^+,K^+-ATPase; Reproductive inhibition; Silver; Sodium balance

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1. Introduction

The 'Biotic Ligand Model' (BLM) is a modeling approach to predict metal toxicity to freshwater organisms, which takes into account the geochemistry of a biological ligand, the gill (Janes and Playle, 1995; Paquin et al., 1999). According to this model, the gill is considered a negatively charged ligand to which Ag^+ can bind. Toxic effects are considered a function of the degree of saturation of 'toxic sites' on the biotic ligand by Ag^+. Current versions of the BLM consider the competition between other cations and Ag^+ for 'toxic binding sites' on the gills as well as the influence of different complexing agents on silver speciation and availability (Paquin et al., 1999; McGeer et al., 2000). Recent studies have demonstrated that these 'key toxic sites' for acute Ag^+ toxicity are the Na^+,K^+-ATPase molecules located at the basolateral membrane of the gill epithelium in both freshwater fish (rainbow trout; Morgan et al., 1997; McGeer and Wood, 1998; Bury et al., 1999) and invertebrates (daphnids and crayfish; Bianchini and Wood, 2002; Grosell et al., 2002). In these animals, the gills are the main organ involved in the active transport of Na^+ and Cl^- from the water into the extracellular fluid, and branchial Na^+,K^+-ATPase activity plays a direct role in this transport (Péqueux, 1995; Flik et al., 1997; Perry, 1997; Castilho et al., 2001). This ultimate mechanism of toxicity may be preceded by an Ag^+-induced blockade of the apical Na^+ channels in ionocytes (Bury and Wood, 1999). The intervening processes (i.e. the pathway by which silver moves through the cytosol from apical to basolateral membranes and its exact actions at the Na^+,K^+ATPase molecule) remain unclear, though recent evidence suggests that Ag^+ competes at the Mg^{2+} site on the enzyme (reviewed by Wood et al., 1999). Thus, the initial inhibition at the apical Na^+ channels and the final inhibition of branchial Na^+,K^+-ATPase activity induced by acute Ag^+ exposure leads to lower sodium uptake across the gills and causes ionoregulatory failure and death in these animals.

To date, the bulk of information on silver toxicity has been restricted to acute exposures (reviewed by Ratte, 1999; Wood et al., 1999). There have been only a few chronic investigations, even in the most studied fish species for silver effects, the rainbow trout (e.g. Davies et al., 1978; Nebeker et al., 1983), and just three that have addressed possible mechanism(s) of toxicity (Galvez et al., 1998; Guadagnolo et al., 2001; Brauner and Wood, 2002). These have suggested that ionoregulatory disturbance is again involved, but the exact mechanism(s) remain unclear. Furthermore, attempts to describe the physiological mechanism of chronic silver toxicity in the most acutely sensitive freshwater organisms—i.e. cladocerans and amphipods (Ratte, 1999; Bianchini et al., 2002b)—are completely lacking from the literature. In order to extend the present (acute) version of the silver BLM (Paquin et al., 1999) for the prediction of chronic silver toxicity, it will be critical to understand the key mechanism of chronic toxicity in daphnids and other sensitive freshwater invertebrates, and specifically to assess whether it is the same as that of acute toxicity—i.e. inhibition of active Na^+ uptake by blockade of Na^+,K^+-ATPase. However, this has never been demonstrated. Therefore, the main objective of the present study was to analyze the mechanism of chronic silver toxicity in *Daphnia magna*.

