Physiological interactions of silver and humic substances in *Daphnia magna*: effects on reproduction and silver accumulation following an acute silver challenge

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Abstract

Silver (Ag) in aquatic environments mediates its toxic actions by inhibiting sodium influx. Humic substances protect against silver toxicity by complexing the toxic, ionic form of the metal, but may also directly stimulate sodium influx in aquatic organisms. This study investigated the effects of silver and humic substances on the water flea *Daphnia magna*. Acute silver challenge (24 h; 1 μg L^{-1}) and the chronic exposure to humic substances (Aldrich humic acid; 7 mg C L^{-1}) had considerable influence on daphnid physiology and reproduction. In particular silver exposure in the absence of humic substances stimulated reproduction, resulted in enhanced adult mass, and altered both the response of the animal to subsequent silver exposure and a physiological surrogate measure of silver toxicity (whole body sodium concentration). The presence of humic substances countered the effects on adult mass and reproduction, returning these parameters to control levels. Humic substances also lowered silver body burden, but with significantly improved whole body sodium status than previously silver-exposed animals. These changes may distort the correlation between silver body burden and indicators of toxic action, an important tenet of site-specific risk assessment tools such as the biotic ligand model.
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1. Introduction

The ionic form of silver (Ag^+) is highly toxic to freshwater organisms. In rainbow trout, Ag^+ passes across the branchial epithelia via a sodium channel (Bury and Wood, 1999), and subsequently inhibits cytosolic carbonic anhydrase and basolateral Na^+, K^+-ATPase resulting in compromised sodium uptake (Morgan et al., 1997, 2004). For aquatic organisms that rely on the active scavenging of ions from a dilute medium for maintenance of body solute levels, sodium influx inhibition can prove fatal (Wood et al., 1996). In fact the greater the sodium turnover rate, the more

toxic Ag^+ is, explaining the high silver sensitivity of the freshwater cladoceran *Daphnia magna* (Bianchini et al., 2002b; Grosell et al., 2002). Recent studies have shown that the mechanism of silver toxicity in *D. magna* resembles that in fish with inhibition of sodium uptake and whole body Na^+, K^+-ATPase activity (Bianchini and Wood, 2003). Furthermore, silver toxicity over chronic exposure periods is similar in nature to that during acute exposures in both daphnids and fish (i.e. ionoregulatory disturbance; Guadagnolo et al., 2001; Bianchini and Wood, 2002; Galvez and Wood, 2002).

Humic substances ameliorate acute silver toxicity to both freshwater fish (e.g. Bury et al., 1999; Van Genderen et al., 2003) and invertebrates (e.g. Karen et al., 1999). This effect is primarily attributed to humic substances binding, sequestering, and hence rendering biologically unavailable, the toxic free metal ion (Janes and Playle,

1995). The ubiquity of humic substances in natural waters and their metal complexation effects ensures that acute silver toxicity is essentially a laboratory phenomenon. Traditionally, humic substances, and other important silver ion complexors, such as reduced sulfides (Rozan et al., 2000), have been excluded from test media. Consequently, silver toxicity in reconstituted waters is likely to greatly overestimate toxicity in field conditions. For example, environmentally realistic humic substance concentrations (<10 mg C L^{-1}) can raise *D. magna* 48 h median lethal concentration (LC_{50}) values by more than 50-fold (unpublished data). As a result silver may only reach acutely toxic concentrations at the point of anthropogenic discharges, where the exposure level may be only transiently high, and the system is temporarily in disequilibrium (e.g. Shafer et al., 1998).

Recently it has been discerned that humic substances can directly influence ion homeostasis in freshwater animals (Wood et al., 2003; Matsuo et al., 2004; Glover et al., in press). In *D. magna*, for example, humic substances significantly increased the maximal rate of sodium influx and elevated whole body sodium concentrations (Glover et al., in press). Such an outcome is in direct contrast to the sodium depleting actions of silver (Bianchini and Wood, 2003). It is possible, therefore, that humic substances could protect against silver toxicity by promoting enhanced ion status, in addition to the protective effects resulting from complexation of the toxic silver ion.

The current study sought to investigate the effect of an acute silver challenge (1 μg L^{-1}) on reproductive and physiological parameters of the water flea *D. magna*. Such an exposure resembles a high, transient exposure to a silver point source. In addition, the effect of long-term exposure to humic substances on *D. magna* physiology and reproduction were investigated. The experimental design consequently permitted an analysis of interactions between silver and humic substances. This was of particular interest given the fact that both these entities act upon sodium metabolism, but with contrasting effects.

