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HETEROGENEITY OF NATURAL ORGANIC MATTER AMELIORATION OF SILVER TOXICITY TO *DAPHNIA MAGNA*: EFFECT OF SOURCE AND EQUILIBRATION TIME

CHRIS N. GLOVER,*† RICHARD C. PLAYLE,‡ and CHRIS M. WOOD†
†Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada
‡Department of Biology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada

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Abstract—Despite the heterogeneity of natural organic matter (NOM) in the aquatic environment, current models that predict metal toxicity to aquatic biota treat these important metal-complexing agents in a homogeneous manner. In this investigation, the ability of 11 commercial and naturally isolated NOM samples to ameliorate silver toxicity to the freshwater crustacean *Daphnia magna* was examined. The commercially available Aldrich humic acid (AHA) increased the 48-h median lethal concentration for daphnid neonates from nominally NOM-free levels of 0.29 to 3.80 μg/L (at 6.9 mg C/L) in a concentration-dependent manner. Three of the tested samples exhibited similar protective effects, but the additional seven NOM samples displayed significantly stronger ameliorative actions. In fact, four samples of both commercial and naturally isolated origin demonstrated greater than fourfold increases in protection compared to that of AHA. Additional investigations showed that increased silver–AHA equilibration time resulted in decreased toxicity. Increased equilibration time also decreased whole-body silver accumulation at NOM levels less than 1 mg C/L. The present results suggest that heterogeneity of NOM and silver–NOM equilibration time will have to be accounted for in future models of silver toxicity to *D. magna* and that laboratory toxicity testing using NOM and metals should account for the effects of metal–NOM equilibration time.

Keywords—Silver Biotic ligand model Dissolved organic carbon Humic substances Invertebrate

INTRODUCTION

In recent years, the development of a mechanistic understanding of silver (Ag) toxicity and Ag speciation in natural waters has offered a means to predict the potential hazard posed to aquatic organisms from environmental Ag. Silver is highly toxic to freshwater organisms, but it is only the free ionic form (Ag^+) that causes toxicity [1,2]. In rainbow trout, Ag^+ appears to enter branchial cells via an apical sodium channel [3]. Once in the cell, Ag^+ primarily causes toxicity via the rapid inhibition of cytosolic carbonic anhydrase and the subsequent impairment of basolateral Na^+,K^+-adenosine triphosphatase [4,5], which are two key enzymes in branchial ion transport. The net effect of these processes is the inhibition of active branchial sodium and chloride uptake [4], processes that are essential for the maintenance of ion homeostasis and, consequently, for survival in the hypo-osmotic, freshwater environment. This mechanism of Ag action appears to be conserved among freshwater organisms, with similar ionoregulatory disturbances being reported in freshwater crayfish [6] and *Daphnia magna* [7].

Knowledge regarding the toxic mechanism of Ag has been used to generate models [8–11] that predict Ag toxicity in natural waters. Ionic elements or compounds in water act both to bind Ag^+ (e.g., Cl^- and natural organic matter [NOM]) or to compete with Ag^+ for binding (i.e., other cations) at the site of toxic action (the biotic ligand). The ability of these complexing and competing agents to prevent Ag from binding to the biotic ligand is a function of their affinity for Ag and for the biotic ligand, respectively. These affinities, along with the binding affinity and binding capacity of the biotic ligand for Ag^+, can be entered into computational models in which the amount of Ag binding and, hence, the Ag toxicity can be predicted. This is the basis for the biotic ligand model (BLM) [8–11]. The success of such models depends on knowledge of toxic mechanisms and metal speciation in the natural environment.

Natural organic matter affects Ag speciation and, hence, Ag toxicity (see, e.g., [12]). These complex organic molecules are characterized by their ubiquity and heterogeneity, with the latter property resulting from diverse precursor molecules and the effects of biological and physical conversion processes [13]. The high affinity of NOM for Ag ($\log K = 9.0$–9.2 [12]) is likely responsible for their observed protective effects regarding freshwater organisms in toxicity tests (see, e.g., [11,14–16]). In fact, the magnitude of the protection offered by NOM is considerably greater than that offered by increased water hardness [15,16]. Current water-quality guidelines established by the U.S. Environmental Protection Agency account only for the effect of hardness, which may have been misinterpreted as a covariant of chloride, a more protective complexing agent [1]. They do not consider the potential ameliorative effects of NOM [17]. In contrast, the BLM offers a more realistic approach to assessing the environmental risk of Ag in the aquatic environment, because it accounts for the important influence of NOM as well as all relevant individual ions in the water column, such as calcium, magnesium, sodium, and chloride.