2. Materials and methods

2.1. Daphnia maintenance

Colonies (ARO strain, lot #090600 DM) of adult gravid *Daphnia magna* (Crustacea, Cladocera) were obtained from Aquatic Research Organisms (ARO, Hampton, NH, USA). The brood origination of these colonies was United States Environmental Protection Agency (Cincinnati, OH, USA) and the daphnids had been reared in a freshwater static renewal system with water saturated in dissolved oxygen, pH 7.5, hardness ~150 mg $CaCO_3$/l and 25 °C. *D. magna* were fed phytoplankton and YCT (a slurry of yeast, Cerophyll and trout chow). Upon arrival at our laboratory, the *D. magna* colonies were gradually acclimated to synthetic water in an incubator. To ensure standardized conditions for water chemistry, synthetic, moderately hard water used for all tests was prepared as a single batch employing 1000 l of reverse osmosis purified water in a food-grade polyethylene tank. This water was reconstituted to the following composition: 1.0 mM $CaCO_3$, 0.15 mM $MgSO_4$ and 0.6 mM NaCl. It was bubbled with pure CO_2 for 24 h to ensure that $CaCO_3$ went into solution and then was bubbled with air for 48 h to ensure removal of excess CO_2 and atmospheric equilibration. Water was then left to stand

for at least 2 weeks prior to use. During the acclimation period, as well as during subsequent experiments, *D. magna* were fed algae (*Ankistrodesmus convolutus*; 1.82×10^8 cells/l = 33 mg dry weight/l) and YCT (18.5 mg dry weight/l). Water was not aerated, but renewed daily, as was food. Temperature was maintained at 22–23 °C for the first 24 h of the acclimation period and was then fixed at 20 °C. Photoperiod was fixed at 16.00 L:8.00 D.

After *D. magna* acclimation, reproductive rate was measured to ensure it met established criteria for a healthy population (15–20 neonates per adult every 3–4 days). Provided that the reproductive rate was satisfactory, we proceeded with the particular colony. Neonates (<24 h) were collected for experiments using plastic pipettes.

2.2. Chronic silver exposure

Neonate daphnids were chronically exposed to a single concentration (total measured = 5.0 ± 0.04 μg Ag/l) of silver as $AgNO_3$ or maintained in control conditions, for 21 days. Test procedures followed the ASTM standard guide for conducting *Daphnia magna* life-cycle toxicity tests (ASTM, 1997). It is important to note that the total silver concentration employed in this study is close to the 48h-LC50 (6.9 μg Ag/l) calculated based on measured total silver and reported for neonates *D. magna* under the same experimental conditions, including the same feeding regime (Bianchini and Wood, 2001a). Also, it should be noted that the presence of food greatly elevates the 48 h-LC50 value relative to that value (0.26 μg Ag/l) derived from tests where food is absent (Bianchini et al., 2002a). This was because food binds Ag^+, rendering it less bioavailable.

Two parallel experiments were run, both employing a static-renewal exposure regime. In the first experiment, 10 neonates were individually exposed, each in a separate plastic beaker containing 50 ml of synthetic water, pre-equilibrated to 20 °C. Another ten neonates were individually transferred to 50 ml beakers under the same experimental conditions, but without silver.

In the second experiment, two groups of thirty neonates were used. One group was maintained under control conditions (without silver) and the other was chronically exposed to silver in beakers containing 1 l of synthetic water, again with daily water renewal and feeding.

All plastic ware used was new and was acid-washed in 1% HNO_3 (trace metal grade; Merck Chemicals) and rinsed thoroughly with synthetic water prior to use.

$AgNO_3$ (SigmaUltra, Sigma Co., St. Louis, MO) was added into the test solution 3 h prior to introduction of *D. magna* employing a stock solution (1 mg/l) acidified with 1% HNO_3. Total silver concentration in the stock solution was checked by graphite furnace atomic absorption spectrometry (GF-AAS; Varian AA-1275 with GTA-9 atomizer). Every week, total and filtered (Acrodisc 0.45 μM polyethersulfone in-line filters; Gelman) silver concentrations, as well as total organic carbon (TOC) concentrations in the exposure solutions were followed over the experiment (time zero and 24 h). Silver concentration in filtered and non-filtered water samples (2 ml) was determined using the GF-AAS described above. Samples for total organic carbon (TOC) analyses (10 ml) were stored in acid-cleaned borosilicate vials. Measurement was conducted on a Dohrmann Organic Carbon Analyzer after removing inorganic carbon by acidification with one drop of concentrated nitric acid and purging for >5 min with a stream of nitrogen gas.

