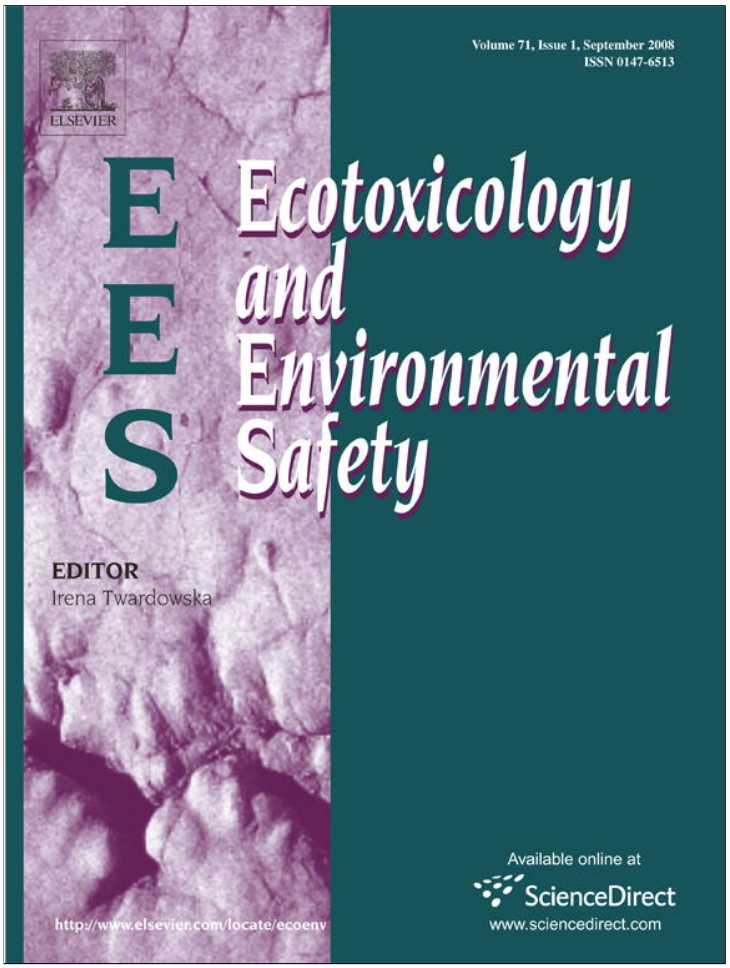

Ecotoxicology and Environmental Safety 71 (2008) 32–40

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Highlighted Article

Does sulfide or water hardness protect against chronic silver toxicity in *Daphnia magna*? A critical assessment of the acute-to-chronic toxicity ratio for silver ☆

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ARTICLE INFO

Article history:
Received 24 May 2007
Received in revised form
13 March 2008
Accepted 24 March 2008
Available online 9 May 2008

Keywords:
Acute-to-chronic ratio
Acute toxicity
Biotic Ligand Model
Chronic toxicity
Daphnia magna
Growth
Hardness
Silver
Reproduction
Sulfide

ABSTRACT

The protective effects of sulfide and water hardness against acute and chronic silver toxicity in *Daphnia magna* were assessed in the presence of food. Results showed that both sulfide and water hardness protected against lethal acute and chronic silver toxicity in terms of mortality. However, only sulfide showed a protective effect against the sub-lethal chronic silver effects on growth and reproduction. These findings suggest that both reactive sulfide and water hardness must be taken into account in the development of a chronic version of the Biotic Ligand Model (BLM) for waterborne silver. Furthermore, acute-to-chronic ratio values for silver toxicity showed that only small increases in toxicity are seen over the chronic exposure relative to the acute toxicity. Mortality is the most sensitive endpoint in moderately hard water and in the presence of sulfide. Reproduction, measured as the number of neonates produced per adult per reproduction day, is the most sensitive one in hard water in the absence of sulfide.

1. Introduction

The Biotic Ligand Model (BLM) is an approach to estimate metal bioavailability and predict toxicity to freshwater organisms, taking into account the geochemistry of a biological ligand, which is usually considered to be the gill (Janes and Playle, 1995; Paquin et al., 1999; McGeer et al., 2000). According to this model, the gill represents a negatively charged ligand to which ionic silver (Ag^+) can bind. Toxic effects are considered as a function of the degree of saturation of "sites of toxicity" on the biotic ligand by Ag^+. The BLM considers the competition between other cations and Ag^+ for the "sites of toxicity" on the gills as well as the influence of different complexing agents on silver speciation and availability (Paquin et al., 2002).

The BLM was originally developed based on acute toxicity data from freshwater animals, especially fish. More recently, this model was calibrated for invertebrates and researchers pointed to the need for its extension for chronic conditions (Paquin et al., 2002). In fact, BLM versions for chronic exposure of freshwater daphnids (*Daphnia magna*) to zinc (Heijerick et al., 2005) and copper (De Schamphelaere and Janssen, 2004), and rotifers (*Brachionus calyciflorus*) to copper (De Schamphelaere et al., 2006) are already available in the literature. However, a BLM version for chronic silver toxicity is not still available. In order to extend the present (acute) versions of the silver BLM (Paquin et al., 1999; McGeer et al., 2000) to the prediction of chronic silver toxicity, it will be critical to understand how the different major ligands and competitors present in the water can influence the chronic effects of this metal.

In the aquatic environment, silver complexing agents may include inorganic ligands (e.g., chloride, bicarbonate, thiosulfate), simple organic ligands (e.g., amino acids, EDTA) and complex polydisperse organic ligands such as humic and fulvic acids. It has also been demonstrated that metastable sulfide complexes commonly occur in oxic surface freshwaters (Rozan et al., 2000). Zinc sulfide clusters (e.g., $Zn_4S_6^{x-}$) are one example of a reactive sulfide complex (for review, Bianchini and Bowles, 2002). Generally, it is considered that ligands present in the water will reduce bioavailability and toxicity of metals to aquatic animals by reducing the free metal ion concentrations (Morel, 1983;

☆ Studies involving daphnids in the present study were strictly conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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0147-6513/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2008.03.006

Campbell, 1995). In fact, it has been shown that reactive sulfide, as zinc sulfide clusters, effectively exerts a protective effect against acute silver toxicity in both daphnids (*D. magna*; Bianchini et al., 2002a) and rainbow trout (*Oncorhynchus mykiss*; Mann et al., 2004). However, only few studies have considered the possible protective effect of the reactive sulfide against chronic silver toxicity in aquatic animals (Kolts et al., 2006; Naddy et al., 2007).

Regarding the competition between Ag^+ and other cations for the binding sites at the biotic ligand, water hardness has been recently shown to have a modest protective effect against chronic silver toxicity in rainbow trout (Morgan et al., 2005). Similar results were also reported for *D. magna* after acute exposure to silver (La Point et al., 1996; Karen et al., 1999). However, no published studies have investigated the potential protective effects of water hardness against the chronic silver effects in the most sensitive freshwater organisms, i.e., cladocerans and amphipods (Ratte, 1999; Bianchini et al., 2002a, b).

In light of the above, the main objectives of the present study were to analyze the possible protective effects of reactive sulfide (as zinc sulfide clusters) and water hardness against chronic silver toxicity in daphnids (*D. magna*).