2. Materials and methods

2.1. D. magna culture

A laboratory culture of *D. magna* was established from gravid females originally acquired from Aquatic Research Organisms (ARO strain; Hampton, N.H.). The culture was maintained in synthetic laboratory water resembling that of Lake Ontario (synthetic Lake Ontario water (SLOW): 1 mM $CaCO_3$, 0.15 mM $MgSO_4 \cdot 7H_2O$, 0.6 mM NaCl), reconstituted from reverse osmosis water. *D. magna* were kept under constant light (16L:8D) and temperature (20–22 °C) conditions and were fed a slurry of yeast, alfalfa, and digested trout chow daily (~6 mL L^{-1}), with water renewal every two days.

2.2. Initial silver exposure

Seven-day old *D. magna* were transferred into one of two different silver exposure conditions: humic (Aldrich Humic Acid (AHA): 7.1±0.1 mg C L^{-1}) or control (humic-free; 0.3±0.1 mg C L^{-1}). Each exposure chamber consisted of an acid-washed glass beaker (Pyrex) containing 50 mL of SLOW and, where appropriate, AHA added from a ~750 mg C L^{-1} stock. Silver was added to each exposure solution as ^{110m}Ag (RISØ National Laboratory, Roskilde, Denmark) to give a final concentration of 1 μg L^{-1}. The metal was added to solution 24 h prior to daphnid introduction to allow for appropriate equilibration with the humic substance. The 24 h LC_{50} value for neonate *D. magna* in SLOW is ~0.5 μg L^{-1} as total silver (unpublished results).

Individual daphnids were added to each beaker. After 24 h animals were removed, placed in 250 mL of SLOW (2×30s) to flush out radiolabelled carapace water, and then transferred to 1.5 mL of SLOW in a 2 mL microcentrifuge tube for γ-counting of ^{110m}Ag activity (Canberra-Packard, Minaxi Auto-gamma 5000). Time in the counting chamber was kept to a minimum (<20 min) to reduce any stress, and the animals were not weighed at this time. *D. magna* were then transferred to an appropriate holding solution (see below).

Concomitantly, a second group of *D. magna* were removed from stock cultures, and housed in silver-free exposure chambers of identical composition to those described above (i.e. humic or humic-free). These animals were handled in an identical manner to silver-exposed animals during and following the 24 h "exposure". As with their silver-exposed counterparts these animals were then transferred to an appropriate holding solution (see below).

2.3. Holding regime

Holding chambers were acid-washed 250 mL beakers containing 50 mL of SLOW. Silver- and control-exposed *D. magna* were transferred individually to either humic (Aldrich Humic Acid: ~7 mg C L^{-1}) or control (humic-free) holding chambers. Consequently, four different exposure/holding regimes were examined: 1) control (humic-free) exposure, control (humic-free) holding (C,C); 2) control exposure, humic holding (C,H); 3) humic exposure, control holding (H,C); and 4) humic exposure, humic holding (H,H). Taking into account the two different exposure treatments (silver- or control-exposed) this resulted in eight different treatment groups (see Table 1). In all cases, these groups remained silver-free for the remaining 14 days of the experiment.

D. magna were fed 1 mL of a yeast, alfalfa, digested trout chow slurry two out of every three days, with water renewal every two or three days. Reproduction was monitored on a daily basis, for two weeks following the initial silver exposure.

Table 1
D. magna mortality and final mass in treatment groups

Treatment group	Mortality (%)	Daphnid mass (mg wet mass)	n
C,C +Ag	25	8.38±0.25	6
C,C −Ag	0	6.72±0.46*	8
C,H +Ag	37.5	7.31±0.48*	5
C,H −Ag	0	6.83±0.26*	8
H,C +Ag	37.5	7.27±0.32*	5
H,C −Ag	0	7.35±0.21*	7
H,H +Ag	0	7.41±0.28*	8
H,H −Ag	0	7.57±0.15	8

The first letter of the treatment group refers to initial 24 h exposure conditions where "C" is control (humic-free) and "H" is humic (7.1 mg C L^{-1}). The second letter corresponds to the holding regime where "C" and "H" again represent control or humic conditions. The +Ag or −Ag designation refers to the absence or presence of silver (1 μg L^{-1}) in the initial exposure. The n column records the number of animals used in all reported values for each treatment group, and represents the post-mortality count (all groups started with n=8). Daphnid wet mass is reported as the mean±S.E.M., at the conclusion of the experiment. Significant differences (*p<0.05) between the C,C +Ag group and other groups was determined by one-way ANOVA. No other significant differences were noted.