After 3 h pre-equilibration, neonates were then placed in the assigned beakers. All neonates were maintained without aeration and under the feeding regime adopted during the acclimation period. Measurements were made to ensure that water remained sufficiently oxygenated (90 ± 8% saturation) and the water pH constant (8.20 ± 0.07) over the 24-h period between each water renewal. Temperature and photoperiod were maintained at 20 °C and 16.00 L:8.00 D, respectively. After 24 h, daphnids were transferred to a new set of test solutions prepared 3 h prior to transfer as previously described. Every 24 h and for 21 days, daphnids were removed from the original beakers and transferred using plastic pipettes.

2.3. Survival, growth and reproduction

Every day during the 21-day test, survival and reproduction in daphnids from the first experiment were checked. The death criteria adopted were a change to milky coloration and lack of movement even after mild stimulation. To assess reproduction, neonates produced in each beaker were counted and discarded at the moment of daily water change. After 21 days of test, the following reproductive

parameters were compiled: total number of neonates produced; time to first brood; number of broods; number of young produced per brood; number of reproduction days; and number of young produced per adult per reproduction day.

At the end of the test, living daphnids from the first experiment were: collected in each treatment (with and without silver) using plastic pipettes; washed (15 s) in deionized water; dried on filter paper (Whatman no. 1); transferred to pre-weighed pieces of aluminum foil; and dried (60 °C) until constant weight. Aluminum foil and daphnid dry weight were determined using an electronic microscale (Mettler UMT2; 0.001 mg accuracy).

2.4. Whole body Na^+,K^+-ATPase activity, Na^+ uptake and Na^+ concentration measurements

After 21 days of exposure, measurements of whole body Na^+,K^+-ATPase activity, Na^+ uptake, and Na^+ concentration were made in living daphnids from the second experiment.

In order to analyze the effect of silver on whole body Na^+,K^+-ATPase activity, eight daphnids were: randomly collected in each treatment (with and without silver) using plastic pipettes; washed (15 s) in deionized water; dried on filter paper (Whatman no. 1); transferred to plastic assay tubes; and frozen (−70 °C) until Na^+,K^+-ATPase activity measurement. For the enzyme activity assay, samples were thawed and kept on ice throughout the analysis. They were homogenized in 0.5 ml ice-cold buffer solution (150 mM sucrose, 10 mM EDTA, 50 mM imidazole and 11.5 mM sodium deoxycholate) and centrifuged at $5000 \times g$ for 30 s, at 4 °C. Enzyme activity was measured in the supernatant using the method described by McCormick (1993) with modifications according to Wheatly and Henry (1987). Two reaction mixtures were assayed. The reaction mixture A consisted of 20 μl of sample, 50 μl of salt solution A and 150 μl of working solution A. The reaction mixture B consisted of 20 μl of sample, 50 μl of salt solution B and 150 μl of working solution B. Salt solution A contained 100 mM NaCl, 10.5 mM $MgCl_2$, 30 mM KCl and 50 mM imidazole, pH adjusted to 7.5. In the salt solution B, NaCl replaced KCl at the same concentration. Working solution A contained 4 u/ml lactate dehydrogenase, 5 u/ml pyruvate kinase, 2.8 mM phosphoenolpyruvate, 3.5 mM ATP, 0.22 mM NADH and 50 mM imidazole, pH adjusted to 7.5. To obtain the working solution B, 1 mM ouabain was added. Kinetic assays were then run in duplicate at 25 °C in a temperature-controlled microplate reader (Molecular Devices, Menlo Park, CA, USA), for 10 min. An ADP standard curve (0–20 nmoles/10 μl) was also run. Na^+,K^+-ATPase activity was then calculated considering the difference in ADP production between the two reaction mixtures (A and B). Protein content in the homogenate was measured using Bradford reagent (Bio-Rad, Richmond, CA, USA). Enzyme activity was then expressed as μmoles ADP/mg protein/h.