2. Materials and methods

2.1. Daphnid culture

D. magna (ARO strain, Aquatic Research Organisms, Hampton, NH, USA) employed in all experiments was collected from a laboratory established culture. Daphnids were reared as previously described (Bianchini et al., 2002a, b, 2003, 2005; Bianchini and Wood, 2002). Briefly, they were maintained in moderately hard water with a composition (100 mg/L $CaCO_3$, 0.15 mM $MgSO_4 \cdot 7H_2O$, and 0.6 mM NaCl) similar to that of the Lake Ontario water. To ensure standardized conditions for water chemistry, the synthetic 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.

During the maintenance period, as well as during experiments, *D. magna* were fed algae (*Ankistrodesmus convolutus*; 1.82×10^8 cells/L = 33.0 ± 3.4 mg dry weight/L) and YCT (18.5 ± 2.2 mg dry weight/L). Water was not aerated, but the experimental medium, including food, was renewed daily. Temperature and photoperiod were fixed at 20 °C and 16 L:8 D, respectively.

After *D. magna* acclimation, reproductive rate was measured to ensure that 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 culture. Neonates (<24 h) were collected for experiments using plastic pipettes.

2.2. Silver exposure

All toxicity tests (acute and chronic) were conducted using a standard static-renewal system (renewal of the test solution every 24 h) and under the same conditions described for the daphnids culture. They were performed in the presence of food because, for several aquatic organisms, the presence of food greatly elevated the acute silver 48 h LC50 values relative to that derived from tests where food was absent (Ratte, 1999; Pedroso et al., 2007a). This probably happens because food binds up Ag^+, rendering it non-bioavailable. Also, it has been demonstrated that food enhances the capability of aquatic invertebrates to deal with the ionoregulatory impairments induced by ionoregulatory toxicants, such as silver and copper (Pedroso et al., 2007b; Pinho et al., 2007).

Neonate daphnids (<24 h) were acutely (48 h) or chronically (21 days life-cycle) exposed to different concentrations of silver ($AgNO_3$) in moderately hard water prepared as described above. Final nominal silver concentrations ranged from 0.75 to 23 μg Ag/L (7.0–213 nM) and were obtained from $AgNO_3$ stock solutions (1 and 10 mg Ag/L) acidified with 1% HNO_3. Total silver concentrations in stock solutions were checked by graphite furnace atomic absorption spectrometry (GF-AAS; AA-1275 with GTA-9 atomizer, Varian, Toronto, ON, Canada). In all tests, unlabeled $AgNO_3$ (SigmaUltra, Sigma, St. Louis, MO, USA) containing a proportion of radioactive ^{110m}Ag (RISOE National Laboratory, Roskilde, Denmark) to facilitate analyses of silver concentrations in the test solutions was added into the test solutions 3 h prior to introduction of *D. magna* neonates. The final specific activity of radiolabeled silver in all test solutions was 0.72 μCi/μg total silver. Analytical problems in measuring silver by GF-AAS in samples containing sulfide have been discussed in detail in an earlier publication (Bowles et al., 2002a). Silver (total and filtered) concentrations were followed over 24 h of exposure. The ^{110m}Ag radioactivities in filtered (representative of dissolved silver) (Acrodisc 0.45 μM polyethersulfone in-line filters; Pall Gelman Laboratory, Ann Arbor, MI, USA) and non-filtered water samples (2 mL) were determined using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra-Packard, Toronto, ON, Canada).

As an additional check on water quality, measurements were made to ensure that water remained sufficiently oxygenated (90.1 ± 7.9% saturation) and the water pH constant (8.16 ± 0.07) over the 24-h period between each water renewal. Water ion (Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^-) composition was also monitored throughout the experiment. Analytical techniques employed are described in detail in a previous publication (Bowles et al., 2002a). Total organic carbon (TOC) was also monitored throughout the experiment. Samples for total organic carbon 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.

Silver exposure was performed in moderately hard water either in the absence or the presence of sulfide, which was spiked as zinc sulfide (ZnS) clusters from stock solutions (64–128 μg S/L; 2–4 μM) to achieve a nominal concentration of 1.0 μg S/L (32 nM). Using this nominal concentration, a mean final concentration of ~0.8 μg S/L (25 nM) over 24 h of exposure would be expected (Bowles et al., 2002a). Zinc sulfide clusters were prepared and measured as previously described (Bowles et al., 2002a). Sulfide concentrations in test solutions were measured in the beginning and the end of each 24-h period of exposure in the acute toxicity tests. In chronic toxicity tests, sulfide concentrations were determined every week, at the beginning and the end of a 24-h period of exposure. Reactive sulfide was quantified by formation and spectrophotometric measurement of methylene blue sulfide (Cline, 1969).

Neonate daphnids were also acutely or chronically exposed to the different silver concentrations in hard synthetic water (4.0 mM $CaSO_4$, 0.6 mM $MgSO_4$ and 0.6 mM NaCl in reverse osmosis water) in the absence of reactive sulfide.

Control tests were conducted under the same experimental conditions to check on possible toxicity due to ZnS clusters and high water hardness themselves. Possible Zn toxicity from the Zn displaced by silver from the ZnS clusters was checked by using $ZnSO_4$ (Anachemia Canada, Montreal, QC, Canada; analytical grade) at a final concentration of 1.7 mg Zn/L (26 μM). Possible toxicity due to sulfate from $CaSO_4$ and $MgSO_4$ added to make up the hard synthetic water was also checked using Na_2SO_4 (Merck Chemicals, Darmstadt, Germany) at a final concentration of 4.6 mM.

All glass and plastic ware used in the toxicity tests was new and was acid washed in 1% HNO_3 (trace metal grade; Merck Chemicals, Darmstadt, Germany) and rinsed thoroughly with synthetic water prior to use.

2.3. Acute toxicity tests

Test procedures followed the OECD standard guidelines, except for the provision of food (OECD, 1984). Acute (48 h) toxicity tests were performed in borosilicate glass beakers containing 250 mL of experimental medium, pre-equilibrated to 20 °C. In all cases, food was first introduced to the experimental media and then silver was added. When sulfide was tested, it was added to the experimental medium before the silver addition. After 3 h pre-equilibration of the test solutions and food, 10 *D. magna* neonates were then placed in each beaker and maintained without aeration. Triplicates for each silver concentration were provided.

After 24 h, *D. magna* neonates were transferred to a new set of test solutions prepared 3 h prior to transfer as previously described. For the second 24 h of exposure, living *D. magna* was held (<5 s) in plastic pipettes during solution changeover. After 48 h of exposure, mortality (%) in each experimental beaker was recorded. The death criteria adopted were a change to milky coloration and lack of movement even after mild stimulation. The 48 h LC50 values and the respective 95% confidence intervals were estimated based on cumulative mortality data using Probit analysis (Finney, 1971) and compared using the 95% confidence interval. The 48 h LC50 values were estimated based on both mean measured total and filtered silver concentrations over the respective period of test.