2.4. Subsequent silver exposure

After two weeks in its holding regime, each individual daphnid was exposed to silver in a similar manner to that described earlier. Each daphnid was transferred to an exposure chamber containing 50 mL of humic-free SLOW and 1 μg L^{-1} silver as ^{110m}Ag, and uptake was again measured over a 24 h period. Any reproduction that occurred during the silver exposure was recorded. *D. magna* were removed from exposure chambers, rinsed in a silver displacement solution (~1 mg L^{-1} AgNO$_3$) for 15 s, then in two SLOW rinses for a total of 1 min, before being blotted dry, weighed (UMT2, Mettler Toledo) and counted for γ-activity. Following counting, daphnids were digested in 50 μl of concentrated H$_2$SO$_4$, diluted and assayed for whole body sodium content by flame atomic absorption spectrometry (220FS, Varian).

2.5. Silver and humic substance measurement

Silver concentrations of the radiolabelled stock were measured by graphite furnace atomic absorption spectrometry (GFAAS; Varian AA-1275 with GTA-9 atomizer). Humic substance concentration was determined by spectrophotometric correlation of samples to standards of known organic carbon content as described previously (Glover et al., in press).

2.6. Data handling

Values reported throughout are the means±S.E.M. of n observations, where the n value is that recorded in Table 1. Data from daphnids that died during the experiment were excluded from subsequent statistical analyses (Hamilton, 1986). Statistical differences were determined at α=0.05,

using one-way ANOVA, and post-hoc Fisher LSD analysis (Statistica 5.1, StatSoft).

3. Results

All *D. magna* exposed to silver survived the initial 24 h exposure period. Mortality rates up to 37.5% were noted in the following 24 h (Table 1), but no deaths were recorded in any treatment group thereafter. All mortality occurred prior to first brood. The final adult mass of the surviving daphnids was significantly greater in silver-exposed animals, that were never exposed to humic substances (C,C +Ag), compared to silver-free and/or humic-exposed groups.

In the initial silver exposure, the presence of humic substances during the exposure significantly reduced silver accumulation (Fig. 1). Accumulation in the absence of humic substances was ~0.34 ng silver animal^{-1}, but only ~0.09 ng silver animal^{-1} when humic substances were present. Complete depuration of silver from all silver-exposed daphnids was achieved after 96 h (data not shown). There were no differences in initial silver accumulation between animals that were held for the ensuing two weeks in humic or control (humic-free) conditions.

Silver-exposure had a significant positive effect on reproduction, but only in those animals exposed and subsequently held in humic-free conditions (Fig. 2A,B). Total neonate production per adult and neonates produced per brood were significantly elevated over the two-week holding period. Each adult in the C,C +Ag group produced
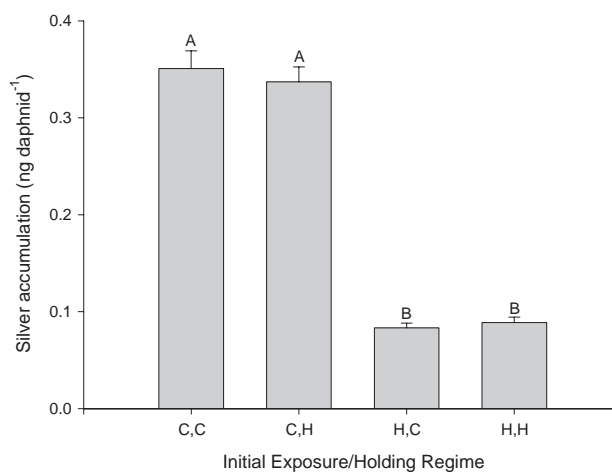