The effect of silver on whole body unidirectional sodium influx was analyzed in eight daphnids which were randomly collected in each treatment (with and without silver) and transferred to 250 ml of fresh experimental medium prepared as previously described and containing ^{22}Na (10 μCi/l, Amersham, specific activity 303 Ci/g Na^+). Water samples for measurement of ^{22}Na radioactivity and total sodium were taken at 0 and 1 h. These samples were used for ^{22}Na radioactivity measurement using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra–Packard, Toronto, ON, Canada) and total Na^+ measurement using the Varian AA-1275 atomic absorption unit operated in flame emission mode. After the 1-h flux period, daphnids were collected using plastic pipettes, washed for 15 s in a concentrated (600 mM) NaCl solution to displace ^{22}Na loosely bound to the surface, dried on filter paper, weighed on an electronic microscale (Mettler UMT2; 0.001 mg accuracy) and transferred to plastic vials. The ^{22}Na radioactivity in the whole body was then measured as described for water samples. Na^+ uptake rate was calculated based on the incorporation of ^{22}Na in the whole body during the 1-h flux period, the mean specific activity of the ^{22}Na in the water, the animal's body weight and the elapsed time.

Whole body sodium concentrations were analyzed in eight daphnids randomly collected in each treatment (with and without silver) using plastic pipettes. Daphnids were then washed (15 s) in deionized water, dried on filter paper (Whatman no. 1), weighed in the electronic microscale, transferred to plastic assay tubes, and digested in 50 μl of concentrated (95–97%) H_2SO_4 (Merck Chemicals) overnight at 60 °C. After digestion, 1.5 ml of deionized water was added to the digested

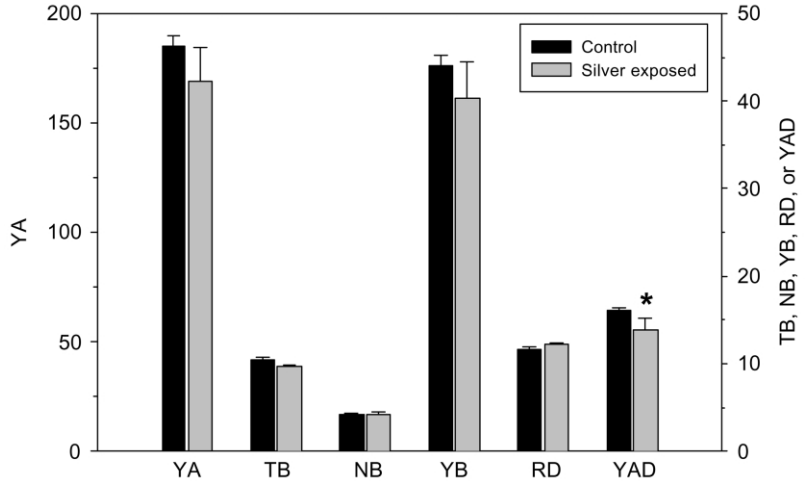

Fig. 1. Reproductive parameters in *Daphnia magna* maintained in control conditions or exposed to 5.03-μg total Ag/l, for 21 days. YA = mean number of young produced per adult; TB = time to first brood (days); NB = number of broods produced; YB = mean number of young per brood; RD = mean number of reproduction days; and YAD = mean number of young produced per adult per reproduction day. Data are expressed as mean ± 1 standard error (control $n = 10$; silver exposed $n = 8$). *Indicates significant different mean between treatments (control and silver exposed).

sample. Sodium concentration was then measured as described for water samples.

2.5. Data presentation and statistical evaluation

All values were expressed as mean ± 1 standard error of mean (S.E.M.). Significant differences ($P < 0.05$) in reproduction, growth, whole body Na^+,K^+-ATPase activity and Na^+ concentration between treatments were assessed by Student's two-tailed *t* test

3. Results

Measured mean silver concentrations in the two experiments were 5.0 ± 0.04 μg Ag/l (total) and 2.0 ± 0.01 μg Ag/l (after 0.45-μm filtration), indicating a high (60%) but variable fraction of non-dissolved silver, presumably associated with the presence of food. Mean TOC value over 24 h of test (i.e. between water renewals) was 4.80 ± 1.32 mg/l.

