SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 34, No. 9, pp. 2023–2032, 2015

Printed in the USA

ECOTOXICITY OF BARE AND COATED SILVER NANOPARTICLES IN THE AQUATIC MIDGE, *CHIRONOMUS RIPARIUS*

Sun-Young Park,† Jiwoong Chung,† Benjamin P. Colman,‡§ Cole W. Matson,§‖ Younghun Kim,#
Byung-Cheon Lee,†† Phil-Je Kim,†† Kyunghee Choi,†† and Jinhee Choi*†

†School of Environmental Engineering, Graduate School of Energy and Environmental System Engineering, University of Seoul, Seoul, South Korea
‡Department of Biology, Duke University, Durham, North Carolina, USA
§Center for the Environmental Implications of NanoTechnology, Duke University, Durham, North Carolina, USA
‖Department of Environmental Science and Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas, USA
#Department of Chemical Engineering, Kwangwoon University, Seoul, South Korea
††Risk Assessment Division, National Institute of Environmental Research, Incheon, Korea

(Submitted 4 October 2014; Returned for Revision 20 November 2014; Accepted 10 April 2015)

Abstract: Although sediment is generally considered to be the major sink for nanomaterials in aquatic environments, few studies have addressed the ecotoxicity of nanomaterials in the presence of sediment. In the present study, the ecotoxicity of silver nanoparticles (AgNPs) with a range of organic coatings was examined in a freshwater sediment-dwelling organism, *Chironomus riparius*, using acute and chronic ecotoxicity endpoints, including molecular indicators. The toxicity of AgNPs coated with different organic materials, such as polyvinylpyrrolidone, gum arabic, and citrate, to *C. riparius* was compared with that of bare-AgNPs and $AgNO_3$ (ionic silver). Total silver concentration was also measured to monitor the behavior of the AgNPs in water and sediment and to determine how ion dissolution affects the toxicity of all AgNPs. The coated- and bare-AgNPs caused DNA damage and oxidative stress-related gene expression. In addition, the bare-AgNPs and $AgNO_3$ had a significant effect on development and reproduction. The surface coatings generally mitigated the toxicity of AgNPs to *C. riparius*, which can be explained by the reduced number of ions released from coated-AgNPs. Citrate-AgNPs caused the most significant alteration at the molecular level, but this did not translate to higher-level effects. Finally, comparing previously conducted studies on AgNP-induced gene expression without sediments, the authors show that the presence of sediment appears to mitigate the toxicity of AgNPs. *Environ Toxicol Chem* 2015;34:2023–2032. © 2015 SETAC

Keywords: Nanoecotoxicology Sediment toxicity Invertebrate toxicology Genotoxicity

INTRODUCTION

Silver nanoparticles (AgNPs) are widely used in consumer and medical products owing to their antimicrobial properties [1,2]. The release of antimicrobial AgNPs into aquatic environments through a range of pathways has been documented, giving rise to increasing concerns about their environmental impacts [3]. To prevent aggregation, AgNPs are often coated with organic compounds [4] such as carboxylic acids (e.g., citrate, carboxylic acids with an alkyl chain), polymers (e.g., polyvinylpyrrolidone, polyacrylate, poly[vinyl-alcohol], polyacrylamide, and thiol-modified oligonucleotides), polysaccharides (e.g., gum arabic [GA], sophorolipids), and surfactants [2,4]. These various coatings modify the surface charge, aggregation, and toxicity of AgNPs in the environment [4]. Although AgNPs with different coatings have been applied widely, the toxicity of AgNPs with surface modifications is still poorly understood. Information on the comparative toxicity of coated- and bare-AgNPs is limited [4,5] with much less knowledge in an ecotoxicological context [6,7].

Although sediment is generally the predicted [8] and observed [9] final sink for nanomaterials introduced into aquatic environments, the majority of studies focus on pure culture aqueous exposures and many fewer studies have addressed the toxicity of nanomaterials to invertebrates in sediment [10–12]. In addition to being the dominant fate of many contaminants, sediments provide an essential habitat for aquatic communities [13]. In aquatic environments, benthic fauna are of great importance because they represent an important link in the aquatic food web, one which can accumulate metals from both aqueous and sediment sources [14]. Therefore, a thorough investigation of the toxicity of NPs in sediment-dwelling organisms is of great importance for predicting the impacts of these emerging contaminants.

The aquatic midge, *Chironomus riparius*, is a benthic invertebrate which is both widely used as an ecotoxicological model species and uniquely suited for assessing the toxicity of sediment as their larvae dwell in freshwater sediment [15]. In the present study, the ecotoxicity of AgNPs with various organic coatings and sizes was examined in *C. riparius*. Specifically, the toxicity of bare AgNPs was compared with that of particles coated with either polyvinylpyrrolidone (PVP), gum arabic (GA), or citrate. To look for size-dependent effects both 8-nm and 38-nm PVP-AgNPs were compared. The toxicity of ionic Ag ($AgNO_3$) was also tested, to determine if the observed toxicity was the result of dissolution or was particle-specific as this determination is an important characteristic for determining Ag nanotoxicity [16–19]. The endpoints were mortality (acute), development and reproduction (chronic), and expression of oxidative stress response genes of *C. riparius* [20–26]. The genotoxicity of AgNPs was tested using a comet assay as genotoxicity is a potentially important aspect of nanotoxicity. Finally, total and ionic Ag concentrations were also measured to monitor the behavior of AgNPs in water and sediment and to determine

All Supplemental Data may be found in the online version of this article.
* Address correspondence to jinhchoi@uos.ac.kr.
Published online 18 April 2015 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3019

how the extent of ion dissolution affects the toxicity of coated- and bare-AgNPs.

MATERIALS AND METHODS

Animals

Chironomus riparius were obtained from the Toxicological Research Center of the Korea Institute of Chemical Technology and have been reared in our laboratory for more than 10 yr. The larvae were reared on an artificial diet of fish food flakes (Tetramin; Tetrawerke) in glass chambers containing dechlorinated tap water and acid-washed sand, with aeration at 20 ± 1 °C under a 16:8-h light:dark photoperiod.