Fig. 1. Silver accumulation (ng daphnid^{-1}) in 7-day old *D. magna* following a 24 h exposure to 1 μg L^{-1} silver, in the presence (7.1 mg C L^{-1}) or absence (<0.3 mg C L^{-1}) of humic substances (Aldrich humic acid). First letter of group designation refers to the absence (C) or presence (H) of humic substances during the exposure, while the second letter refers to the absence or presence of humic substances in the holding regime that followed the exposure. Plotted points represent the mean±S.E.M of n individuals, where n is the value presented in Table 1. Bars sharing the same letter are not significantly different, with significance (p<0.05) determined by one-way ANOVA, followed by post-hoc LSD analysis.
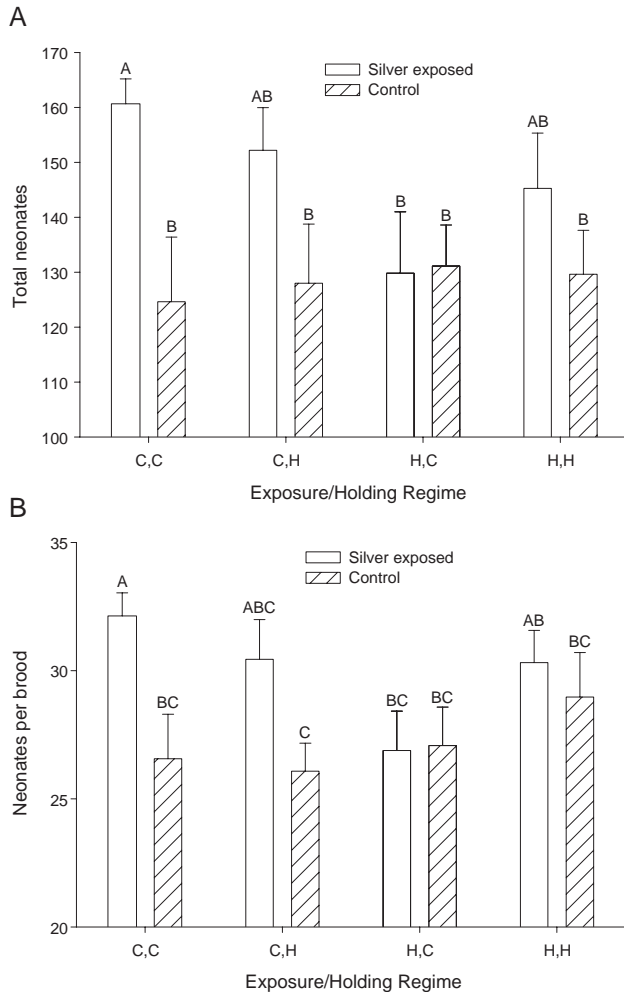
A

B

Fig. 2. Total neonate production per adult (A) and neonates produced per brood (B) in *D. magna* kept for two weeks in humic or humic-free conditions following an acute silver challenge (24 h, 1 µg L^{-1}). Solid bars represent silver-exposed animals, while hatched bars represent animals that were not silver challenged. The first letter of group designation refers to the absence (C) or presence (H) of humic substances during the silver exposure, while the second letter refers to the absence or presence of humic substances in the two-week holding regime that followed exposure. Plotted points represent the mean±S.E.M of *n* individuals, where *n* is the value presented in Table 1. Bars sharing the same letter are not significantly different, with significance ($p<0.05$) determined by one-way ANOVA, followed by post-hoc LSD analysis.

161±5 neonates, compared to 125±12 neonates per adult for the corresponding silver-free group (C,C −Ag). *D. magna* exposed to silver under humic-free conditions but subsequently maintained in humic conditions had total neonate numbers that were similarly elevated, though not statistically different from their control-maintained cohort. There were no significant differences between control-exposed (−Ag) daphnids. Reproductive parameters were not related to differences in final adult mass with only poor positive correlations noted for both total neonates per adult ($r^2=0.04$, $p=0.12$) and neonates per brood ($r^2=0.09$, $p=0.03$) when correlated against mass. There were no significant differences in other reproductive parameters

monitored throughout the experimental period, with time to first brood and the number of broods showing no differences between the treatment groups (not shown). All daphnids in all treatments were considered to be reproductively healthy, maintaining neonate production at a level comfortably in excess of the OECD guidelines (≥60 neonates per female per 21 days; OECD, 2001).

In the "subsequent" silver exposure, daphnids raised in the absence of silver and humic substances accumulated considerably more silver than those that had an exposure history involving either silver or humic substances (Fig. 3). Naïve animals never exposed to silver or humic substances accumulated 1.69±0.36 ng silver daphnid^{-1}, compared to levels in the range of 0.65–1.00 ng silver daphnid^{-1} for the other groups. Silver accumulation was poorly correlated to size ($r^2=0.12$, $p=0.01$), again suggesting the differences in final adult mass were not responsible for the observed patterns. As confirmation, when data were expressed per unit weight of daphnid (data not shown) the significance trends were identical to those shown in Fig. 3.