Data from the individually exposed daphnids ($N = 10$ per treatment) showed survival rates of 80% in the presence of silver and 100% in the absence of silver. No significant effect of silver was observed on the growth of living daphnids (at day 21, control dry weight = 0.98 ± 0.06 mg; silver exposed dry weight = 0.90 ± 0.08 mg).

Although there was no silver effect on growth, significant changes were observed in both reproduction and the physiological and biochemical parameters analyzed. Regarding reproduction, a significant decrease in the total number of neonates produced (control = 1850 neonates; silver exposed = 1352 neonates) and a slight but significant reduction (13.7%) in the number of young produced per adult per reproduction day (YAD) was observed after 21 days of silver exposure. The other five reproductive parameters analyzed were not significantly affected by chronic silver exposure (Fig. 1).

The most remarkable effects of chronic silver exposure were observed in the physiological and biochemical parameters measured. Whole body Na^+ concentration was significantly reduced by 65% after chronic silver exposure (Fig. 2). Measurements of unidirectional Na^+ influx showed that chronic silver exposure significantly inhibited whole body sodium uptake by 81% despite a 60% increase in whole body Na^+,K^+-ATPase activity (Fig. 2).

4. Discussion

4.1. Ionoregulatory responses to chronic silver exposure

Acute silver toxicity is attributable to ionic Ag^+, and is directly related to an inhibition of the

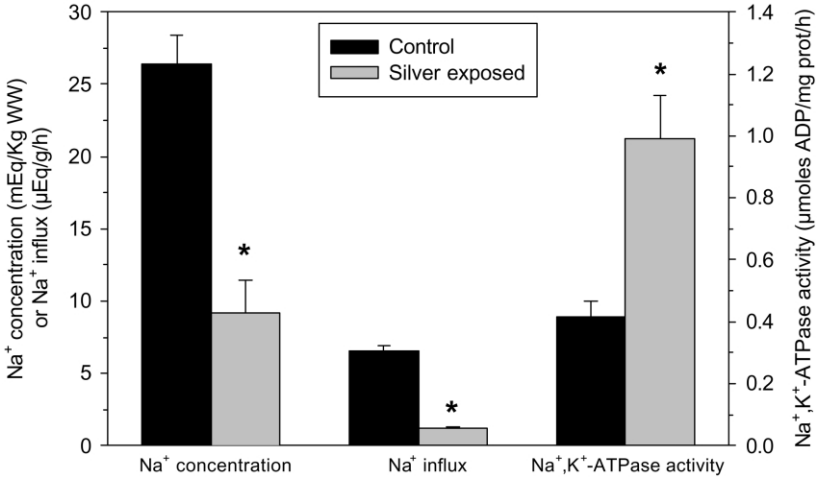

Fig. 2. Whole body sodium concentration, whole body sodium uptake rate and whole body Na^+,K^+-ATPase activity in *Daphnia magna* maintained in control conditions or exposed to 5.03-µg total Ag/l, for 21 days. Data are expressed as mean ± 1 standard error ($n=8$). *Indicates significant different mean between treatments (control and silver exposed).