Culture media

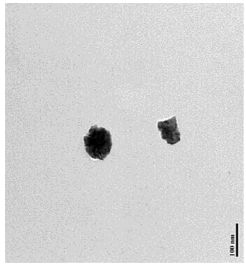
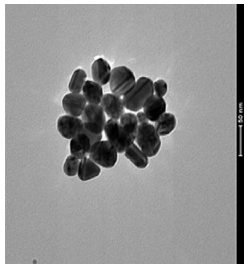
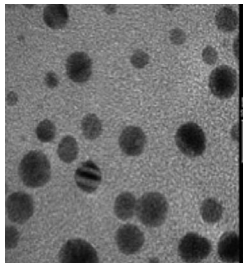
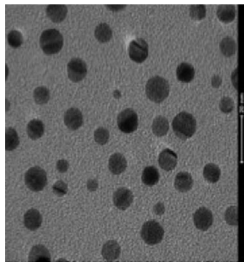
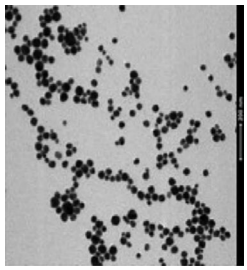
Dechlorinated tap water was used to maintain cultures of *C. riparius*. Acid-washed sand was used as the physical medium for larval *C. riparius*. To ensure greater interexperimental consistency, all experiments were run in US Environmental Protection Agency (USEPA) moderately hard water [27]. Briefly, this standard medium is characterized as having a conductivity of 340 μS/cm to 360 μS/cm and a pH of 7.9 and is representative of many surface waters. For greater realism and consistency with other laboratory [28–30] and large-scale mesocosm [31] experiments, a blended soil from the CEINT Mesocosm Facility at Duke University was used. Briefly, 3 surface soils were blended to yield a final sediment texture of 64% sand, 13% silt, and 23% clay, with a loss on ignition (an index of organic matter) of 5% [31]. These characteristics make it consistent with the composition suggested in the Organisation for Economic Co-operation and Development (OECD) guideline [32]. Specifically, it is close to the suggested 20% clay and 4% to 5% organic matter. In addition, according to trace metal analysis, background Ag in the sediment was below the detection limit (Supplementary Data, Table S1).

Preparation of Ag exposure media and characterization of AgNPs

Bare-AgNPs (described by the vendor as having a size <100 nm; Sigma-Aldrich Chemical) were homogenously dispersed in deionized water by sonication for 13 h (Branson-5210 sonicator; Branson) at maximum power, stirring for 7 d, and filtering through a cellulose membrane (pore size 100 nm, Advantec; Toyo Toshi Kaisha) to remove NP aggregates. The final concentrations of Ag in bare-AgNPs solution were measured using a multitype inductively coupled plasma emission spectrometer (Elan DRCII, detection limit 0.1 ng/L; Perkin Elmer). The particle shape was determined using a LIBRA 120 transmission electron microscope (TEM; Carl Zeiss) at 80 kV to 120 kV, and the hydrodynamic size distribution was evaluated using a Photal dynamic light scattering spectrometer (DLS-7000; Otsuka Electronics). Stock solution of bare-AgNPs was used as test material for 2 wk.

Stock solutions of ionic silver consisted of $AgNO_3$ (AG002; Next Chimica) in deionized water as described [25,33,34]. Coated-AgNPs were synthesized at Duke University, as described [16,35,36,37]. Physicochemical properties have been determined for bare-AgNPs and coated-AgNPs (Table 1) [16,37]. Briefly, hydrodynamic diameters of the bare-AgNPs were between 30 nm and 40 nm according to dynamic light scattering measurements (Table 1). The average core sizes were bare-AgNPs, 31.6 nm; citrate-AgNPs, 7 nm; small PVP-AgNPs, 8 nm (hereafter PVP8-AgNPs); large PVP-AgNPs, 38 nm (hereafter PVP38-AgNPs); GA-AgNPs, 6 nm.

Table 1. Characterization of bare- and coated silver nanoparticles (AgNPs).[a]

	Bare-AgNPs	Citrate-AgNPs[b]	GA-AgNPs[c]	PVP8-AgNPs[b]	PVP38-AgNPs[c]
Transmission electron microscopic image					
Zeta potential (mV)	-26.3 ± 1.2	-30 ± 3	-46 ± 2.5	-5.0 ± 0.2	-10.9 ± 0.4
Hydrodynamic diameter (nm)	31.6 ± 6.7	7 ± 11	6 ± 1.7	8 ± 2	38 ± 8

[a]Particle shape was determined using a transmission electron microscope, and hydrodynamic diameter and zeta potentials were evaluated using a Photal dynamic light scattering spectrometer.
[b]Yang et al. [16].
[c]Yin et al. [37].
GA = gum arabic; PVP8 = 8-nm polyvinylpyrrolidone; PVP38 = 38-nm polyvinylpyrrolidone.

Acute toxicity tests

Mortality test. A mortality test was conducted using a modified OECD guideline [38]. A total of 10 fourth instar larvae were exposed to 5 concentrations (0 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, and 5 mg/L) of Ag in all 6 Ag treatments (5 × AgNPs, 1 × $AgNO_3$) in 100 mL reconstituted moderately hard USEPA water without sediment. Mortality was determined after 24 h of exposure.

DNA damage. As preparation for the comet assay, a total of 10 larvae were collected after 24 h in 100 mL USEPA water without sediment (control) or after exposure to 1 mg/L of Ag in all 6 Ag treatments. An alkaline comet assay was conducted, as described [5,39]. Briefly, treated organisms were placed in 1 mL of phosphate-buffered saline, containing 20 mM ethylenediaminetetraacetic acid and 10% dimethyl sulfoxide, and disintegrated mechanically by mincing. The cell suspension was precipitated by vortexing and then immediately mixed with 100 μL of 1% low–melting point agarose. To prepare slides, 100 μL of 1% low–melting point agarose was spread onto a normal agarose precoated microscope slide and incubated at 4 °C for 5 min to allow for solidification. Cells were lysed in high salt and detergent and subsequently exposed to alkali buffer (pH > 13) for 20 min at 4 °C to allow for DNA unwinding. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min. After electrophoresis, slides were neutralized and dehydrated in 70% ethanol. Slides were stored in a dry place until image analysis. Before analysis, slides were stained with ethidium bromide (20 μg/mL), then analyzed at 400× magnification using a fluorescence microscope (excitation filter, BP 546/12 nm; barrier filter, 590 nm). Approximately 50 cells per slide (3 slides per treatment) were examined. The DNA damage was expressed as the tail moment ([tail length × tail %DNA]/100) using an automated image analysis method (Komet 5.5; Kinetic Imaging).

Two potential artifacts can arise when using the comet assay to examine the genotoxicity of NPs: NP–DNA association can result in modified comet length and NPs such as TiO_2 can cause photochemical DNA damage during the assay, yielding overestimates of genotoxicity [40]. We feel these are unlikely to have influenced the present findings for 3 reasons. First, appreciable NP–DNA association seems unlikely in the present experiments given our low concentrations in the exposure medium and subsequent removal of test organisms from the exposure medium prior to analysis. Second, unlike TiO_2, AgNPs are not photoactive under laboratory conditions [36] and are thus unlikely to cause appreciable genotoxicity as a result of photochemistry. Third, the genotoxicity results described below in *Genotoxicity test* are consistent with the demonstrated genotoxic potential of AgNPs reported in many studies using complementary approaches [41–44].