2.4. Chronic toxicity test

Test procedures followed the ASTM standard guidelines for conducting *D. magna* life-cycle toxicity tests (ASTM, 1997). For each experimental medium or respective control, 10 neonates were exposed to test conditions for 21 days.

Each daphnid was individually exposed in a separate plastic beaker containing 50 mL of the experimental medium, pre-equilibrated to 20 °C. As in the acute toxicity tests, food was first introduced in the test solution and then $AgNO_3$ (SigmaUltra, Sigma) from the radiolabeled stock solution was added 3 h prior to introduction of *D. magna*. When sulfide was tested, it was introduced in the test solution before the silver addition. 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. After 24 h, daphnids were transferred to a new set of test solutions prepared 3 h prior to

transfer as previously described. Every day and for 21 days of exposure, daphnids were removed from the original beakers and transferred using plastic pipettes. Every week, total and filtered (Acrodisc 0.45 μM polyethersulfone in-line filters; Gelman) silver concentrations, as well as total organic carbon and sulfide concentrations in the exposure solutions were followed over the experiment (time 0 and 24 h). Measurements were carried out as described above.

Every day and for 21 days of exposure, survival and reproduction in daphnids were checked. The death criteria adopted were the same as in the acute tests. To assess reproduction, neonates produced in each beaker were counted and discharged at the time of daily water change. However, at the 14th day of exposure, 20 neonates from each experimental condition were randomly collected and reared under the respective control condition for 4 days. The remaining neonates were then discharged as usual. After a 4-day growth period, daphnids from the second generation were collected, counted and weighed (dry weight), as described below.

After 21 days of exposure, living first generation daphnids were collected in each treatment 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 weights were determined using an electronic microscale (Mettler UMT2; 0.001 mg accuracy).

At the end of the exposure period (21 days), the following reproductive parameters of daphnids from the first generation were analyzed: total number of neonates produced (TN), time to first brood (TB), number of broods (NB), number of young produced per brood (YB), number of reproduction days (RD), and number of young produced per adult per reproduction day (YAD).

All reproduction and growth values, except those for the TN, were expressed as mean ± 1 standard error of mean (S.E.M.). Significant effects of silver on growth and reproduction were assessed by analyses of variance followed by the Tukey's test for each treatment. The significance level adopted was 95%.

The 21-day LC50 (mortality) and 21-day EC50 (growth and reproduction) values and their respective 95% confidence intervals were calculated using Probit analysis (Finney, 1971). Values were compared using the 95% confidence interval (APHA, 1999). The 21-day LC20 (mortality) and 21-day EC20 (growth and reproduction) values were also calculated in order to calculate the acute-to-chronic ratio (ACR), as described below. All these values were estimated based on both mean measured total and filtered silver concentrations over the respective period of test.

2.5. Acute-to-chronic ratios

Acute-to-chronic (ACR) ratios for silver toxicity were calculated based on mortality, growth of the first-generation daphnids and reproduction (YAD) data obtained for each experimental condition. For mortality, the ACR value was calculated by dividing the 48-h LC50 value by the corresponding 21-day LC20 value or the 21-day LC50 value. For growth and reproduction (YAD), the ACR value was calculated dividing the 48-h LC50 value by the corresponding 21-day EC20 value or the 21-day EC50 value. In all cases, ACR values were calculated considering the measured total silver or the filtered silver concentrations.

3. Results

3.1. Water chemistry

The ionic composition of the different experimental media employed in both acute and chronic toxicity tests is shown in Table 1. Measured concentrations of major ions in the different experimental media were very close to the nominal ones. As expected, Ca^{2+} and Mg^{2+} concentrations in the hard synthetic water were approximately four-fold higher than those measured in the moderately hard synthetic water (Table 1).

Sulfide concentration in the test media attributable to the food provided to daphnids was 0.064 μg S/L of test media (2 nM). The mean concentration of the total (water plus food) sulfide present in the experimental media over 24 h of test was 0.74 ± 0.06 μg S/L (23.04 ± 1.78 nM). The mean total organic carbon concentration over the 24 h of test was 4.8 ± 1.3 mg/L.

Measured total silver concentration in all experimental media employed ranged from 65.33% to 99.73% of the nominal concentration, indicating on average a relatively low loss (~15%) of silver onto the vessel wall and algae. The percentage loss decreased as the total concentration increased. For all experimental media, measured filtered silver concentrations corresponded to ~50% of the total silver present in the water (Table 2).

Table 1
Ionic composition of the different experimental media employed in the acute and chronic assays with *Daphnia magna*

Medium	Ion				
	Na^+ (mM)	K^+ (μM)	Ca^{2+} (mM)	Mg^{2+} (mM)	Cl^- (mM)
MH	0.769 ± 0.015	2.173 ± 0.040	0.971 ± 0.011	0.144 ± 0.002	0.605 ± 0.008
MHS	0.763 ± 0.007	2.119 ± 0.044	0.942 ± 0.012	0.145 ± 0.002	0.613 ± 0.010
MHZn	0.763 ± 0.013	2.247 ± 0.075	0.956 ± 0.012	0.146 ± 0.003	0.627 ± 0.025
HH	0.765 ± 0.009	2.304 ± 0.031	4.028 ± 0.137	0.690 ± 0.012	0.626 ± 0.011
MHSu	6.513 ± 0.107	2.681 ± 0.041	0.909 ± 0.012	0.141 ± 0.003	0.647 ± 0.036

Data are means ± 1 S.E. ($N = 6$). MH: moderately hard synthetic water without sulfide (<5 nM); MHS: moderately hard synthetic water with sulfide (~25 nM); MHZn: moderately hard synthetic water with zinc; HH: hard synthetic water without sulfide (<5 nM); MHSu: moderately hard synthetic water with sulfate. See text for explanation.

Table 2
Total measured and filtered (0.45 μM) silver concentration in the different experimental media employed in acute and chronic toxicity tests with *Daphnia magna*

Medium	Nominal Ag (μg/L)	Total Ag (μg/L)	Ag loss (%)	Filtered Ag (μg/L)	Filtered Ag (% total Ag)
MH	0.75	0.49 ± 0.01	34.67	0.21 ± 0.001	42.86
	1.50	1.03 ± 0.04	31.33	0.69 ± 0.002	66.99
	3.00	2.02 ± 0.06	32.67	1.16 ± 0.005	57.43
	6.00	4.93 ± 0.08	17.83	1.98 ± 0.002	40.16
	7.00	6.23 ± 0.08	11.00	2.80 ± 0.004	44.94
	7.70	6.57 ± 0.07	14.68	2.95 ± 0.008	44.90
	8.50	7.92 ± 0.10	6.82	3.56 ± 0.007	44.95
	11.5	10.59 ± 0.14	7.91	3.98 ± 0.008	37.58
	23.0	22.48 ± 0.38	2.26	10.11 ± 0.079	44.97
MHS	0.75	0.61 ± 0.03	18.67	0.25 ± 0.005	40.98
	1.50	1.01 ± 0.04	32.67	0.69 ± 0.004	68.32
	3.00	2.32 ± 0.06	22.67	1.16 ± 0.016	50.00
	6.00	5.22 ± 0.08	13.00	1.98 ± 0.025	37.93
	7.00	6.51 ± 0.05	7.00	3.54 ± 0.018	54.38
	7.70	7.67 ± 0.09	0.39	3.61 ± 0.022	47.07
	8.50	7.98 ± 0.07	6.12	3.75 ± 0.011	46.99
	11.5	10.10 ± 0.11	12.17	3.96 ± 0.006	39.21
	23.0	21.41 ± 0.22	6.91	10.10 ± 0.057	47.17
HH	0.75	0.61 ± 0.02	18.67	0.32 ± 0.003	52.46
	1.50	1.16 ± 0.05	22.67	0.69 ± 0.006	59.48
	3.00	2.10 ± 0.05	30.00	1.16 ± 0.007	55.24
	6.00	4.96 ± 0.09	17.33	1.98 ± 0.012	39.92
	7.00	6.52 ± 0.10	6.86	3.54 ± 0.011	54.29
	7.50	7.49 ± 0.06	0.27	3.61 ± 0.012	48.20
	8.50	7.98 ± 0.09	6.12	3.75 ± 0.020	46.99
	11.5	10.63 ± 0.12	7.57	3.98 ± 0.016	37.44
	23.0	21.00 ± 0.23	8.70	10.11 ± 0.033	48.14