Such models are a considerable improvement on the hardness-derived criteria, but they still require refinement. In particular, existing BLMs do not account for the considerable heterogeneity of NOM. The present study sought to determine whether the heterogeneity of NOM is reflected in a heterogeneous protective response to waterborne Ag toxicity to the

* To whom correspondence may be addressed (chris.glover@nifes.no).

freshwater cladoceran *D. magna*. Cladocerans are the organisms most sensitive to waterborne Ag, with acute median lethal concentrations (LC50s) of less than 1 μg/L (see, e.g., Bianchini [18]). These small, freshwater hyper-regulators possess a high surface area to volume ratio, resulting in a rapid loss of sodium and other ions to the external medium across the body surface [19,20]. The reliance on efficient active-uptake mechanisms to compensate for these ion losses makes these animals especially susceptible to ionoregulatory toxicants.

The effect of NOM source on Ag toxicity was assessed using NOM collected from natural waters by reverse osmosis and commercially available sources. In addition, the influence of Ag–NOM equilibration time on Ag toxicity and Ag bioaccumulation was examined. The results from the present study may be influential in determining whether NOM source and equilibration time will be important parameters for future calibrations of the BLM in predicting Ag toxicity to *D. magna* and other freshwater biota.

MATERIALS AND METHODS

Daphnia magna *culture*

A laboratory culture of *D. magna* was established from adult gravid animals acquired from Aquatic Research Organisms (ARO strain; Hampton, NH, USA). Daphnids were maintained in constant light (16:8-h light:dark photoperiod), temperature (20–22°C), and culture medium conditions. The culture medium was a moderately hard laboratory water reconstituted from water that had been deionized by reverse osmosis, with a composition based on that of Lake Ontario, North America (1 mM $CaCO_3$, 0.15 mM $MgSO_4 \cdot 7H_2O$, and 0.6 mM NaCl; pH 8.0). Water was bubbled for 24 h with CO_2 to ensure dissolution of $CaCO_3$, then further aerated for 48 h with air to rid excess CO_2. Selenium (2.5 μg/L) and vitamin B_{12} (6 μg/L) were added to culture water to ensure adequate nutrition, but these elements were excluded from toxicity trials.

48-h LC50 determination

Toxicity tests were performed using a semistatic renewal system [19]. Each toxicity testing chamber (new, acid-washed, 250-ml glass beakers) contained the appropriate concentration of NOM and one of five or six Ag concentrations in 250 ml of synthetic Lake Ontario water. Silver was added to test solutions from a stock containing radiolabeled $^{110m}AgNO_3$ (~5–10 kBq/μg Ag; RISØ National Laboratory, Roskilde, Denmark) and unlabeled $AgNO_3$ at a total Ag concentration of approximately 5 mg/L. Silver concentrations in stock solutions were measured by graphite-furnace atomic absorption spectrometry (model AA-1275 with GTA-9 atomizer; Varian, Palo Alto, CA, USA), and the specific activity of the stock solutions was used to determine exposure concentrations by measurement of ^{110m}Ag radioactivity in the test chambers. Based on the results of experiments described below, the chambers were prepared 24 h before test commencement to allow adequate equilibration of Ag with the NOM and were kept in the dark to prevent light-mediated photolysis. Ten daphnid neonates (age, <24 h) were added into each chamber before it was placed back in the dark for 24 h. After 24 h, dead daphnids were counted and removed, and living animals were transferred into a second test chamber that contained water with a composition identical to that in the first chamber, which had again been left for 24 h in the dark to allow for Ag–NOM equilibration to occur. Tests were terminated 48 h after inoculation. The high volume to biomass ratio, the 24-h renewal, and the withholding of food were intended to minimize daphnid contribution to organic carbon levels [21].

Median lethal concentration tests were repeated three or four times for each NOM source and concentration. The results for each test were pooled, and 48-h LC50 values and their 95% confidence intervals were determined using probit analysis (Probit Analysis Program, Ver 1.5; U.S. Environmental Protection Agency, Cincinnati, OH, USA). For all tests, the addition of NOM had no influence on the pH of solutions, with the exception of Dundas Water Treatment Plant NOM (DSN; ON, Canada) and Luther Marsh NOM (LMN; ON, Canada). These solutions were returned to pH 8.0 by the addition of 0.1 N KOH.