Stress response gene expression. Exposure aquariums were prepared by adding 150 mL USEPA reconstituted moderately hard water to 45 g dry weight of sediment. The water column was spiked to give a concentration of 1 mg/L for all 6 Ag treatments. After equilibration for 1 h, 10 fourth instar larvae were introduced. Larvae were collected after 24-h exposure, frozen in liquid nitrogen, and stored at –80 °C for gene expression analysis. Total RNA was extracted from the samples using Trizol (Invitrogen), following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse-transcribing 1 μg of total RNA using an iScript cDNA Synthesis kit (Bio-Rad). To study the stress response gene expression of larvae, quantitative real-time polymerase chain reaction (RT-PCR) was performed using IQ SYBR Green Super Mix (Bio-Rad) and the following reaction conditions: initial denaturing at 95 °C for 7 min, followed by 44 cycles of 95 °C for 15 s, 55 °C for 1 min, and extension of 72 °C for 15 s. Melting curves were calculated from 65 °C to 95 °C with a 0.2 °C increase per cycle using a CFX96 RT-PCR detection system (Bio-Rad). The expression level of each gene was calculated after exposure to the different forms of Ag. The messenger RNA (mRNA) level of each gene was normalized to that of the constitutively expressed *C. riparius* gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Genbank accession EU99991). The cycle threshold values were converted to relative gene expression levels using the CT ($2^{-\Delta Ct}$) method and the analysis software provided with the CFX-96 real-time PCR machine. A list of the tested genes and a description of their function are provided in Supplemental Data, Table S2.

Measurement of ionic silver in water. Ionic silver dissolved from all tested AgNPs was measured. To do this, USEPA water was brought to 1 mg/L of AgNPs for all AgNP treatments; and after 24 h, ionic silver was measured using an Orion 4-star pH/ISE meter (Thermo Scientific) equipped with a silver/sulfide ion-selective electrode (Orion model 9616BNWP, detection limit 10 μg/L; Thermo Scientific) [45]. Calibration was done against a dilution series of $AgNO_3$ solutions.

Chronic toxicity tests

Reproduction and development tests. For the chronic toxicity test, a modified OECD guideline was used [32]. Exposure aquariums were prepared by adding 400 mL of USEPA water to 50 g of sediment. The USEPA waters were spiked to 1 mg/L with either bare-AgNPs, citrate-AgNPs, or $AgNO_3$. After equilibration for 1 h, 30 fourth instar larvae were introduced and their emergence and reproduction monitored for 25 d until all treated and control organisms were dead. The emerging adults were retained with steel-wire mesh until the emergence was complete in all treatments.

To investigate developmental effects, emergent adults from each vessel were counted. For reproduction, the egg masses oviposited by the emerged adults in the control and treated vessels were counted. Every 2 d, 50 mg of Tetramin fish food flakes was supplied to each aquarium. Test solutions were not renewed. All data were recorded at daily intervals.

Silver fate in water and sediment in chronic toxicity tests. To verify the partitioning of bare-AgNPs, citrate-AgNPs, and $AgNO_3$ in water and sediment, silver content was analyzed after 4 h, 12 h, 24 h, 7 d, and 25 d of exposure. Ten milliliters of water and 1.5 g of sediment were sampled from each exposure aquarium for analysis. Sample water and sediment were digested overnight in a closed-type Teflon Digestion Vessel (Savillex) with the extraction solution at 200 °C. The composition of extraction solutions was 10 mL of a mixture of HNO_3 and hydrogen fluoride with a 4:1 ratio for mixed acid digestion. After complete digestion, the remaining acids were evaporated until the hydrofluoric acid had been completely eliminated from the solution. The residue was then diluted with 20 mL of deionized water with 1% to 5% HNO_3 prior to analysis. All samples were frozen immediately after collection until analysis. The metal content in water and sediment was determined using inductively coupled plasma-mass spectrometry (Elan DRC II, detection limit 0.1 ng/L; Perkin Elmer).

Given that the goal of the present experiment was to look at the trend over time of silver concentration in the water column for bare-AgNPs, $AgNO_3$, and citrate-AgNPs, we ran 3 separate experiments with 1 laboratory replicate of each. We then fit the

resulting data using nonlinear regression and a modified version of the formula described in Quik et al. [46]. Specifically, we used Equation 1

$$Ag_t = (Ag_0 - Ag_{res}) \times e^{(-k_{sed} \times t)} + (Ag_{res}) \quad (1)$$

where Ag_t is the percentage of silver in the water column at time t, Ag_{res} is the residual silver in the water column after aggregation and/or precipitation, and k_{sed} is the sedimentation rate constant. The model was iterated to solve for k_{sed} and Ag_{res} given $Ag_0 = 100$, and then from uncertainty in these parameters, 95% confidence intervals were generated for the model fit. Nonlinear regression was done using the nonlinear modeling functionality of JMP Pro Ver 11 (SAS Institute).

Statistical analysis

Statistical differences between the control and treated samples were examined with a one-way analysis of variance using SPSS 12.0KO. All data are reported as means ± standard error of the mean. Toxicological data were assessed for normality using the Shapiro-Wilk test and homogeneity of variance, using Levene's test. One-way analysis of variance was performed on all data, and $p < 0.05$ was considered statistically significant by Tukey's honestly significant difference test.

RESULTS AND DISCUSSION

Through a series of experiments using either acute or chronic exposures, we examined the impacts of 31.6-nm bare particles (bare-AgNPs), 6-nm GA–coated particles (GA-AgNPs), 7-nm citrate-coated particles (citrate-AgNPs), and 8 nm or 38 nm polyvinylpyrrolidone-coated particles (PVP8-AgNPs and PVP38-AgNPs, respectively) on *C. riparius*. Exposures were conducted in USEPA moderately hard water with standardized sediment. Using acute exposures (24 h), we examined mortality, genotoxicity, and stress response gene expression, as well as changes in $AgNO_3$ concentrations. Using chronic exposures (25 d), we examined differences in emergence and reproductive output over the course of a 25-d exposure. We saw little evidence of mortality under acute exposures, but in these same exposures we saw evidence of sublethal toxicity in the form of both genotoxicity and changes in the transcription of genes associated with oxidative stress. In the chronic exposure experiment, we observed decreases in emergence and reproductive output.

Acute mortality test

The potential toxicity to fourth instar larvae of *C. riparius* was compared for $AgNO_3$ and AgNPs with different surface coatings. Mortality tests were conducted for 24 h without sediment, and no mortality was observed for controls. Larvae treated with the AgNPs and $AgNO_3$ showed <10% mortality even at the highest concentrations tested (5 mg/L). Across concentrations, coated-AgNPs on average caused less mortality (<5 %) than bare-AgNPs and $AgNO_3$; however, the mortality rate was not significant compared with control ($p > 0.05$; Supplemental Data, Table S3).