Data are means ± 1 S.E. ($N = 6$). MH: moderately hard synthetic water without sulfide (<5 nM); MHS: moderately hard synthetic water with sulfide (~25 nM); HH: hard synthetic water without sulfide (<5 nM). See text for explanation.

3.2. Lethal toxicity

In all control conditions (without silver addition), i.e., moderately hard synthetic water, moderately hard synthetic water in the presence of sulfide, moderately hard synthetic water in the presence of zinc, moderately hard synthetic water in the presence of sulfate, and in hard synthetic water, data from both acute and

Table 3
Toxicity values based on mortality, growth of first generation and reproduction of *Daphnia magna* exposed to waterborne silver in the presence of food in moderately hard water in the absence or the presence of reactive sulfide (23 nM) and in hard water and the absence of reactive sulfide

Parameter	Treatment					
	Silver		Silver+sulfide		Silver+high hardness	
	Total	Filtered	Total	Filtered	Total	Filtered
48-h LC50 (mortality)	6.88a (6.58–7.23)	3.09a (2.94–3.24)	8.28b (8.15–8.50)	3.21ab (3.02–3.46)	8.20b (8.05–8.44)	3.54b (3.34–3.95)
21-day LC20 (mortality)	2.12a (2.05–2.18)	2.14a (2.07–2.23)	6.11b (5.95–6.32)	3.00b (2.63–3.25)	6.41b (6.17–6.68)	3.31b (3.14–3.50)
21-day LC50 (mortality)	4.70a (4.19–5.31)	2.81a (2.41–3.26)	6.93b (6.53–7.72)	3.02a (2.97–3.08)	6.57b (5.98–7.18)	3.54b (3.33–3.84)
21-day EC20 (growth)	6.42a (6.20–6.68)	2.85a (2.72–2.96)	7.60b (7.51–7.70)	3.04b (3.02–3.07)	7.56b (7.29–7.83)	3.65c (3.58–3.72)
21-day EC50 (growth)	8.02a (7.60–8.92)	3.60a (3.41–4.01)	8.05a (7.95–8.26)	3.42a (3.18–3.70)	8.57a (8.19–9.43)	3.81a (3.60–4.09)
21-day EC20 (reproduction)	5.57a (5.38–5.75)	2.93a (2.50–3.29)	6.51b (6.35–6.68)	2.54a (2.30–2.83)	5.43a (5.21–5.64)	2.27a (2.02–2.58)
21-day EC50 (reproduction)	6.17a (6.00–6.35)	3.02a (2.92–3.13)	7.05b (6.64–7.25)	2.79a (2.61–2.98)	6.10a (5.93–6.26)	2.87a (2.72–3.01)

The number of neonates produced per adult per reproduction day was the endpoint considered for reproduction. All values are expressed in μg Ag/L. Values into brackets represent the 95% confidence interval. Different letters indicate significant difference between treatments for values calculated based on total or filtered silver concentrations.

chronic toxicity tests showed survival rates higher than 90% and 80%, respectively.

When the LC50 values were calculated based on measured total silver concentrations, toxicity in the presence of food was significantly lower in the presence than in the absence of sulfide after both acute (1.20-fold difference in 48-h LC50) and chronic (1.47-fold difference in 21-day LC50) exposure. Furthermore, in the absence of sulfide and presence of food, toxicity was significantly lower in hard synthetic water than in moderately hard synthetic water after both acute (1.19-fold difference in 48-h LC50) and chronic (1.40-fold difference in 21-day LC50) exposure (Table 3).

When the LC50 values were calculated based on filtered silver concentrations, in the presence of food there was no significant difference in either acute or chronic silver toxicity in the absence or the presence of sulfide. However, silver toxicity was significantly lower in hard water than in moderately hard water after both acute (1.15-fold difference in 48-h LC50) and chronic (1.26-fold difference in 21-day LC50) in the presence of food and absence of sulfide (Table 3).

3.3. Sub-lethal toxicity

Under all control conditions (absence of silver), data from the individually exposed daphnids ($N = 10$ per treatment) showed survival rates ranging from 80% to 100%. Similarly, no significant changes in reproductive (Fig. 1A) and growth (Fig. 1B) parameters were detected after 21 days of test, except for a significant ($P < 0.05$) reduction in the time to first brood and an increase in the number of reproductive days in hard synthetic water (Fig. 1A).

Regarding growth, a significant effect of silver was observed in living daphnids of the first generation after 21 days of test in all treatments (Fig. 2A). However, no significant differences were observed between treatments when the 21-day EC50 values were calculated based on either measured total or filtered silver concentrations (Table 3).

In the second-generation daphnids, mortality rates after 4 days in clean water were lower than 20%. Also, no significant differences were observed between treatments. However, in all treatments, a marked growth inhibition was observed in daphnids generated by those exposed to measured total silver concentrations beyond 6 μg Ag/L, which corresponded to ~3 μg of filtered Ag/L (Fig. 2B).

Regarding reproduction, no marked changes were observed in the TB over the experimental period in all silver treatments. However, reductions in TN, YA, NB, YB, RD (data not shown), and YAD were observed (Fig. 3). When total measured silver concentrations were considered, these effects were observed beyond a threshold concentration of about 6 μg Ag/L. When filtered silver concentrations were considered, these effects were observed beyond a threshold concentration of 3 μg Ag/L.

In moderately hard water, 21-day EC50 values calculated based on total measured silver concentrations were significantly higher in the presence than in the absence of sulfide for the following reproductive parameters: TN, YB, RD (Table 4), and YAD (Tables 3 and 4). In comparing hard water with moderately hard water, the only significant difference observed was a lower 21-day EC50 value for RD. Based on measured filtered silver concentrations, no significant differences were observed among the different experimental conditions for any of the reproductive parameters analyzed (Table 4).