Water samples from each test chamber were taken for γ-counting (Minaxi Auto-gamma 5000; Canberra-Packard, Meridian, CT, USA), applying the appropriate counting window [22]. Samples were taken for determination of both total and dissolved Ag (filtered through 0.45-μm syringe filters; Acrodisc; Pall-Gellman; Ann Arbor, MI, USA). Further water samples were taken for measurement of total and dissolved organic carbon levels. Samples (25 ml of water) were collected in glass vials, ensuring no headspace that could result in degradation before measurement. Dissolved samples were collected in a similar manner, except the water sample was passed through a 0.45-μm syringe filter. Filters were initially rinsed with 25 ml of deionized water, followed by 25 ml of the water sample, before the final 25 ml of filtrate were taken for subsequent organic carbon analysis.

Determining the effect of Ag–NOM equilibration time

Median lethal concentration analyses for determination of equilibration time effects were performed identically to the 48-h tests described above with three exceptions. First, toxicity tests ran for only 24 h. Second, only Aldrich humic acid (AHA) was used (~0–4 mg C/L). Third, two equilibration times of NOM with $AgNO_3$ were used (3 and 24 h). Toxicity tests were started immediately after the equilibration period had ended.

Silver accumulation with respect to Ag–NOM equilibration time was examined using 8- to 9-d-old daphnids (~1 mg). Beakers containing synthetic Lake Ontario water and a range of AHA concentrations (~0–7 mg C/L) were equilibrated with 1 μg Ag/L (added as $^{110m}AgNO_3$) for 3 or 24 h before daphnid addition (eight daphnids in 250 ml). After 16 h, daphnids were removed, rinsed (10–20 s) in a high-Ag solution (~200 mg Ag/L) to displace surface-bound Ag, rinsed again (30–45 s) in deionized water, blotted dry, and weighed (precision, 0.001 mg; UMT2; Mettler-Toledo, Greifensee, Switzerland). Daphnids were then counted for Ag accumulation by γ-counting as described above.

NOM source and concentration determination

Nordic Reservoir NOM (NRN), Nordic Reservoir humic acid (NRH), Nordic Reservoir fulvic acid (NRF), and Suwannee River NOM (SRN) samples were all obtained from the International Humic Substances Society (St. Paul, MN, USA) as freeze-dried, reference-grade samples (see Table 1). Aldrich humic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA), also as a freeze-dried powder. The natural or ''real-world'' isolates (Black Creek NOM [BCN; ON, Canada], Grand River NOM [GRN; ON, Canada], Rochester Wastewater Treatment Plant [R(I) and R(II); NY, USA], LMN, and DSN) were collected via reverse-osmosis concentration of natural

Table 1. Sources of natural organic matter (NOM) used

NOM	Code	Physical state	Supplier
Aldrich humic acid (sodium salt)	AHA	Freeze-dried powder	Sigma-Aldrich[a]
Nordic Reservoir NOM	NRN	Freeze-dried powder	International Humic Substances Society[b]
Nordic Reservoir humic acid	NRH	Freeze-dried powder	International Humic Substances Society
Nordic Reservoir fulvic acid	NRF	Freeze-dried powder	International Humic Substances Society
Suwannee River NOM	SRN	Freeze-dried powder	International Humic Substances Society
Black Creek NOM (ON, Canada)	BCN	Reverse-osmosis concentrate	Collected by R.A. Bell
Grand River NOM (ON, Canada)	GRN	Reverse-osmosis concentrate	Collected by R.A. Bell
Rochester Wastewater Treatment Plant (NY, USA)	R(I)	Reverse-osmosis concentrate	Collected by R.A. Bell
Rochester Wastewater Treatment Plant (NY, USA)	R(II)	Reverse-osmosis concentrate	Collected by R.A. Bell
Luther Marsh NOM (ON, Canada)	LMN	Reverse-osmosis concentrate	Collected by R.C. Playle
Dundas Wastewater Treatment Plant (ON, Canada)	DSN	Reverse-osmosis concentrate	Collected by R.C. Playle

[a] St. Louis, Missouri, USA.
[b] St. Paul, Minnesota, USA.

waters. This methodology has been detailed elsewhere [23,24]. The isolates BCN, R(I), R(II), and GRN were not modified postisolation. The LMN and DSN samples were passed through a cation-exchange resin to remove potential trace-element contamination.