Genotoxicity test

The lack of differences in mortality in Ag treatments compared with controls does not indicate that there is a lack of acute toxicity; rather, it should be seen as motivating the examination of other acute toxicity endpoints, including genotoxicity and gene expression. A number of studies [41,42] have suggested that genotoxicity is an important mechanism for AgNP-induced toxicity. We previously reported the genotoxic potential of AgNPs in various systems, such as mammalian cell lines [43,44], in *Caenorhabditis elegans*, in *Daphnia magna*, and in *Chironomus riparius* [33] using the same bare-AgNPs that were used in the present study, as well as the PVP-AgNPs also used (*Caenorhabditis elegans* [5]). We tested for genotoxicity in all Ag treatments. Extending beyond our past results, we found that $AgNO_3$, bare-AgNPs, citrate-AgNPs, and both sizes of PVP-AgNPs caused significant DNA damage (Figure 1), whereas the GA-AgNPs were not significantly different from controls ($p = 0.159$). The citrate-AgNPs were the most genotoxic NPs, increasing DNA damage roughly 3-fold compared with controls. Although there is a trend toward the smaller PVP-AgNPs having a higher genotoxic potential than larger ones—the tail moment measured in the

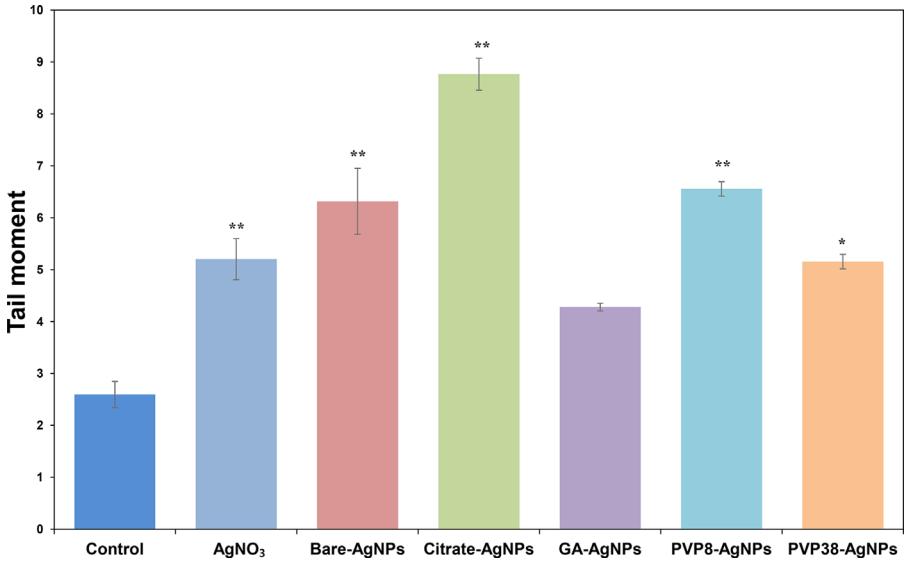


Figure 1. DNA damage as measured by comet assay in bare and coated silver nanoparticles (AgNPs) and $AgNO_3$-exposed *Chironomus riparius*. Results are expressed as tail moment ($n = 3$, mean ± standard error). Asterisks indicate significant difference ($^*p < 0.05$, $^{**}p < 0.01$) compared with the control group using analysis of variance. GA = gum arabic; PVP8 = 8-nm polyvinylpyrrolidone; PVP38 = 38-nm polyvinylpyrrolidone.

PVP8-AgNP-exposed larvae was slightly higher than that of the PVP38-AgNP-exposed larvae—this difference was not statistically significant ($p = 0.332$). Why the different AgNPs and $AgNO_3$ had differential effects on genotoxicity is an open question. Previous studies suggest that oxidative stress is involved in AgNP toxicity to *C. riparius* [23,25,33]. It may be that the observed range of genotoxicity for different forms of AgNPs and $AgNO_3$ was related to differences in the magnitude of Ag-induced oxidative stress. Although ion release of coated-AgNPs was significantly lower than that of bare- AgNPs (Supplemental Data, Figure S1), higher genotoxicity was observed in citrate and PVP-AgNPs than even $AgNO_3$. These results showed that AgNP toxicity could be linked not only to ion effects but also to particle-specific effects, and the coating agent could change the toxic effect of particles. As reported previously, stable AgNPs, such as citrate-AgNPs, are likely to remain in the water column and increase potential toxicity to pelagic organisms [7]. Therefore, we could assume that stable citrate-AgNPs are more genotoxic than other AgNPs in water only exposure.

Stress response gene expression

A previous study investigating stress response gene expression in *C. riparius* reported that bare-AgNPs led to greater induction of genes related to oxidative stress and detoxification relative to $AgNO_3$ [33]. In the present study, we examined the effects of $AgNO_3$ and several different forms of AgNPs on the transcription of a similar set of genes. However, the present experiment was conducted using more environmentally realistic methods (i.e., in the presence of sediment). Ten genes involved in the oxidative stress response were selected: superoxide dismutases (*SOD*: *Cu-ZnSOD*, *MnSOD*), catalase (*CAT*), glutathione *S*-transferase (*GSTs*: *GSTd3*, *GSTs4*, *GSTe1*), phospholipid glutathione peroxidase (*PHGPx*), thioredoxin reductase 1 (*TrxR1*), heme oxyganase-1 (*HO-1*), and transferrin (*TF*). In addition, a gene reflecting general stress response (heat shock protein 70 [*HSP70*]) and a gene involved in developmental regulation (StAR-related lipid transfer domain [*START-1*]) were analyzed. A brief description of these genes and previous publications using these selected oxidative stress–related genes are presented in Supplemental Data, Table S2.

Six of the 10 stress genes showed changes in expression for at least 1 Ag treatment, as did both general stress genes (*START1* and *HSP70*). The most sensitive change was observed in *MnSOD* expression, which showed significant differences from the controls for 4 of the 6 forms of Ag tested, with all 4 notably being NP forms (bare-AgNPs, PVP38-AgNPs, citrate-AgNPs, and GA-AgNPs; Figure 2). Of all the forms of Ag investigated, citrate-AgNPs had the most widespread effects on gene expression, leading to significant increases in the expression of 5 of the genes ($p < 0.05$; Figure 2). All significant changes relative to controls were increases with the exception of those for *CAT* (bare-AgNPs and $AgNO_3$ treatments) and *TF* ($AgNO_3$ and PVP8-AgNPs treatments), which had significantly decreased expression (Figure 2).

Of all Ag forms examined, only GA-AgNPs induced *Cu-ZnSOD* gene expression, whereas bare-AgNPs, PVP38-AgNPs, citrate-AgNPs and GA-AgNPs induced all *MnSOD* gene expression (Figure 2). The mitochondria are often considered to be particularly susceptible to AgNPs, which leads to an excess of oxidative stress [26]. The higher induction of *MnSOD* than *CuZnSOD* might be related to increased mitochondrial activity after exposure to AgNPs. The expression of another important antioxidant enzyme gene, *CAT*, was decreased by $AgNO_3$ and by the bare-AgNPs (Figure 2). The AgNPs–induced *SOD* gene expression would be expected to lead to an increase in *CAT* enzyme activity to counteract the excess formation of H_2O_2 by the action of the SOD enzymes. In contrast to that expectation, exposure to coated-AgNPs did not change the expression of the *CAT* gene, whereas exposure to bare-AgNPs led to a significant decrease in *CAT* gene expression, rather than the expected increase. This might be the result of increased CAT enzyme activity without an increase at the transcriptional level. Alternatively, increased expression of the *SOD* genes may not have led to increased SOD enzyme activity. Another difference between our previous experiment without sediment and the present experiment with sediment was observed in *GST* mRNA expression. In our previous study, the expression of 3 types of *GST* mRNA (i.e., *GST d1*, *s1*, and *e1*) increased dramatically