3.4. Acute-to-chronic toxicity ratios

Acute-to-chronic toxicity ratios (ACR) were calculated based on mortality, growth and reproduction (YAD) data shown in Table 3. ACR values (Table 5) were lower than 1.6, except for mortality when values were calculated considering the 21-day LC20 (ACR = 3.25). The lowest ACR value in each experimental condition was generally observed when calculated based on growth, being close to 1.0. Of course ACR values were always higher when calculated based on 21-day LC20 or 21-day EC20 than when calculated based on 21-day LC50 or 21-day EC50, though the differences were usually lower than 25%. In moderately hard water, ACR values calculated based on 21-day LC20 and 21-day LC50 (i.e., mortality) were generally higher than those calculated based on 21-day EC20 and 21-day EC50 (i.e., growth and reproduction), respectively. In hard water, the reverse was true for reproduction—i.e., ACR values calculated based on 21-day EC20 and 21-day EC50 (i.e., YAD) were higher than those calculated based on 21-day LC20 and 21-day LC50 (i.e., mortality), respectively. When ACR values were calculated based on total silver concentrations, the highest value (ACR = 3.25) was obtained in moderately hard water, in the absence of sulfide, and when calculated based on the 21-day LC20 values. When ACR values were calculated based on filtered silver concentrations, the highest value (ACR = 1.56) was obtained in hard water, in the absence of sulfide, and when calculated based on a reproductive endpoint (YAD).

4. Discussion

In the present study, we assessed the possible protective effects of reactive sulfide and water hardness against both acute

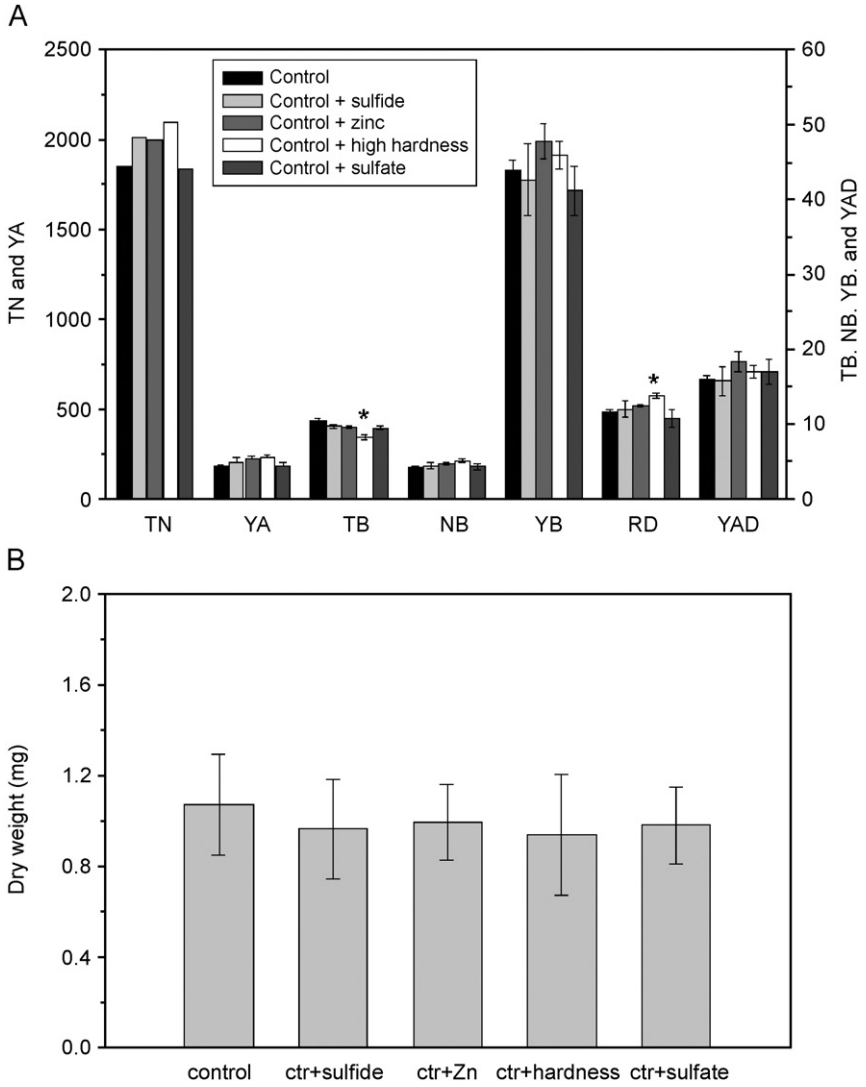

Fig. 1. Reproductive (A) and growth (B) parameters in *Daphnia magna* maintained in the different control conditions (see text for explanation), for 21 days. TN: total number of neonates produced; YA: mean number of young produced per adult; TB: time to first brood (days), NB: number of broods produced, 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 S.E. Asterisk (*) indicates significant different mean from the mean in control neonates ($P < 0.05$).

and chronic waterborne silver toxicity in *D. magna*, the most sensitive aquatic organism when silver is added to the water as $AgNO_3$ (Ratte, 1999; Bianchini et al., 2002a, b). As observed in fish, Ag^+ has been shown to be a potent ionoregulatory toxicant in freshwater invertebrates, including daphnids (Bianchini and Wood, 2002, 2003; Grosell et al., 2002a, b). In *D. magna*, waterborne Ag^+ affects whole body ionoregulation after both acute (Bianchini and Wood, 2003) and chronic exposure (Bianchini and Wood, 2003).

Regarding reactive sulfide, we have previously demonstrated that zinc sulfide clusters at a high level (100 nM) and at an environmentally realistic level (~25 nM) were able to protect against acute waterborne Ag^+ toxicity in rainbow trout (*O. mykiss*; Mann et al., 2004) and daphnids (*D. magna*; Bianchini et al., 2002a), respectively. However, these previous acute toxicity studies were performed in the absence of food, following the ASTM guidelines (ASTM, 1997). In fact, addition of food, timing of adding food to exposure solutions, and equilibration time were shown to be important factors affecting waterborne Ag^+ toxicity to *Ceriodaphnia dubia* (Kolts et al., 2006; Naddy et al., 2007). Data obtained in the present study with *D. magna* clearly indicate that reactive sulfide at an environmentally realistic level (23 nM) is also able to protect against acute waterborne Ag^+ toxicity in the presence of food in the water. Furthermore, they also clearly indicate that reactive sulfide protects to a small but significant extent against the chronic waterborne silver toxicity when mortality, growth and reproductive parameters of the first-generation daphnids are taken into account. This finding is in agreement with a very recent study performed on *C. dubia*, which showed that sulfide (chromium reactive sulfide = 75.4 nM) prepared as humic acid combined with CuS raised the chronic EC20 by 36% and the MATC by 42% (Naddy et al., 2007). The protective effect of reactive sulfide against waterborne Ag^+ toxicity is probably associated with the fact that silver binds strongly to sulfide with a conditional constant for Ag:HS of $\log K' \sim 8.9$, thus reducing silver bioavailability (Bowles et al., 2002a) and toxicity (Bianchini et al., 2002a) by reducing the free metal ion concentration (Morel, 1983; Campbell, 1995).