Na^+,K^+-ATPase located at the basolateral membrane of the gill epithelium in freshwater fish (Morgan et al., 1997; Wood et al., 1999) and crustaceans (Bianchini and Wood, 2002; Grosell et al., 2002). The enzymatic inhibition induced by acute silver exposure in these animals leads ultimately to ionoregulatory failure and death. In the present study, we provide the first evidence as to the nature of the chronic mechanism of silver toxicity in daphnids, the most acutely sensitive freshwater organisms (Ratte, 1999; Bianchini et al., 2002b). Note that the acute LC50 (6.9 µg Ag/l) and chronic EC50 (4.7 µg Ag/l) values for silver are very similar in the presence of food (Bianchini and Wood, 2001a), and much higher than the acute LC50 value (0.26 µg Ag/l) in the absence of food (Bianchini et al., 2002a). The chronic mechanism appears to be clearly associated with ionoregulatory disturbance, though in fine detail it may differ from the acute mechanism. After 21 days exposure in the presence of food to a total silver concentration approximating the EC50, the ionoregulatory disturbance was characterized by a decrease of 65% in whole body Na^+ concentration, an 81% decrease in unidirectional Na^+ uptake, yet a 60% increase in whole body Na^+,K^+-ATPase activity. This may be compared and contrasted with our earlier observations of approximately a 40% decrease in whole body Na^+ concentration associated with a 40% decrease in unidirectional Na^+ uptake and a 90% decrease in whole body Na^+,K^+-ATPase activity in daphnids after 48-h exposure to the acute LC50 level of silver in the absence of food (Bianchini and Wood, 2002). Clearly, daphnids are capable of surviving much larger losses of Na^+ during chronic exposure when food is present, and are capable of mounting compensatory measures. The importance to the development of a chronic BLM is the finding that chronic toxicity appears to be exerted through an ionoregulatory mechanism, an issue whose regulatory implications are summarized below.

We speculate that the very large Na^+ uptake inhibition during chronic exposure is explained by an inhibition of the Na^+ channels at the apical 'gill' membrane. This statement is based on three findings. Firstly, in the present study, there was no inhibition but rather a 60% increase in whole body Na^+,K^+-ATPase activity in chronically silver-exposed *D. magna*. In this context, it is important to mention that synthesis of new Na^+,K^+-ATPase units induced by environmental Na^+ depletion, which if not compensated leads to a whole body Na^+ depletion, has been widely reported in the crustacean literature (see Péqueux, 1995 for review). Thus, the increased whole body Na^+,K^+-ATPase activity could be a compensatory response to the whole body Na^+ depletion induced by silver. Secondly, *D. magna* acutely exposed to ionic Ag^+ exhibited a competitive inhibition of the whole body Na^+ uptake rate within 1 h, probably too fast to be explained by basolateral

Na^+,K^+-ATPase inhibition, and pointing to a second potential site of action, the apical Na^+ channel (Bianchini and Wood, 2002). In this regard, it is interesting to note that Bury and Wood (1999) showed in rainbow trout that silver, probably as Ag^+, enters the branchial epithelial cells via the Na^+ channel coupled to the proton ATPase in the apical membrane. Thirdly, we have recently demonstrated in non-contaminated *D. magna* that more than 50% of the whole body Na^+ uptake rate in both neonates and adults can be inhibited by phenamil, a specific inhibitor of Na^+ channels (Bianchini and Wood, 2001b). Therefore, the three findings reported above support the idea that in *D. magna*, Ag^+ is acting as an analogue of Na^+ in the apical Na^+ channels and an inhibition of this Na^+ entry site, rather than an inhibition of basolateral Na^+,K^+-ATPase, is the key site for chronic silver toxicity in *D. magna.*

However, it must be pointed out that at present we have no knowledge of the actual level of ionic free Ag^+ in our chronic exposure media (or indeed if it exists at all), or any way of reliably calculating it because of: the complication of the presence of food; associated high levels of TOC; a high but variable fraction of non-dissolved silver as revealed by 0.45 μm filtration; and current uncertainties as to how to deal with the partitioning of silver under these circumstances. Nevertheless, according to BLM theory (Janes and Playle, 1995; Paquin et al., 1999; McGeer et al., 2000), regardless of whether or not Ag^+ exists free in solution, it can conceivably be transferred from weaker waterborne and particulate ligands to stronger ligands such as the 'toxic sites' on the daphnid 'gill'. An additional complication (discussed further below) is that the effects observed could reflect the impact of chronic dietary as well as waterborne silver exposure, because the algae and YCT undoubtedly complexed some of the silver present, and were ingested by the daphnids. This, of course, will also be the situation in the real world, though absolute total silver concentrations in even the most polluted real world situations will likely be several orders of magnitude lower than the concentrations used here (e.g. Flegal et al., 1997; Adams and Kramer, 1999; Kramer et al., 1999; Herrin et al., 2000).