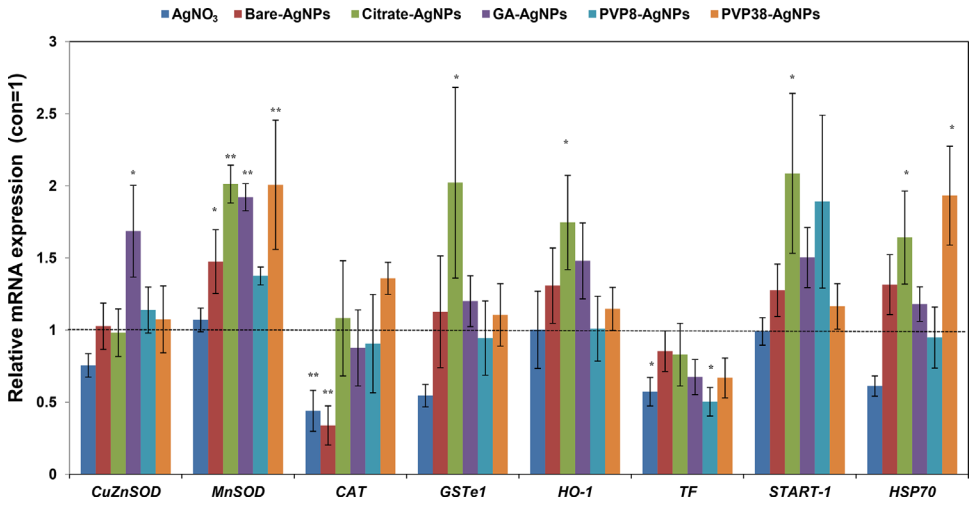


Figure 2. Stress response gene expression after treatment with 1 mg/L of bare and coated silver nanoparticles (AgNPs) and $AgNO_3$ for 24 h in the presence of sediment. Gene expression levels (*CuZnSOD*, *MnSOD*, *CAT*, *GSTe1*, *HO-1*, *TF*, *START-1*, *HSP70*) were calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and are shown as mean ± standard error (control = 1, $n = 5$). The *Chironomus* GAPDH gene was used as a reference gene. Asterisks indicate significant difference ($^{*}p < 0.05$, $^{**}p < 0.01$) compared with the control group using analysis of variance. GA = gum arabic; PVP8 = 8-nm polyvinylpyrrolidone; PVP38 = 38-nm polyvinylpyrrolidone.

after exposure to bare-AgNPs and $AgNO_3$ [21,26], whereas such a tendency was not observed in the present study. The only case in which there was significantly increased *GST* expression was observed with *GSTe1* and was caused by citrate-AgNPs (Figure 2; Supplemental Data, Figure 2). Much like *GSTe1*, the expression of *HO-1* was increased only after citrate-AgNP exposure (Figure 2). When taken together, these results suggest that citrate-AgNPs may possess higher oxidative stress–inducing potential than other particles.

The expression of *TF* was decreased by $AgNO_3$ and PVP8-AgNPs, which suggests that these Ag forms may affect the expression of *TF* through unknown mechanisms (Figure 2). In our previous study, mRNA expression of *PHGPx1* and *TrxR1* in *C. riparius* was reported to be up-regulated as a consequence of oxidative stress [21,24,25]. However, in the present study, none of the Ag forms tested affected the expression of these 2 antioxidant enzyme genes (Supplemental Data, Figure S2). The increased expression of the steroidogenesis-related gene *START1* by citrate AgNPs suggests that the potential exists for chronic developmental effects (Figure 2). The increased expression of *HSP70* mRNA by PVP38-AgNPs and citrate-AgNPs may be explained as a general stress response (Figure 2).

Among the coated-AgNPs, citrate-AgNPs had the most pronounced effect on many of the genes tested (i.e., *MnSOD*, *GSTe1*, *HO-1*, *START-1*, *HSP-70*), which indicates that citrate-AgNPs have a higher oxidative stress–inducing potential than the other types of AgNPs or Ag ion. A size-dependent effect was observed in *MnSOD*, *CAT*, and *HSP70* gene expression as a stronger response was found in their expression with the PVP38-AgNPs than its smaller counterpart.

When the current gene expression results from the exposure to bare-AgNPs and $AgNO_3$ in the presence of sediment were compared with those from a previous study without sediment [26], the present study showed evidence of a less sensitive response in the presence of sediment (Table 2). In an aquatic system with sediment, the lower oxidation reduction potential from organic matter present in sediment could decrease AgNP toxicity [47]. According to the present acute effects results, the presence of sediment during exposure should play a critical role in mitigating toxicity. The dramatic increase in the expression of genes in a previous study might reflect the larvae's response to contaminant exposure in a harsh environment, whereas the attenuated response in the presence of sediment might reflect a more restricted physiological response. Therefore, we can conclude that the presence of sediment may affect the toxicity of AgNPs to *C. riparius*, but how this factor affects oxidative stress mechanisms requires further study.

One of the main advantages of using molecular indicators of gene expression is their predictive power for higher-level effects by providing an indicator of the mechanism of toxicity for contaminants [48]. However, higher-level effects such as mortality, growth, development, and reproduction should involve a variety of gene- and protein-level mechanisms. In addition, organisms may exhibit different patterns of molecular-level responses under more complex environmental conditions. The dramatically different sensitivity of transcriptional responses in the presence and absence of sediments, as shown in Table 2, reinforces the idea that care should be taken when using gene expression to diagnose chemical contamination in complex, environmentally relevant systems, because of the various potential confounding factors.

Extent of dissolution of AgNPs in water

Given that the present results suggest that the presence or absence of a surface coating may affect the toxicity of AgNPs to *C. riparius*, we looked at the quantity of Ag ions released by coated-AgNPs relative to the bare-AgNPs after spiking 1 mg/L of the bare-Ag and coated-AgNPs into USEPA water (Supplemental Data, Figure S1). The results clearly document reduced dissolution for the coated-AgNPs, indicating that the reduced toxicity of the coated-AgNPs compared with the bare-AgNPs is likely at least in part related to ion release. However, we did not find a difference in extent of dissolution between citrate-AgNPs and PVP-AgNPs despite differences in their toxicity, suggesting that dissolution is only part of the equation. We suggest that the differences in gene expression responses for the various coated-AgNPs (Figure 2) may be specific to the individual particles, and thus, the effect of coating on oxidative stress mechanisms requires further study.