In aquatic environments, metastable sulfide complexes commonly occur in oxic surface freshwaters at picomolar to nanomolar concentrations (Rozan et al., 2000), while silver is generally present in these media at concentrations lower than those of sulfide. Thus, these observed sulfide concentrations are high enough to remove Ag^+ from other ligands and potentially

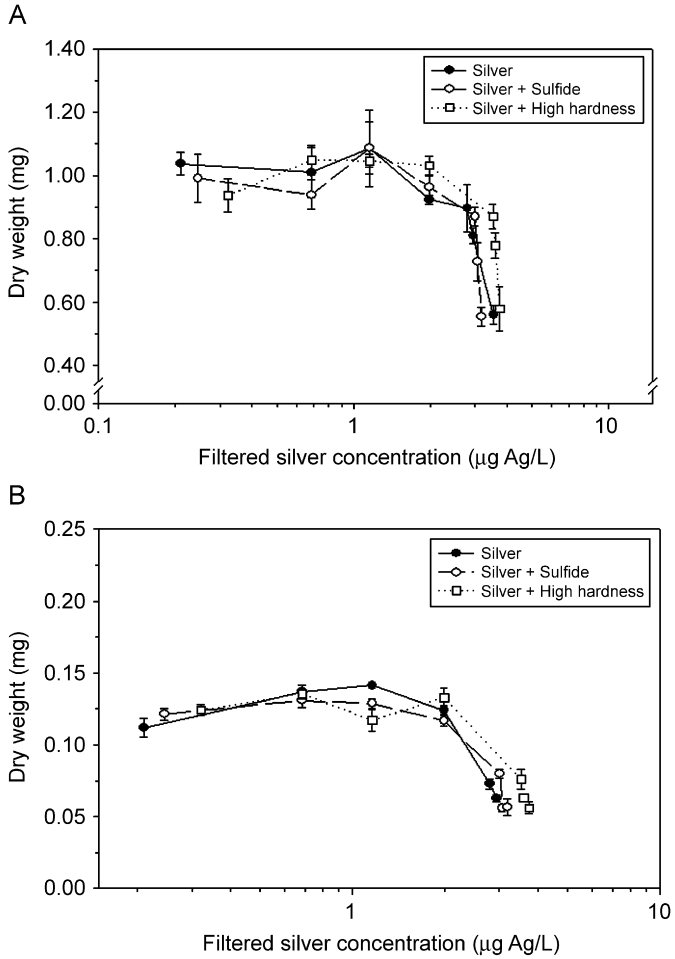

Fig. 2. Dry weight (mg) of first (A) and second-generation (B) *Daphnia magna* chronically (21 days) exposed to silver (added as $AgNO_3$) in moderately hard synthetic water in the absence (<5 nM) of sulfide, in moderately hard synthetic water in the presence (23 nM) of reactive sulfide (zinc sulfide clusters), and in hard synthetic water in the absence of sulfide (<5 nM). Results were expressed based on filtered silver concentrations. Data are expressed as mean±1 S.E.

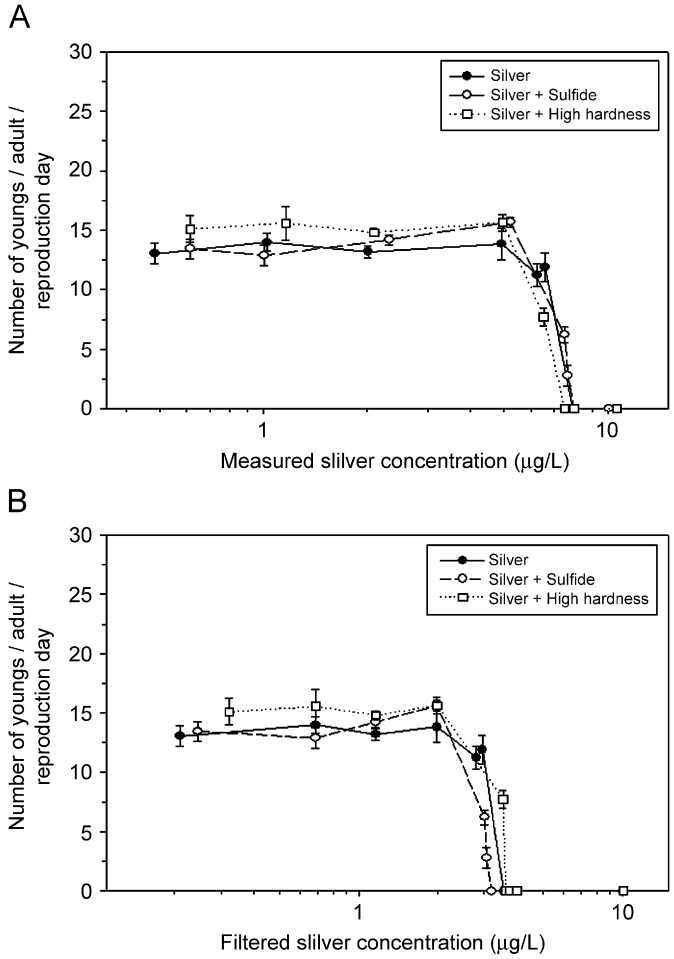

Fig. 3. Number of neonates produced per adult per reproduction day (YAD) in *Daphnia magna* chronically (21 days) exposed to silver in moderately hard synthetic water in the absence (<5 nM) of sulfide, in moderately hard synthetic water in the presence (23 nM) of reactive sulfide (zinc sulfide clusters), and in hard synthetic water in the absence of sulfide (<5 nM). Results are expressed considering measured total silver (A) or filtered silver (B) concentrations. Data are expressed as mean±1 S.E.

exert a detoxifying effect on Ag^+ (for review, Bianchini and Bowles, 2002). Considering a 1:1 binding ratio of silver to sulfide (Bowles et al., 2002a), it would be expected that reactive sulfide added to the water as zinc sulfide clusters in the experimental media would be enough to bind up to ~23 nM of Ag^+ in the present study. Based on measured total silver concentrations, the difference between the LC50 values in the presence and the absence of sulfide is 13.0 nM (1.4 μg Ag/L) and 20.7 nM (2.23 μg Ag/L) after acute (48-h LC50 values) and chronic (21-day LC50 values) exposure, respectively. When we consider the sub-lethal effect (21-day EC50 values) of Ag^+ on reproduction (YAD), this value is 15.3 nM (1.65 μg Ag/L). Taken all these values together, a general mean value is 16.3 nM (1.8 μg Ag/L). The similarity of 16.3 nM change in Ag toxicity to the 23 nM of sulfide present in the experimental medium argues strongly for 1:1 binding ratio of silver to sulfide. Furthermore, the difference in test duration relative to the difference between LC50 (13 nM) and average EC50 (16 nM) values may also be attributed to the fast and slow binding sites on the sulfide clusters.