Exposure and holding conditions also significantly influenced whole body sodium levels following silver exposure (Fig. 4). Previously silver-exposed animals, without a humic exposure history (C,C +Ag) exhibited a reduced whole body sodium concentration compared to most other groups. The whole body sodium level of 29.9±0.8 mg kg wet weight^{-1} in these daphnids, was significantly lower than the level of 33.2±1.1 mg kg wet weight^{-1} determined in the corresponding silver-free control group (C,C −Ag). Within previously exposed animals a humic exposure time response was noted. Those animals with short humic exposures (H,C) exhibited whole body sodium concentrations that did not differ significantly from the C,C +Ag group. Whole body sodium levels in longer humic
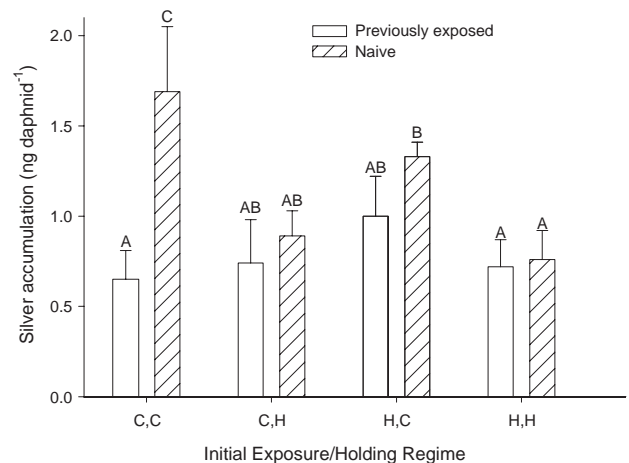

Fig. 3. Silver accumulation (ng daphnid^{-1}) in *D. magna* exposed to a subsequent acute silver challenge (24 h, 1 µg L^{-1}). Solid bars represent animals that were previously exposed to silver two weeks previously, while hatched bars represent those animals that had never been exposed to silver. Please refer to the legend accompanying Fig. 2 for further details.

Fig. 4. Whole body sodium (mg kg wet weight^{-1}) in *D. magna* exposed to a subsequent acute silver challenge (24 h, 1 μg L^{-1}). Solid bars represent animals that were previously exposed to silver two weeks previously, while hatched bars represent those animals that had never been exposed to silver. Please refer to the legend accompanying Fig. 2 for further details.
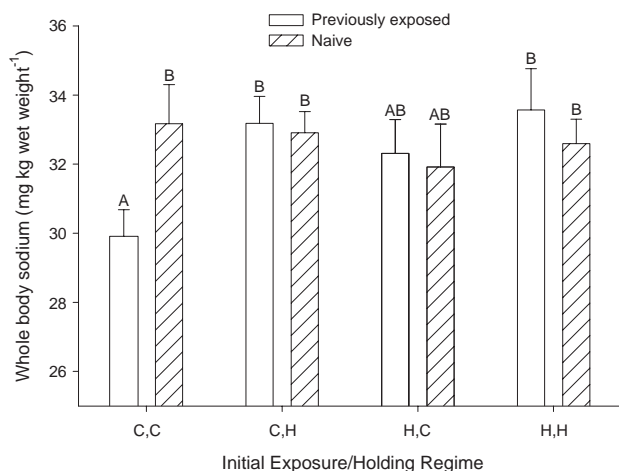
exposures (>2 weeks) all differed significantly from the C,C +Ag treatment.

4. Discussion

4.1. A hormetic effect of silver?

The deleterious effects of ionic silver on aquatic life are well described. In laboratory settings median acute lethal concentrations (48 h LC$_{50}$) for the highly sensitive cladoceran *D. magna* are less than 1 μg L^{-1} (e.g. Bianchini et al., 2002a). In chronic exposures mortality occurs at similar levels to that in acute exposures (e.g. Nebeker, 1982; Nebeker et al., 1983), but this is likely an artifact of food binding to waterborne silver and rendering it less toxic (Wood et al., 2002). Chronic exposure via the dietary route may also cause sublethal effects, such as reproductive impairment (*e.g.* Hook and Fisher, 2001). The present study demonstrated that over a chronic time-frame the exposure of daphnids to silver for 24 h—i.e. in a manner similar to that which may occur close to a point source—can have a beneficial effect. In particular this acute challenge appeared to significantly promote reproduction and adult mass. Interestingly, a hormetic phenomenon in response to silver exposure has been noted previously, with Brauner and Wood (2002b) describing enhanced growth and ionoregulatory development in rainbow trout early life stages during chronic exposures to waterborne silver.