The NOM concentration was determined in the following manner. A standard curve for each NOM was created covering the experimental concentration range, and samples were analyzed for total organic carbon using a Shimadzu total organic carbon analyzer (model 5050A; Mandel Scientific, Guelph, ON, Canada). Before analysis, each sample was acidified with a single drop of 16 N HNO_3 and sparged with N_2 gas to remove inorganic carbon. An identical set of samples was used to generate a standard curve of absorbance at 300 nm using a benchtop spectrophotometer (Ultrospec 4054; LKB, Bromma, Sweden). Linear correlations were established between organic carbon level and absorbance. Consequently, organic carbon concentrations during tests were monitored by measuring absorbance at 300 nm. Available stock for the BCN, GRN, R(I), and R(II) samples was limited; thus, the concentrations in test solutions were relatively low. For these samples, the test concentrations recorded were nominal, based on organic carbon analysis of stock solutions. A similar difficulty existed for DSN. The optically light nature of this stock meant the correlation between absorbance and organic carbon concentration was poor. However, because the level of DSN tested was relatively high, the organic carbon to absorbance ratio for five samples with organic carbon in the range of that employed in the DSN toxicity tests was used to generate a single value (absorbance per unit organic carbon) by which DSN levels could be monitored.

Statistical analysis

Statistical differences between 48-h LC50 values in the presence of different NOM sources could not be determined directly because of differences in tested organic carbon concentrations among NOM samples. Consequently, the 95% confidence intervals for the linear regression of the AHA data (48-h LC50 vs NOM concentration) were plotted. The LC50 values for other NOMs having 95% confidence intervals that overlapped the AHA 95% confidence interval were considered to offer protection not significantly different from that afforded by AHA [25]. The significance of differences in Ag accumulation was assessed by two-way analysis of variance using equilibration time and concentration as independent factors with a post-hoc, least-significant-difference analysis.

RESULTS

The addition of AHA to test waters decreased Ag toxicity in a concentration-dependent manner (Fig. 1). This effect was especially prominent at low levels of added AHA, where the LC50 for 0.6 mg C/L was 1.48 μg total Ag/L, up from a nominally NOM-free value of 0.29 μg total Ag/L. A linear

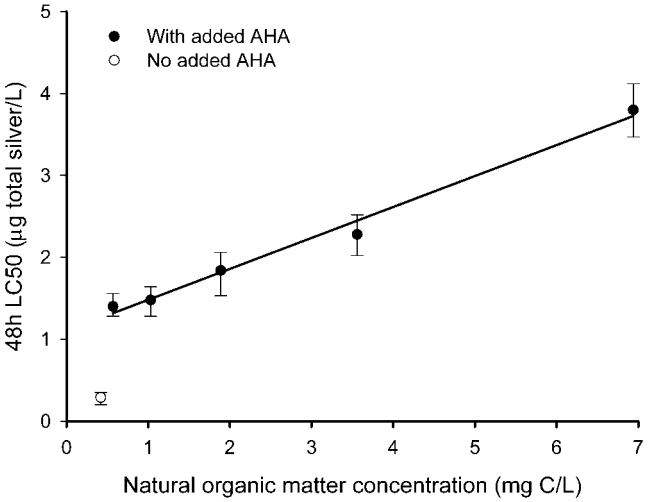

Fig. 1. Effect of increasing Aldrich humic acid (AHA) concentration (mg C/L) on median lethal Ag toxicity (48-h LC50; μg total Ag/L) to *Daphnia magna* neonates (age, <24 h). Plotted values represent the mean ± 95% confidence interval for three or four tests as described in *Materials and Methods.*