The lack of significant differences in waterborne Ag^+ toxicity in the absence and the presence of sulfide when 48-h LC50 or 21-day EC50 values were calculated based on filtered silver concentrations and can be explained by the fact that zinc sulfide clusters bind to filtration membranes. When sulfide is present, filtration is not appropriate as a mean of size discrimination, but it can be used to assess the proportion of the silver (I) that is bound to the sulfide clusters (Bowles et al., 2002b). Thus, the fact that no significant differences were observed among the 48-h LC50 or 21-day EC50 values calculated based on filtered silver concentrations in the absence and the presence of sulfide indicates that all the toxic silver was in the dissolved phase and all silver bound to sulfide (retained in the 0.45 μM-mesh filter) was not toxic. Interestingly, it has been shown that complexed silver can be ingested and accumulated in the digestive tract of *D. magna*, enhancing the whole body silver burden in the presence of sulfide (Bianchini et al., 2005). These findings on the effect of reactive sulfide on silver accumulation, together with those recently reported for the effect of the natural organic matter on silver accumulation in daphnids (Glover and Wood, 2005) and fish (Mann et al., 2004) clearly indicate that silver body or gill burden is a poor indicator of silver toxicity in natural environments. A better indicator is the BLM-modeled load relative to the inferred lethal accumulation (LA50) at the true toxic sites, as is now used routinely in BLM's for other metals with daphnids (De Schamphelaere and Janssen, 2004; De Schamphelaere et al., 2006).

Regarding water hardness, which is a function primarily of the Ca^{2+} and Mg^{2+} concentrations in the water, results from the present study clearly indicate that elevating water hardness in the

Table 4
Toxicity values (21-day EC50) based on reproduction of *Daphnia magna* exposed to waterborne silver in the presence of food in moderately hard water in the absence or the presence of reactive sulfide (23 nM) and in hard water and the absence of reactive sulfide

Parameter	Treatment					
	Silver		Silver+sulfide		Silver+high hardness	
	Total	Filtered	Total	Filtered	Total	Filtered
TN	5.51a (4.95–6.14)	2.48a (2.29–2.69)	6.47b (6.27–6.64)	2.53a (2.44–2.61)	5.87a (5.72–6.02)	2.71a (2.57–2.83)
YA	6.63a (6.48–6.78)	2.71a (2.53–2.94)	6.52a (6.32–6.69)	2.55a (2.46–2.63)	6.42a (6.19–6.65)	2.48a (2.30–2.65)
NB	6.96a (6.85–7.12)	2.93a (2.88–3.00)	7.25a (7.06–7.42)	2.88a (2.79–2.95)	6.66a (6.36–6.97)	2.73a (2.45–2.99)
YB	6.61a (6.47–6.76)	2.95a (2.87–3.04)	7.47b (7.39–7.52)	2.99a (2.96–3.01)	6.52a (6.33–6.71)	3.23a (2.93–3.55)
RD	6.93a (6.81–7.12)	3.11a (3.06–3.20)	7.64b (7.61–7.66)	3.06a (3.04–3.07)	6.04c (5.51–6.70)	3.09a (2.84–3.36)
YAD	6.17a (6.00–6.35)	3.02a (2.92–3.13)	7.05b (6.64–7.25)	2.79a (2.61–2.98)	6.10a (5.93–6.26)	2.87a (2.72–3.01)

All values are expressed in μg Ag/L. Values into brackets represent the 95% confidence interval. Different letters indicate significant difference between treatments for values calculated based on total or filtered silver concentrations. See caption of Fig. 1 for details on the abbreviations of reproductive parameters.

Table 5
Acute-to-chronic ratio for silver toxicity based on mortality, growth and reproduction (YAD: number of neonates produced per adult per reproduction day) of *Daphnia magna* exposed to waterborne silver in the presence of food in moderately hard water in the absence or the presence of reactive sulfide (23 nM) and in hard water and the absence of reactive sulfide

Concentration	Treatment	Endpoint					
		Mortality		Growth		Reproduction	
Total silver	Silver	3.25[a]	1.46[b]	1.07[a]	0.86[b]	1.24[a]	1.12[b]
	Silver+sulfide	1.36	1.19	1.09	1.03	1.27	1.17
	Silver+high hardness	1.28	1.25	1.08	0.96	1.51	1.34
Filtered silver	Silver	1.44	1.10	1.08	0.86	1.05	1.02
	Silver+sulfide[c]	1.07	1.06	1.06	0.94	1.26	1.15
	Silver+high hardness	1.07	1.00	0.97	0.93	1.56	1.23

Data were calculated based on measured total or filtered silver concentrations.
[a] Values were calculated considering the 21-day LC20 (mortality) or EC20 (growth and reproduction) values.
[b] Values were calculated considering the 21-day LC50 (mortality) or EC50 (growth and reproduction) values.
[c] Values must be considered with caution because filtration is not appropriate as a means of size discrimination, but can be used to assess the proportion of the silver (I) that is bound to the sulfide clusters (Bowles et al., 2002b).

presence of food protected to a small but significant extent against both acute and chronic silver lethality. However, this protection is small, reducing the acute and chronic mortality by about 1.2- and 1.3-fold, respectively. In the absence of food, similar small or negligible protection of water hardness against the lethality induced by waterborne silver has been previously reported in both fish and *D. magna* after acute waterborne silver exposure (Davies et al., 1978; La Point et al., 1996; Bury et al., 1999a, b; Karen et al., 1999). After chronic exposure, a small protection against mortality was also observed in early life stages of rainbow trout (Morgan et al., 2005).

Despite the protective effect of water hardness against acute and chronic mortality of daphnids exposed to waterborne silver in the presence of food, there was no statistically significant effect of hardness on growth and reproduction. This finding is in agreement with those previously reported for daphnids, where no apparent effect of water hardness against chronic toxicity endpoints was observed (US-EPA, 1980), and a small protective effect of water hardness against the developmental effects of silver was observed in early life stages of rainbow trout (Morgan et al., 2005). Lack of effect or only small protective effects against chronic toxicity when fish and daphnids were exposed to silver have also been reported for other water chemistry parameters, such as dissolved organic carbon (Brauner and Wood, 2002) and chloride (Brauner et al., 2003). The small protective effect of elevating water hardness against acute and chronic toxicity in daphnids would likely be associated with the relatively weak competition of Ca^{2+} ($\log K = 3.3$) and Mg^{2+} ($\log K = 3.0$) with silver for the binding at the sites causing toxicity, i.e., the biotic ligand (Janes and Playle, 1995; Schwartz and Playle, 2001). Also, the important role of Ca^{2+} and Mg^{2+} on permeability of epithelial cells (Hunn, 1985; McDonald, 1983; Wood, 2001) could be also contribute to the modest protection of elevating water hardness against the lethality induced by silver.

In addition to the chronic effects on mortality, growth and reproduction induced by waterborne Ag^{+} exposure in the first-generation daphnids, growth inhibition of the second-generation daphnids raised in silver-free water was observed in the present study. All these effects were observed at total silver exposures beyond 6 μg Ag/L (~3 μg filtered Ag/L) to the parents. Silver effects on the second-generation daphnids could be associated to neonate exposure to silver present in the test media used to expose their parents. This statement is based on the facts that neonates were not transferred immediately upon release, and there is the possibility of latent effects. In fact, daphnid neonate exposure to silver for 1–3 h can lead to biological effects or even death (Kolts et al., 2006; Naddy et al., 2007). Another possible explanation for the observed silver effects on the second-generation daphnids is associated with the possible effects of silver accumulated in offspring due to either maternal transfer during egg production or embryo development in the brood chamber. This statement is based on the fact that silver accumulated in neonates after hatching would be eliminated very rapidly from *D. magna* (Bianchini et al., 2005; Glover and Wood, 2005). Furthermore, maternal transfer of other metals such as Hg and Cd has been reported to be an important pathway for elimination from the parent, inducing transgenerational physiological effects in offspring of freshwater invertebrates, including *D. magna* (Craig et al., 1998; Munger et al., 1999; Tsui and Wang, 2004). In addition, Tsui and Wang (2004) demonstrated in *D. magna* that the percentage of Hg retention by the second generation is generally higher than that by the parental generation after 20 days of depuration. Unfortunately, silver concentration was not measured in the second-generation daphnids in the present study.