A biologically favourable outcome in response to a potentially toxic insult is well described in the Cladoceran literature. Positive effects of metal exposures to daphnid species have been noted in laboratory studies for copper (De Schampelaere and Janssen, 2004) and cadmium (Elnabar-

awy et al., 1986; Bodar et al., 1988) and also in investigations where animals were exposed to naturally contaminated sediments containing metal mixtures (Marti-nez-Madrid et al., 1999). This latter study, in addition to detailing stimulated reproduction at low metal levels, also described a significant effect of such conditions on final adult mass, similar to the patterns observed herein. An important difference to note is that in all of these studies the animals were exposed to the metal over a chronic time frame, whereas the positive effects of silver exposure on daphnid adult mass and reproduction described here result from a single 24 h exposure to the metal.

4.2. Effects on reproduction

Stimulation of neonate production and brood size were greatest in silver-exposed animals that were never exposed to humic substances. In two of the three other humic exposure regimes, silver-exposed daphnids exhibited higher reproduction measures, although these values were not significantly different from their non-silver-exposed controls. The observed effects are consequently likely an effect of silver, albeit one that is ameliorated by the presence of humic substances.

There are reports of impaired reproduction in chronically silver-exposed aquatic organisms in the marine environment (Brown et al., 2003), and such findings have also been described for freshwater *Ceriodaphnia dubia* in laboratory experiments (Hook and Fisher, 2001; Bielmyer et al., 2002). The study of Hook and Fisher (2001) reported control reproduction of 16.1 neonates for each adult each week. This is a level less than that considered healthy for brood *C. dubia* (US EPA, 2002), and barely exceeds the US EPA test criteria of 15 young per adult in a 7–8 day study (US EPA, 2002). The study of Bielmyer et al. (2002) reports an effect of silver (both as a nitrate salt and liganded to sulfide) on adult fecundity, but did not provide reproduction rates. It is therefore possible that the observed deleterious effects on reproduction reported in these studies, at very low silver levels, may only be indicative of impacts on physiologically compromised animals. In physiologically more robust animals, reproductive inhibition is less prevalent (Bianchini and Wood, 2002) or even absent (this study).

The mechanism behind silver-mediated stimulation in reproduction is unknown, but it may be a secondary effect resulting from silver exposure. The anti-bacterial actions of ionic silver are well-described (e.g. Schierholz et al., 1998). It is possible that the positive actions of silver observed in the present study could be associated with changes in microbiological fauna caused by silver exposure. The high, single dose of silver may have eliminated bacterial competition for food resources and thus promoted daphnid nutrition. Evidence of gut microflora exists for *D. magna* (King et al., 1991), and silver-mediated effects on the gut microbial community could persist long after the silver has depurated from the animal. Enhanced nutritional status

would increase energy resources and thus contribute to the observed effects on both reproduction and adult mass.

In chronic exposure to silver levels of 0.1 and 1 $\mu g \ L^{-1}$ (as silver nitrate), positive effects on larval length, and weight were noted in rainbow trout early life stages (Brauner and Wood, 2002b). These results could again be attributed to an anti-microbial action of silver.

The applicability of these findings to the natural environment remains unknown. *D. magna* consume bacteria as part of their diet (e.g. Langenheder and Jurgens, 2001) so an anti-bacterial action of silver may seem to argue against a positive health effect, at least in the field where exposure to silver and food is likely to occur simultaneously (in contrast to the controlled conditions herein). However in natural waters the toxic, ionic form of silver is virtually eliminated due to complexation with natural ligands (e.g sulfides; Shafer et al., 1998). Thus anti-bacterial actions of silver may be restricted to the gut environment where extracellular digestive processes (Peters, 1987) may desorb silver from strong ligands, impacting gut fauna and potentially resulting in physiological and reproductive effects.