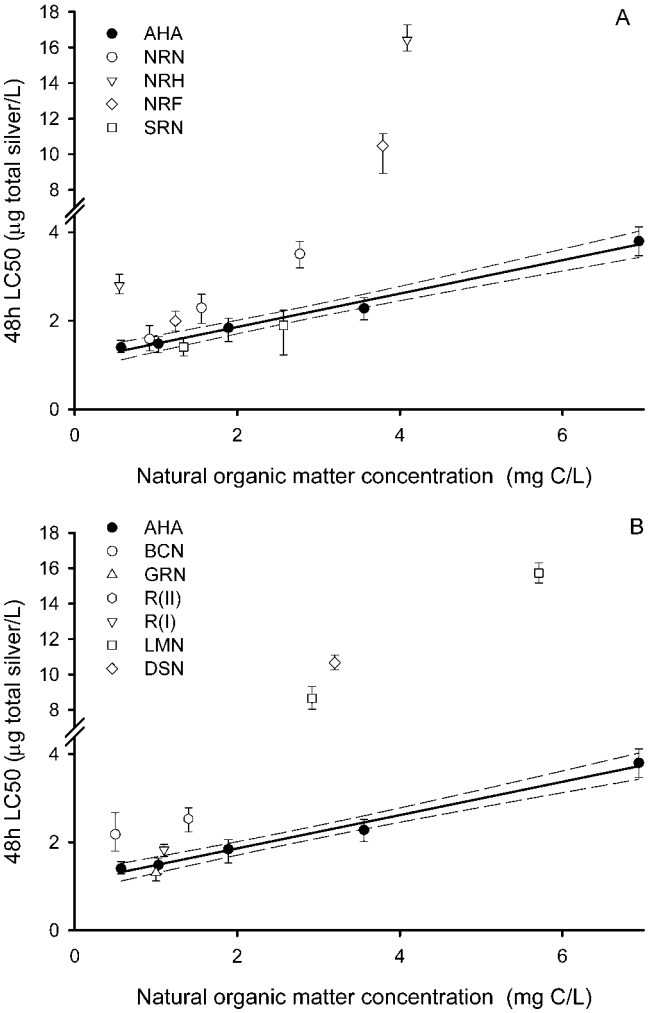

Fig. 2. Effect of commercially available (**A**) and reverse osmosis (**B**) natural organic matter source and concentration on median lethal Ag toxicity (48-h LC50; µg total Ag/L) to *Daphnia magna* neonates (age, <24 h). Plotted values represent the mean ± 95% confidence interval for three or four tests. Three-letter codes are those detailed in Table 1. The black circles with fitted regression line (±95% confidence intervals) represent the values recorded for Aldrich humic acid.

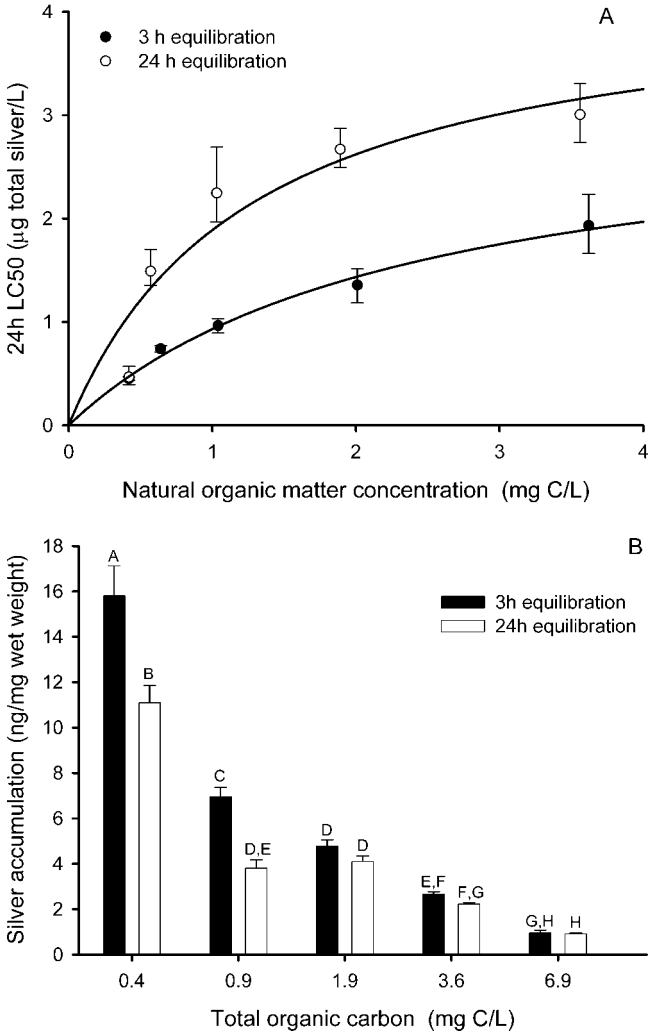

Fig. 3. Effect of Ag–natural organic matter equilibration time on (**A**) median lethal Ag toxicity (24-h LC50; µg total Ag/L) and (**B**) whole-body Ag accumulation (ng Ag/mg wet wt) in *Daphnia magna*. Plotted values for LC50 data represent the mean ± 95% confidence interval for three or four tests on *D. magna* neonates (age, <24 h), whereas plotted accumulation data represent the mean ± standard error of four (3-h equilibration; 0.4 mg C/L) or eight (all other values) 8- to 9-d-old *D. magna* following a 16-h exposure to 1 µg Ag/L. Statistical significance was determined by two-way analysis of variance followed by post-hoc, least-significant-difference test at a significance level of $\alpha = 0.05$. Bars sharing letters are not significantly different. The overall effect of equilibration time ($p < 0.001$) was significant.

regression fit of these values yielded the following equation: 48-h LC50 = 1.10 + 0.38[NOM], $r^2 = 0.99$.

The ameliorative effects of the tested NOM samples are summarized in Table 2. Dissolved (<0.45 µm) Ag and organic carbon levels were very similar to the values for total Ag and organic carbon, indicating that the Ag and organic matter were in the dissolved phase. The single exception to this pattern was the 48-h LC50 dissolved Ag value for DSN, which was considerably lower than the 48-h LC50 derived from total Ag values (6.12 vs 10.65 µg Ag/L).