Chronic toxicity and fate of Ag in water and sediment

The acute toxicity results provide insights into the comparative toxic potential between different AgNPs and $AgNO_3$ but do not fully reflect Ag impacts on organism fitness. Indeed, in a previous study with *C. riparius* exposed to bare-AgNPs, up to 2 mg/L of AgNPs caused no mortality; however,

Table 2. Comparison of stress response gene expression results between previous (water only condition) and current (in presence of the sediment) studies[a]

	AgNPs			$AgNO_3$		
Exposure condition	Water only condition	Sediment condition	*p*	Water only condition	Sediment condition	*p*
CuZnSOD	0.93 ± 0.09[b]	1.03 ± 0.16	0.67	1.48 ± 0.06[b]	0.76 ± 0.08	8.3 E-04
MnSOD	0.67 ± 0.03[b]	1.48 ± 0.22	0.030	0.87 ± 0.05[b]	1.07 ± 0.08	0.13
CAT	1.15 ± 0.15[b]	0.34 ± 0.14	8.7 E-03	0.65 ± 0.03[b]	0.44 ± 0.14	0.32
PHGPx	3.56 ± 0.03[b]	1.18 ± 0.18	3.7E-05	1.47 ± 0.21[b]	1.27 ± 0.19	0.55
GST d3	11.64 ± 0.29[b]	1.05 ± 0.01	1.2E-07	2.79 ± 0.13[b]	1.22 ± 0.12	1.3 E-04
GST s4	11.37 ± 0.35[b]	1.4 ± 0.16	9.5E-08	2.67 ± 0.21[b]	0.82 ± 0.18	6.4E-04
GST e1	9.15 ± 0.32[b]	1.13 ± 0.39	7.8E-06	2.93 ± 0.20[b]	0.55 ± 0.08	1.1E-05
1.1TrxR1	1.55 ± 0.04[b]	1.17 ± 0.2	0.19	1.16 ± 0.12[b]	1.05 ± 0.21	0.74
HO-1	1.63 ± 0.13	1.31 ± 0.26	0.41	2.67 ± 0.14	1.00 ± 0.27	4.2 E-03
TF	0.73 ± 0.01	0.85 ± 0.14	0.55	2.45 ± 0.10	0.57 ± 0.10	1.7E-05
START-1	0.65 ± 0.09	1.28 ± 0.18	0.048	0.99 ± 0.05	0.99 ± 0.10	0.99
HSP70	3.50 ± 0.19	1.32 ± 0.21	4.11 E-04	3.81 ± 0.09	0.61 ± 0.07	1.3E-07

[a]Gene expression levels were measured in the 4th instar larvae exposed to 1 mg/L of bare silver nanoparticles (AgNPs) or $AgNO_3$ for 24 h, calculated relative to each control group (control = 1), and shown as mean ± standard error (n = 3 [water only] or 5 [sediment]). Expression levels of selected genes were compared between water only and sediment conditions. The *Chironomus* GAPDH gene was used as a reference.

[b]Results from Nair et al. [33]. *p* values showed difference between water only condition and sediment condition using *t* test (<0.05 significant).

chronic toxicity was observed (i.e., pupation, emergence failure) at concentrations as low as 0.2 mg/L [33]. Therefore, low prevalence of acute toxicity should not be used to conclude that AgNPs are safe materials.

To determine if AgNPs cause chronic toxicity, developmental and reproductive parameters were monitored for 25 d after fourth instar *C. riparius* larvae were exposed to bare-AgNPs, citrate-AgNPs, or $AgNO_3$. The effect on development was measured by counting the number of emerged adults per larvae introduced, whereas effects on reproduction were quantified by counting the number of egg masses per larvae introduced. The citrate-AgNPs were selected from among the coated-AgNPs because they produced the most notable changes in gene expression and DNA damage. We also examined $AgNO_3$ to determine how dissolution or the particle-specific effects of AgNPs affect chronic toxicity.

The bare-AgNPs provoked a significant decrease in emergence and reproduction potential (Figure 3), which was comparable to that previously observed using sand as sediment [33]. Exposure to $AgNO_3$ also led to a decreased emergence, but the magnitude of the decrease was smaller than that observed for bare-AgNPs. Reproduction was not affected by $AgNO_3$ exposure, which suggests the potential for particle-specific effects of AgNPs, as was observed for acute effects. The citrate-AgNPs did not have a significant effect on adult emergence or reproduction. Although we only examined the chronic toxicity of 1 of our 4 coated-AgNPs, the results with citrate-AgNPs suggest that bare-AgNPs were more toxic than either citrate-AgNPs or $AgNO_3$. It is also of note that, although citrate-AgNPs caused strong short-term molecular-level responses in our acute toxicity tests, they had modest chronic effects, suggesting a potential disconnect between the 2 measures of organismal impact.

To better understand potential drivers of this disconnect in the short- and long-term toxicity of $AgNO_3$, bare-AgNPs, and citrate-AgNPs, we examined the fate of Ag in water and sediment compartments over a 25 d period (Figure 4). The bare-AgNPs spiked to the water column moved quickly to the sediment compartment. Consistent with previous observations [33], <10% of the total spiked Ag was found in the water column 12 h after spiking, and this pattern persisted until the end of the experiment (25 d). A similar trend was observed in the $AgNO_3$-spiked samples; however, the citrate-AgNPs behaved

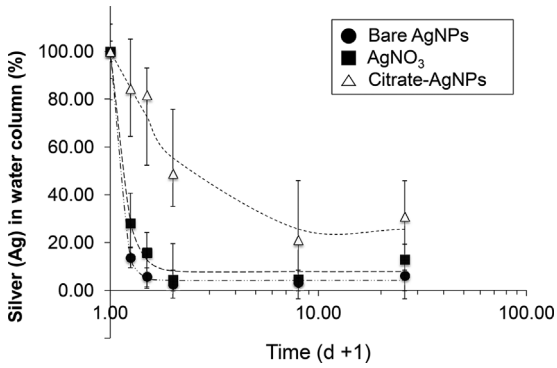


Figure 4. Inductively coupled plasma-mass spectrometric analysis of silver (Ag) content in water and sediment after spiking 1 mg/L of bare silver nanoparticles (AgNPs), $AgNO_3$, and citrate-AgNPs to water. Analysis was conducted on 4 h, 12 h, 24 h, 7 d, and 25 d exposed samples. The data were fit to a nonlinear regression model using a modified version of the formula described in Quik et al. [46]. The figure shows the model fit (dashed lines), 95% interval around the model fit (error bars), and measured values (bare-AgNPs [●], $AgNO_3$ [■], and citrate-AgNPs [△]). Time is represented as d + 1 and then presented on a log10 axis for clearer representation of the data.

completely differently. Precipitation of total Ag to the sediment was insignificant at early time points, and even at 24 h 50% of the citrate-AgNPs remained in the water column. The citrate-AgNPs gradually accumulated in the sediment. Thus, the Ag content in the water compartment was higher in the citrate-AgNP exposures than for either bare-AgNPs or $AgNO_3$ exposures, which was also evidenced by the observation of a suspension of particles in the water column (Supplemental Data, Figure S3).