4.3. Influence of humic substances

The results from the present study suggest that humic substances antagonise the positive effects of silver on both adult mass (Table 1) and to a lesser extent reproduction (Fig. 2). Humic substances themselves, however, had no significant actions on these parameters. The observed patterns cannot be attributed to the decreased silver accumulation in the presence of humic substances during the initial silver exposure regime (Fig. 1). For zebra mussels a positive effect of humic substances has been equated with enhanced nutrition, with the humic materials acting as a source of carbon, while also potentially enhancing the bioavailability of micronutrients, via complexation by the organic matrix (Roditi et al., 2000). Contrastingly, in early life stage exposures in rainbow trout, humic substances slowed growth rate (Brauner and Wood, 2002a). It is possible that these effects were due to micronutrient binding, whereby the complexation of important micronutrients by humic materials reduced bioavailability and thus countered any positive actions of silver-exposure. Differences between this study and that of Roditi et al. (2000) could be explained by the different feeding strategies.

A further possibility to account for the ability of humic substances to counter the positive actions of silver on growth and reproduction may involve alterations in ion metabolism. Exposure of *D. magna* to humic substances (AHA) results in an increase in sodium influx and whole body sodium status (Glover et al., in press). This perturbation could promote a switching of energy resources away from reproduction and growth, to dealing with the ionoregulatory challenge posed by the humic matter. Changes in ion regulation in response to humic materials in *D. magna* occur rapidly (Glover et al., in press), and appear to be much

faster than ion regulation perturbation promoted by silver in animals of equivalent size in similar water chemistry (unpublished data). This may also explain why humic effects are observed even after only 24 h exposure to humic substances, albeit of a lesser magnitude than the more extended humic exposure regime.

The importance of the humic effect in natural waters is not known. The effects noted here were at a relatively high concentration (7 mg C L^{-1}), albeit one that is bracketed by environmental levels (1–15 mg C L^{-1}; Thurman, 1985). It remains to be determined if similar actions are caused by lower humic concentrations, or humic substances other than AHA.

4.4. Acclimation effects

Exposure of *D. magna* to silver engenders an acclimation response, such that a subsequent silver exposure results in a lower silver body burden, compared to naïve animals (Fig. 3). Exposure to humic substances resulted in a similar effect. Acclimation to waterborne silver insult has been demonstrated in rainbow trout (Galvez and Wood, 2002). In that study physiological acclimation (recovery of ionoregulatory parameters), preceded toxicological acclimation (increase in LC_{50}). The reduction in silver body burden in previously exposed animals could be explained by modification of the ionoregulatory apparatus as described previously. Rainbow trout showed recovery in sodium influx, plasma sodium levels and activity of Na^+, K^+-ATPase (Galvez and Wood, 2002). Humic substances also cause modifications in ionoregulation. In *D. magna* this was manifested as altered membrane permeability (Glover et al., in press). It is possible that the different stimuli have similar inhibitory effects on silver uptake.

While humic substances and previous silver exposure have similar effects on silver body burden, they have diverse actions on whole body sodium levels, an important indicator of silver toxicity. Whereas silver-exposed animals in the absence of humic substances showed a significantly reduced whole body sodium level, those animals that were exposed to humic substances, irrespective of silver exposure, demonstrated sodium levels equivalent to control (C,C −Ag) animals. The major mode of action of silver is to inhibit sodium influx, resulting in a reduction in whole body sodium (Bianchini and Wood, 2003). Conversely, while Aldrich humic acid complexes silver ion and thus reduces silver bioavailability, it also stimulates sodium influx and results in enhanced whole body sodium level (Glover et al., in press). Such differences could explain the effect of subsequent silver exposure on whole body sodium levels, with the humic-exposed animals starting from a higher whole body sodium level, and the silver-exposed (but humic-free) daphnids already having a reduced sodium level.

The physiological perturbation (reduced whole body sodium) in previously silver-exposed daphnids was actually

greater than control animals despite a lessened silver body burden. Whether this translates to a greater toxicological sensitivity (i.e. LC_{50}) is currently unknown. It does however suggest that the relationship between silver body burden and toxicological surrogate measures may not hold in acclimated animals, a conclusion which has also been drawn from a number of studies on fish acclimated to other metals (reviewed by Niyogi and Wood, 2003). Current regulatory models rely on a predictable relationship between silver accumulation and toxic effects (e.g. the biotic ligand model; Di Toro et al., 2001).

Acknowledgements

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