Some NOM sources offered considerably greater protection against Ag toxicity to *D. magna* than that afforded by AHA (Fig. 2). In fact, of all the NOMs tested, only three (GRN, R(I), and SRN) consistently offered protection on the same magnitude as AHA, whereas none was less protective. Low levels of NRN and NRF (<1.5 mg C/L) also offered protection equivalent to that from AHA, but these NOMs were more protective at higher levels. The most protective samples of NOM were NRH, NRF, LMN, and DSN, all of which required Ag levels fourfold greater than the equivalent AHA Ag concentration to cause 50% mortality over 48 h.

The effect of Ag–NOM equilibration time (3 or 24 h) on Ag toxicity to *D. magna* neonates is illustrated in Figure 3A. A prominent decrease in Ag toxicity (increase in 24-h LC50 value) occurred with increased equilibration time. This effect was significant overall but most prominent at 1 mg C/L, where 24-h equilibration resulted in a 48-h LC50 value more than twofold higher than that after 3-h equilibration.

Two trends are exhibited in Figure 3B. As the NOM concentration increased, the accumulation of Ag deceased, mirroring the decrease in toxicity observed as NOM levels were raised (Fig. 3A). An effect of equilibration time also was observed, whereby increased equilibration time resulted in a reduction in daphnid whole-body Ag burden. This effect was noted at all NOM levels tested and was significant overall, but it was only individually significant at NOM concentrations less than 1 mg C/L.

DISCUSSION

Heterogeneity in humic substances protection against Ag toxicity to D. magna

A clear, ameliorative effect of NOM on Ag toxicity to *D. magna* was observed. In the present study, the 48-h LC50 in the nominal absence of NOM was 0.29 μg Ag/L, which is very similar to the 48-h LC50 values of 0.18 to 0.26 μg Ag/L for *D. magna* reported by Bianchini et al. [18,19] in an identical water. All of these 48-h LC50 values are lower than those recorded by most other researchers (~0.5–1 μg Ag/L [11,14,16,26,27]). This discrepancy is likely a result of the low background NOM levels and lower alkalinity (100 mg/L as $CaCO_3$) of the synthetic water used in the present study and those of Bianchini et al. [18,19] as well as the use of measured Ag levels that account for loss of Ag to toxicity chamber walls because of binding (see, e.g., [19]). In addition, differences in LC50 values may reflect the longer equilibration time (24 h) between Ag and the trace amounts of NOM in the water used during the present study (see below).

The addition of AHA to the test chamber decreased Ag toxicity to *D. magna* in a concentration-dependent manner. This decrease in Ag toxicity was the result of NOM complexation, rendering Ag unavailable to interact with the toxic site of action, as indicated by decreased Ag accumulation (Fig. 3B). At the highest AHA concentration tested (6.9 mg C/L), the 48-h LC50 was 3.80 μg/L, a 13-fold decrease in toxicity over nominally NOM-free conditions. This is a considerably higher protection than the 6.0- to 6.5-fold ameliorative effect reported for higher apparent concentrations of AHA in previous studies [11,16]. However, the poor solubility of AHA in water is such that 10 mg C/L (the nominal level reported by Bury et al. [11]) equates to a measured total organic carbon level of 1.9 mg C/L, as determined by the method described in *Materials and Methods*. At this total organic carbon level in the present study, a protection of 6.3-fold was discerned, which is in line with the protection reported in previous studies. The study of Karen et al. [16] reported only nominal NOM values, although measurement of NOM concentrations was described. Assuming the 10 mg C/L concentration reported by those authors is a measured value, the toxicity of the unusual chemical composition of the water itself, which contained little if any sodium, probably accounts for the lower protective effect of AHA reported in that study.

The protective effect of AHA was most prominent at low concentrations. The addition of 0.6 mg C/L of AHA increased the 48-h LC50 by nearly fivefold. To our knowledge, this high protection at very low levels of added NOM has not been noted previously. This could be a function of the low control LC50 value, leading to a greater scope for increase, or a function of the greater sensitivity of daphnids to Ag. At the low concentrations of Ag required for toxicity, relatively lower concentrations of NOM would be required to chelate the toxic free ion (Ag^+); thus, amelioration would occur at low NOM concentrations. In support of this, further additions of AHA had lesser ameliorative actions, albeit still in a concentration-dependent manner (Fig. 1). The slope of the linear regression fitted to the increase in LC50 values (in μg total Ag/L) with increasing AHA concentrations (mg C/L) was 0.38. This result is in agreement with other studies that showed only a modest increase in protection with increasing NOM concentration [14,16,28]. In fact, a slope of 0.40 was determined for the protective effect of site-1 NOM against Ag toxicity to fathead minnows [28], corresponding very closely to that described the present study.