Though we cannot provide an exact explanation for this disconnect in toxicity between acute and chronic exposures, we hypothesize that it is the result of both the interplay of particle and larval behavior—leading to different exposures to the 3 forms of Ag at different timescales—and compensatory physiological mechanisms that lead to disconnects between molecular biomarkers and individual/population effects on fitness. We hypothesize that exposure at different timescales may differ because the larvae tend to stay in the water column in the early phase of the exposures (up to 24 h), then burrow into the sediment (Supplemental Data, Table S4). The behavior of

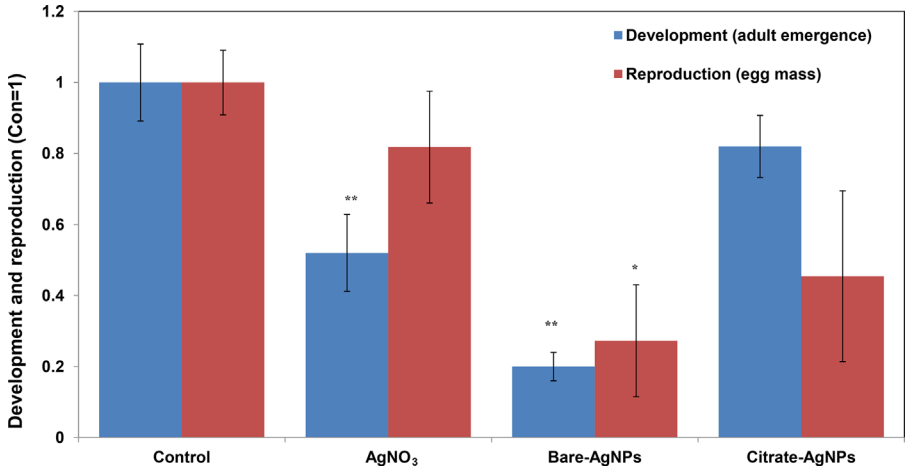


Figure 3. Development (adult emergence) and reproduction (number of egg masses) parameters in $AgNO_3$ and bare and citrate silver nanoparticles (AgNPs) exposed fourth instar larvae of *Chironomus riparius*. The results are expressed as relative values compared with the control (control = 1, $n = 3$, mean ± standard error, $p < 0.05^*$, $p < 0.01^{**}$).

citrate-AgNPs showed them to be more stable in the USEPA water as demonstrated by the observation that 50% of the citrate-AgNPs remained in the water column after 24 h, whereas bare-AgNPs and $AgNO_3$ were quickly associated with the sediment (Figure 4; Supplemental Data, Figure S3). Therefore, we might conclude that short-term toxicity of citrate-AgNPs was induced by a relatively high particle concentration in the water column and the presence of larvae in the water column, whereas low chronic toxicity was the result of lower precipitation to the sediment and, thus, low exposure to the larvae which were in the sediment (Figure 4). Additionally, as described earlier in the *Stress response gene expression* section, it is difficult to extrapolate from biomarker responses to physiological/individual/population effects because of compensatory mechanisms and confounding factors that regulate physiological/individual fitness and population dynamics [49].

Effect of coating, size, and sediment

Particle size, coating, and the presence of sediment all affected AgNP behavior and toxicity to *C. riparius* (Supplemental Data, Table S5). The most marked differences were observed for coatings. Surface coatings are known to contribute to differences in the stability of nanomaterials in aquatic media, which in turn can affect their bioavailability and toxicity [4,7,29]. In our previous study with *C. elegans*, bare-AgNPs were more toxic than PVP-coated AgNPs [6]. In another *C. elegans* study with AgNPs of similar sizes coated with either citrate, PVP, or GA, all had significantly different growth inhibitory effects, with the GA-AgNPs being more toxic than the PVP-AgNPs, whereas the PVP-AgNPs were more toxic than the citrate-AgNPs [16]. The authors suggested differences in dissolution as a potential mechanism for this differential toxicity [16]. Another study, with Japanese medaka, reported GA-AgNPs to be more toxic than PVP- and citrate-AgNPs, but all coated-AgNPs were significantly less toxic than $AgNO_3$ [7].

In the present study, we found that coated-AgNPs had different effects compared with bare-AgNPs in both acute toxicity (gene expression; Figure 2) and chronic toxicity (emergence and reproduction; Figure 3) and different fates of silver as reflected in dissolution (Supplemental Data, Figure S1) and precipitation to the sediment (Figure 4). Bare-AgNPs were reported to aggregate quickly and were more likely to settle onto the sediment, thereby enhancing the risk to benthic organisms. Unlike the bare particles, stable AgNPs, such as citrate-AgNPs, are likely to remain in the water column and increase potential toxicity to pelagic organisms [7]. Our measurements of total Ag content and ion release also suggest that citrate-AgNPs in the water column existed as particles rather than ions. In the absence of aggregation, the adverse effects of AgNPs to aquatic species are thought to be maximized as a result of increased residence times in the water column, leading to increased bioavailability [50,51]. Though Ag ions largely contribute to the toxicity of AgNPs, particle-specific effects of AgNPs have been reported in our previous studies as well as those of other groups (Supplemental Data, Table S6). In the present study, relative to bare-AgNPs, coated-AgNPs had reduced dissolution (Supplemental Data, Figure S1) and similarly reduced toxicity (Figures 1–3).

The particle size of the PVP-AgNPs had an effect on *HSP70* gene expression (Figure 2), with the larger 38-nm particles yielding higher expression. In contrast, although the difference was not statistically significant, there was a trend suggesting that smaller PVP-AgNPs may have been more genotoxic than larger PVP-AgNPs ($p = 0.332$; Figure 1), which is in agreement with our previous study with *C. elegans* and these same PVP-AgNPs [5]. In that study, PVP8-AgNP–exposed *C. elegans* had an increase in 8-hydroxydeoxyguanosine adducts, an oxidative DNA damage indicator, whereas PVP38-AgNP–exposed *C. elegans* showed no such increase.

We clearly saw the effect of sediment in moderating the gene expression of *C. riparius* in response to AgNPs and $AgNO_3$ exposure (Table 2). Eight out of 12 genes exhibited differential sensitivity in their expression responses to AgNP exposure, suggesting that sediment mitigated the short-term toxicity of AgNPs, though the mechanism is not clear. This is consistent with the reduction of Ag toxicity to zebrafish and *Daphnia* in the presence of plants and the same sediment as in the present study [29], which was correlated to reduced Ag concentrations in the water. Other studies also suggest that sediment altered the transformation and biological activity of AgNPs in aquatic environments, which would likely affect their toxicity [10,48].

CONCLUSION

The present study examined the effects of coatings, size, and the presence of sediment on the toxicity of AgNPs to *C. riparius*. The toxicity of AgNPs depends on the interplay between intrinsic particle characteristics, particle interactions with the environment, and the life history of the organism being studied. Because *C. riparius* live in/on the sediment, the presence of AgNPs in the sediment likely had stronger effects on *C. riparius* than it would on similar organisms that live in the water column. Coated- and bare-Ag NPs caused DNA damage and alteration of the expression of oxidative stress–related genes. In particular, the presence of surface coatings affected the toxicity of AgNPs to *C. riparius*, with the reduced release of ionic silver from coated-AgNPs likely playing an important role. The presence of sediment also seemed to affect toxicity as it mitigated the initial toxicity of AgNPs to *C. riparius*. Focusing solely on acute toxicity, one may overestimate the concentrations at which meaningful biological effects may happen at the organism level. This can be observed in our finding of chronic toxicity at a concentration for which no acute toxicity (mortality) was observed. Gene expression can be a sensitive indicator of organismal stress and modes of toxicity and may align with toxicity. However, changes in gene expression do not necessarily beget changes in toxicity; in the present study citrate-AgNPs did not have stronger toxicity than other particles, but altered expression of various genes. Thus, while changes in gene expression may indicate a stress on organisms, such results must be interpreted with caution as they may or may not align with acute or chronic toxicity.