Taken all together, data reported in the literature and those described in the present study in the presence of food in the water clearly indicate that reactive sulfide has a protective effect against both acute and chronic silver effects in both fish and daphnids. Thus, reactive sulfide must be taken into account in the refinement of the acute BLM version for silver, as well as in the development of a chronic BLM version for this metal in freshwater environments. Furthermore, data available in the literature and

those described here in the presence of food in the water clearly indicate that water hardness has only a small protective effect on chronic toxicity endpoints in aquatic animals. Nevertheless, it should also be taken into account in the development of a chronic BLM version for silver considering its small but significant protective effect on the chronic lethality induced by silver in both fish and daphnids. Since we are dealing with chronic exposure, food provision is essential, just as in the real world. Therefore, calibration of chronic BLM's must be done in the presence of food. In this context, further measurements of the binding capacity for the most relevant food for the tested species are necessary.

The ACR values reported here (Table 5) are markedly higher than those reported previously in the literature for *D. magna*, the most sensitive aquatic organism to waterborne Ag^+ (Ratte, 1999; Bianchini et al., 2002a, b). Using the species mean acute (SMAV) and chronic (SMCV) values reported in the literature based on measured total silver concentration (Wood et al., 2002), the ACR value calculated is 0.15. However, this value is calculated with an acute SMAV being estimated in the absence of food while the SMCV is always determined in the presence of food. In fact, the fact that the ACR value is *below 1.0* is because the acute LC50 value for *D. magna* is ~10-fold higher in the presence than in the absence of food when tests are performed under the same experimental conditions (Nebeker et al., 1983; Erickson et al., 1998; Bianchini et al., 2002a, b). Therefore, if we correct the ACR value mentioned above (0.15) by a factor of 10, the new calculated ACR value would be 1.5, which matches with the ACR value reported in the present study for mortality in moderately hard water and reproduction (YAD) in hard water (Table 5). This undoubtedly reflects, at some extent, silver complexation by food, reducing its toxicity. In the present study, the total organic carbon concentration in the experimental media, mostly attributable to food addition, was ~5 mg/L. In fact, it has been demonstrated that small amounts of dissolved organic carbon in freshwater almost increase 3–4 fold the 48-h LC50 in *D. magna* exposed to silver (Glover and Wood, 2005). In addition to the protection associated to silver complexation by organic matter, the effect of food helping animal's physiology, increasing its tolerance to metal exposure must also be considered. For example, we recently showed that food has important protective effects against silver effects on ionic regulation in marine copepods, increasing their tolerance to copper exposure (Pedroso et al., 2007b; Pinho et al., 2007). It must be stressed that both silver and copper are ionoregulatory toxicants to freshwater animals (Grosell et al., 2002b). In fact, the presence of food in the water has been shown to significantly increase tolerance of several aquatic invertebrates to both silver and copper exposure (Ratte, 1999; Bianchini et al., 2003; Kolts et al., 2006; Naddy et al., 2007; Pedroso et al., 2007a; Pinho et al., 2007).

While not currently accepted in regulations, it is clear from data reported in the present study that it is not appropriate to calculate ACR values based on unfed (acutely exposed) and fed (chronically exposed) animals because the calculated values would be well below *1.0*. This procedure is illogical from the scientific and regulatory point of view and it is not also ecologically relevant. As discussed above, it is well known that the impact of feeding on the sensitivity of aquatic animals to acute toxicity of metals, including silver (Ratte, 1999; Bianchini et al., 2003; Kolts et al., 2006; Naddy et al., 2007; Pedroso et al., 2007a, b; Pinho et al., 2007). For environmental regulations purposes, we thus suggest that ACR values reported here for *D. magna* clearly show that only small increases in toxicity are seen over the chronic exposure relative to the acute toxicity when growth or a reproductive parameter (YAD) were used as the chronic endpoints. This statement is based on the fact that the highest ACR value observed in these cases for all experimental conditions is only 1.56, i.e., the silver toxicity increased from acute-to-chronic exposure by only 1.56-fold, despite the varieties of water chemistry variables tested (sulfide and hardness). However, a much higher ACR value (ACR = 3.25) is observed when measured total silver concentrations (as opposed to measured filtered silver concentrations) were employed to calculate the toxicity values and the 21-day LC20 value was used to calculate the ACR value (Table 5).

When comparing ACR values calculated based on the different endpoints, data reported in the present study clearly showed that mortality would be the most sensitive endpoint to detect increments in toxicity during chronic exposure relative to the acute toxicity. Mortality was the most sensitive endpoint in moderately hard water, in the presence of food, and in either the absence or the presence of a low level (23 nM) of sulfide in the water. Reproduction, measured as YAD, would be the most sensitive one in hard water, in the presence of food and absence of sulfide, although there were not marked differences in ACR values for mortality and reproduction. These findings can be observed when ACR values were derived from toxicity values calculated based on either total or filtered silver concentrations. However, when filtered silver concentrations were considered to calculate the toxicity values, the reproduction endpoint (YAD) was more sensitive than mortality (Table 5). This last finding should be considered with caution due to possible biases introduced by sulfide chemisorptions onto the filter (Bowles et al., 2002b). It is also important to note that ACR values calculated for growth did not reveal greater toxicity during chronic exposure at any experimental condition tested—i.e., ACR values remained close to 1.0. Of course, ACR values calculated based on 21-day LC20 (mortality) or 21-day EC20 (reproduction) values were always more sensitive than those calculated based on 21-day LC50 (mortality) or 21-day EC50 (reproduction) values.

Finally, data reported here and other evidence from the literature suggest that possible maternal transfer of silver may be an important pathway of metal elimination in daphnids. This possibility deserves further experimental attention, specifically to evaluate whether a maternally transferred silver body burden correlates with toxicity in the first-generation daphnids.

Acknowledgments

We acknowledge the financial support of KODAK Canada Inc. and the NSERC CRD Program. We also wish to thank Joseph W. Gorsuch (formerly of Eastman Kodak, now of Gorsuch Environmental Management Services), and Dr. Trevor Smith (Kodak Canada) for excellent project management, Dr. Russ Bell, Dr. Jim Kramer, and Mike J. Ernste (McMaster University) for assistance with sulfide analyses, and Dr. David Rollo (McMaster University) for loan of the micro-scale. A. Bianchini is a fellow of the Brazilian CNPq (Proc. # 300906-2006-4) and C.M. Wood is supported by the Canada Research Chair Program.

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