Considerably greater increases in protective effect were noted with other NOMs. In fact, of all samples tested, only SRN (of the commercially available samples) and GRN and R(I) (of the naturally isolated samples) had protective effects equivalent to that of AHA. All other samples tested had significantly greater ameliorative effects at one or more tested concentrations. Traditionally, AHA is the NOM of choice in laboratory testing studies because of its availability and low cost. The present study, however, clearly shows that it may not be representative of NOM in all natural waters.

The influence of NOM source in aquatic toxicology has been described previously. Effects of NOM source on the toxicity of copper to *Ceriodaphnia dubia* [29] and *D. magna* [30] have been reported, with effects on Ag toxicity to fathead minnows also observed [28]. Richards et al. [31] showed that LMN was the most effective of three NOMs for protecting against mixed-metal toxicity to rainbow trout, corroborating the present findings with *D. magna*. However, neither LMN nor the other NOM sources succeeded in decreasing gill Ag burden, but both lead and copper binding were NOM-source dependent [31].

The influence of NOM source appears to depend on differing species-dependent binding affinities of Ag for the biotic ligand (see below). Furthermore, metals appear to bind to distinct moieties within the NOM; consequently, NOM may have different protective abilities for the toxicity of different metals. Silver, for example, likely binds to nitrogen-containing groups [32] and, possibly, to organic thiols [33], whereas a metal, such as copper, tends to bind to carbon-containing moieties, such as phenolic groups [34]. Clearly, the influence of NOM source on metal toxicity is likely to depend on both the metal and the organism under investigation as well as on the nature of the NOMs themselves. The correlation of toxicity amelioration with the physicochemical properties of the various NOMs used in the present study is the focus of the companion study [35].

The similarity between total and filtered toxicity values (Table 1) suggests that Ag bound to NOM in the present study was fully dissolved or colloidal and, thus, able to pass through 0.45-μm filters. This result is consistent with the pattern of Ag speciation in natural waters [36]. The exception was Ag in the presence of DSN. In these tests, the 48-h LC50 decreased from 10.65 to 6.12 μg Ag/L when calculated on the basis of dissolved Ag. This decrease in dissolved Ag was not correlated with a decline in total organic carbon, which remained at 2.3 to 2.4 mg C/L. This suggests the presence of Ag complexation to another component of DSN. This NOM sample contained very high concentrations of Cl^- [35], suggesting that the formation of insoluble Ag–chloride complexes may have been responsible for this anomaly.

Effect of Ag–NOM equilibration

A longer Ag–NOM equilibration time before addition of animals to test solutions resulted in both decreased Ag toxicity and decreased Ag accumulation (Fig. 3). This contrasts with the results of Hollis et al. [23], who found no effect of NOM–metal equilibration time on cadmium and copper accumulation on rainbow trout gill. The NOM levels tested in that study were 5 mg C/L and, therefore, corresponded to a concentration at which virtually no effect of equilibration time was observed in the present investigation, suggesting that the effects of equil-

Table 2. Effect of natural organic matter (NOM) on 48-h median lethal concentration (48-h LC50) of silver to *Daphnia magna*[a]