The only other physiological data during chronic silver exposures to freshwater organisms was obtained from early life stage (Guadagnolo et al., 2001; Brauner and Wood, 2002) and juvenile (Galvez et al., 1998) rainbow trout. Those data again suggest ionoregulatory disturbance as the mechanism of toxicity, with primary effects manifested as inhibited Na^+ uptake and homeostasis. Interestingly, Brauner and Wood (2002) reported that either inhibited or elevated Na^+,K^+ATPase activity could occur in embryonic trout, depending on the exact developmental stage.

4.2. Growth and reproductive responses to chronic silver exposure

Growth, a commonly used endpoint in many chronic tests, does not appear in this study to be a useful endpoint for chronic silver BLM modeling. In the present study, a total silver concentration (5.0 μg Ag/l) causing 20% mortality and 60% loss of whole body Na^+ did not significantly affect growth of *D. magna*. Growth was also unaffected in the epibenthic amphipod, *Hyalella azteca,* exposed for 10 days or in the oligochaete, *Lumbriculus variegatus*, exposed for 28 days to 440 mg/kg silver sulfide-spiked sediments (Hirsch, 1998a,b). However, reproductive endpoints may be more suitable. A decrease in the total number of neonates produced over the life-cycle test (21 days) was observed. The lower number of neonates produced by daphnids chronically exposed to silver was partly explained by the 20% mortality observed during the test. A small but significant reduction in the mean number of neonates produced per adult per reproduction day (YAD) was also observed and this parameter was certainly not affected by the mortality rate observed during the test, since mortality occurred in the first days of test, before the subsequently dead daphnids had produced any neonates. A 60% loss of whole body Na^+ would probably be fatal to most species, so it is perhaps not surprising that reproduction of daphnids was impaired 13.7%.

Sublethal effects of silver on reproduction of freshwater zooplankton species such as cladocerans and copepods have been reported previously in the literature. Nebeker et al. (1983) reported significant effects on reproduction (YAD) of *D. magna* chronically (21 days) exposed to silver nitrate. However, in that study, nominal concentrations causing significant mortality (21-day LOEC = 4.1 μg/l) were as low or lower than those affecting reproduction (21-day EC50 = 10.5 μg/l). Recently, Hook and Fisher (2001) also demonstrated that silver affected reproductive success

in both freshwater cladocerans (*Simocephalus* sp. and *Ceriodaphnia dubia*) and marine copepods (*Acartia tonsa* and *A. hudsonica*). Their studies indicated that the threshold concentrations of total silver in the water to induce these effects were much lower (indeed approx. 0.1 μg/l) when the algal food was contaminated by the waterborne silver than when exposure was just via the dissolved phase. Again, this emphasizes that the effects observed in the present study may have been partly or wholly induced by exposure to silver through the dietary route. Regardless, it is clear that the ionoregulatory disturbance (Fig. 2) was a much more marked effect than the reproductive disturbance (Fig. 1) in the present study.

4.3. Regulatory perspective

Overall, the present results with daphnids, together with earlier data on fish (Galvez et al., 1998; Guadagnolo et al., 2001; Brauner and Wood, 2002) provide encouragement for efforts to extend and modify the present acute version of the silver BLM (Paquin et al., 1999; McGeer et al., 2000) so as to create a model which successfully predicts chronic silver toxicity for environmental regulation and risk assessment. Had the toxic mechanism been entirely different during chronic exposure than the ionoregulatory disturbance associated with inhibited Na^+ uptake, modeling would have had to start over again from first principles. Since the toxic mechanism appears similar but differs in fine details, it seems likely that the acute model can be simply be recalibrated for chronic prediction by fitting different binding constants and endpoints. Overall, we conclude that Na^+ uptake inhibition is the best tool to determine sensitivity to both acute and chronic toxicity in future versions of the Biotic Ligand Model for silver.

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