NOM source	Total NOM concentration (mg C/L)	Dissolved NOM concentration (mg C/L)	48-h LC50 (μg/L)	
			Total Ag(I)	Dissolved Ag(I)
NOM-free			0.29 (0.20–0.35)	0.23 (0.17–0.29)
AHA	0.57 ± 0.03	0.57 ± 0.03	1.40 (1.28–1.56)	1.35 (1.23–1.50)
	1.03 ± 0.03	0.99 ± 0.03	1.48 (1.28–1.64)	1.41 (1.22–1.56)
	1.89 ± 0.03	1.86 ± 0.04	1.84 (1.53–2.06)	1.72 (1.46–1.91)
	3.56 ± 0.04	3.52 ± 0.06	2.28 (2.02–2.52)	2.12 (1.88–2.35)
	6.94 ± 0.03	6.71 ± 0.10	3.80 (3.47–4.12)	3.56 (3.26–3.87)
NRN	0.92 ± 0.07	0.89 ± 0.15	1.59 (1.32–1.89)	1.56 (1.29–1.88)
	1.56 ± 0.07	1.91 ± 0.17	2.29 (1.94–2.60)	2.33 (1.95–2.66)
	2.77 ± 0.08	2.64 ± 0.06	3.51 (3.20–3.80)	3.69 (2.96-4.28)
NRH	0.55 ± 0.05	0.46 ± 0.08	2.80 (2.61–3.05)	2.84 (2.67–3.10)
	4.09 ± 0.11	4.23 ± 0.21	16.42 (15.80–17.26)	16.65 (16.00–17.54)
NRF	1.24 ± 0.06	1.34 ± 0.08	2.00 (1.77–2.22)	2.08 (1.83–2.32)
	3.79 ± 0.09	3.85 ± 0.10	10.47 (8.92–11.17)	9.14 (7.19–9.88)
SRN	1.34 ± 0.10	1.26 ± 0.09	1.41 (1.21–1.62)	1.41 (1.21–1.62)
	2.57 ± 0.10	2.82 ± 0.10	1.90 (1.23–2.24)	1.97 (1.31–2.32)
BCN	0.5		2.18 (1.80–2.67)	2.14 (1.74–2.63)
GRN	1.0		1.31 (1.12–1.45)	1.29 (1.11–1.43)
R(I)	1.4		2.53 (2.23–2.78)	2.54 (2.29–2.74)
R(II)	1.1		1.83 (1.68–1.95)	1.85 (1.72–1.95)
DSN	2.37 ± 0.17	2.34 ± 0.16	10.65 (10.27–11.09)	6.12 (5.43–6.97)
LMN	2.92 ± 0.12	3.03 ± 0.11	8.65 (8.04–9.32)	7.57 (7.01–8.21)
	5.71 ± 0.11	5.83 ± 0.21	15.73 (15.17–16.31)	13.91 (13.44–14.39)

[a] Values represent the mean ± standard error (NOM concentrations) or the mean ± 95% confidence intervals (LC50 values). Refer to *Materials and Methods* for descriptions of NOM measurement and LC50 analysis. Organic carbon concentrations in the NOM-free treatment were not measured directly but were less than 0.4 mg C/L as Aldrich humic acid equivalents. Three-letter codes for NOM sources are given in Table 1.

ibration are likely concentration dependent (Fig. 3B). In addition, both the test organisms and the metals were different, and the metal levels were much higher (>500 nM in the study of Hollis et al. [23] vs 10 nM in the present study). A major mitigating factor explaining the difference between these studies may be the sensitivity of the experimental organism used, which in turn dictates the tested concentration range of metal. Copper–NOM equilibration time influenced both the accumulation and toxicity of copper in another cladoceran species, *C. dubia* [29,37]. Furthermore, Erickson et al. [14] found a significant, 30 to 40% increase in the Ag 48-h LC50 value for *D. magna* in aged solutions but no corresponding increase in the protective effects of aged waters for fathead minnows.

Physicochemical properties also may dictate the interaction of Ag with organisms in the presence of NOM. For example, the time-dependent kinetics of Ag complexation with NOM likely is important. The reaction time of humic materials with lanthanide metals, for example, is very rapid, with complete metal complexation occurring within minutes, as determined by ultrafiltration [38]. However, after 15 min, only 4% of the metal remained bound following passage through a cation-exchange resin, in contrast to the 40% binding observed after 48 h [38]. This result suggests that whereas initial weak binding is almost instantaneous, the formation of strong binding, perhaps to functional groups sterically hindered from surface interactions, is slower to develop. The lowered accumulation and toxicity observed after 24 h may result from the slow development of strong-bond formation between Ag and NOM, resulting in a higher biologically unavailable fraction of complexed Ag.

These findings also indicate that the initial period of exposure to Ag likely dictates Ag toxicity. For the effect of Ag–NOM equilibration, toxicity was determined over 24 h. Consequently, for 3-h equilibration, daphnids were exposed to Ag that had been equilibrating for 3 to 27 h versus 24 to 48 h for 24-h equilibration (i.e., overlapping time frames of metal–NOM equilibration). It therefore can be assumed that Ag toxicity is mediated early in the 3-h equilibration.

The present results have considerable implications for the modeling of Ag toxicity to aquatic organisms. The effects of both NOM source and Ag–NOM equilibration time will need to be taken into account. Previously, it has been suggested that the effect of NOM source could be accounted for by a weighting factor that incorporates a property of the NOM that correlates with toxicity [24,30,31]. Physicochemical correlates of toxicity amelioration are the focus of the companion study [35]. Equilibration time also may be an important factor to consider in laboratory calibrations of future BLMs. In most natural waters, metals will be in equilibrium with NOM. Unless laboratory experiments account for equilibration time, they may overestimate the potential risk posed by environmental metals, which could result in overly protective water-quality criteria.

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