SUPPLEMENTAL DATA

Tables S1–S6. (185 KB DOC).
Figures S1–S3. (93 KB PDF).

Acknowledgment—S.-Y. Park, J. Chung, and B. Colman contributed equally to the present study. This research was supported by the Basic Science Research Program through the National Research Foundation of Korea, funded by the Ministry of Education (2012R1A1A2041679), and by a grant from the Korean Ministry of the Environment through the National Institute of Environmental Science and the Environmental Health R&D Program (2014001370004 and 2012001370009). The present research was also supported by the US National Science Foundation and the USEPA under Cooperative Agreements EF-0830093 and DBI-1266252 and the Center for the Environmental Implications of NanoTechnology.

Data availability—Data, associated metadata, and calculation tools are available on request. Please contact J. Choi (jinhchoi@uos.ac.kr).

REFERENCES

1. Luoma S. 2008. Silver nanotechnologies and the environment: Old problems or new challenges. Project on Emerging Nanotechnologies, Pew Charitable Trusts, PEN-15. Woodrow Wilson International Center for Scholars, Washington, DC.
2. Tolaymat TM, El-Badawy AM, Genaidy A, Scheckel KG, Luxton TP, Suidan M. 2010. An evidence-based environmental perspective of manufactured silver nanoparticle in syntheses and applications: A systematic review and critical appraisal of peer-reviewed scientific papers. *Sci Total Environ* 408:999–1006.
3. Benn TM, Westerhoff P. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environ Sci Technol* 42:4133–4139.
4. Levard C, Hotze EM, Lowry GV, Brown GE, 2012. Environmental transformations of silver nanoparticles: Impact on stability and toxicity. *Environ Sci Technol* 46:6900–6914.
5. Ahn JM, Eom HJ, Yang X, Meye JN, Choi J. 2014, Comparative toxicity of silver nanoparticles on oxidative stress and DNA damage in the nematode *Caenorhabditis elegans*. *Chemosphere* 108:343–352.
6. Wu Y, Zhou Q, Li H, Liu W, Wang T, Jiang G. 2010. Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquat Toxicol* 100:160–167.
7. Kwok KW, Auffan M, Badireddy AR, Nelson CM, Wiesner MR, Chilkoti A, Liu J, Marinakos SM, Hinton DE. 2012. Uptake of silver nanoparticles and toxicity to early life stages of Japanese medaka (*Oryzias latipes*): Effect of coating materials. *Aquat Toxicol* 120–121:59–66.
8. Gottschalk F, Sun T, Nowack B. 2013. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. *Environ Pollut* 181:287–300.
9. Lowry GV, Espinasse BP, Badireddy AR, Richardson CJ, Reinsch BC, Bryant LD, Bone AJ, Deonarine A, Chae S, Therezien M. 2012. Long-term transformation and fate of manufactured Ag nanoparticles in a simulated large scale freshwater emergent wetland. *Environ Sci Technol* 46:7027–7036.
10. Waissi-Leinonen GC, Petersen EJ, Pakarinen K, Akkanen J, Leppänen MT, Kukkonen JV. 2012. Toxicity of fullerene (C60) to sediment-dwelling invertebrate *Chironomus riparius* larvae. *Environ Toxicol Chem* 31:2108–2116.
11. Li S, Wallis LK, Ma H, Diamond SA. 2014. Phototoxicity of TiO_2 nanoparticles to a freshwater benthic amphipod: Are benthic systems at risk? *Sci Total Environ* 466–467:800–808.
12. Stanley JK, Coleman JG, Weiss CA Jr, Steevens JA. 2010. Sediment toxicity and bioaccumulation of nano and micron-sized aluminum oxide. *Environ Toxicol Chem* 29:422–429.
13. Batley GE, Maher WA. 2001. The development and application of ANZECC and ARMCANZ sediment quality guidelines. *Australas J Ecotoxicol* 7:79–90.
14. Lucan-Bouché ML, Habets F, Biagianti-Risbourg SV. 2000. Toxic effects and bioaccumulation of cadmium in the aquatic oligochaete *Tubifex tubifex*. *Ecotoxicol Environ Saf* 46:246–251.
15. US Environmental Protection Agency. 1989. Section 7 Dilution water. Methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 2nd ed. EPA/600/4-89/001. Office of Research and Development, Cincinnati, OH.
16. Yang X, Gondikas AP, Marinakos SM, Auffan M, Liu J, Hsu-Kim H, Meyer JN. 2012. Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. *Environ Sci Technol* 46:1119–1127.
17. Cong Y, Banta GT, Selck H, Berhanu D, Valsami-Jones E, Forbes VE. 2011. Toxic effects and bioaccumulation of nano-, micron- and ionic-Ag in the polychaete, *Nereis diversicolor*. *Aquat Toxicol* 105:403–411.
18. Hoheisel SM, Diamond S, Mount D. 2012. Comparison of nanosilver and ionic silver toxicity in *Daphnia magna* and *Pimephales promelas*. *Environ Toxicol Chem* 31:2557–2563.
19. Eom HJ, Ahn JM, Kim Y, Choi J. 2013. Hypoxia inducible factor-1 (HIF-1)-flavin containing monooxygenase-2 (FMO-2) signaling acts in silver nanoparticles and silver ion toxicity in the nematode, *Caenorhabditis elegans*. *Toxicol Appl Pharmacol* 270:106–113.
20. Nair PM, Choi J. 2011. Identification, characterization and expression profiles of *Chironomus riparius* glutathione *S*-transferase (GST) genes in response to cadmium and silver nanoparticles exposure. *Aquat Toxicol* 101:550–560.
21. Nair PM, Choi J. 2012. Characterization and transcriptional regulation of thioredoxin reductase 1 on exposure to oxidative stress inducing environmental pollutants in *Chironomus riparius*. *Comp Biochem Physiol B Biochem Mol Biol* 161:134–139.
22. Nair PM, Choi J. 2012. Effects of cadmium chloride and nonylphenol on the expression of StAR-related lipid transfer domain containing protein (*START1*) gene in aquatic midge, *Chironomus riparius*. *Comp Biochem Physiol C Toxicol Pharmacol* 155:369–374.
23. Nair PM, Park SY, Choi J, 2011. Expression of catalase and glutathione *S*-transferase genes in *Chironomus riparius* on exposure to cadmium and nonylphenol. *Comp Biochem Physiol C Toxicol Pharmacol* 154:399–408.
24. Nair PM, Park SY, Choi J. 2012. Characterization and expression analysis of phospholipid hydroperoxide glutathione peroxidase cDNA from *Chironomus riparius* on exposure to cadmium. *Comp Biochem Physiol B Biochem Mol Biol* 163:37–42.
25. Nair PM, Park SY, Lee SW, Choi J. 2011. Differential expression of ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene in silver nanoparticles exposed *Chironomus riparius*. *Aquat Toxicol* 101:31–37.
26. Park SY, Nair PM, Choi J. 2012. Characterization and expression of superoxide dismutase genes in *Chironomus riparius* (Diptera, Chironomidae) larvae as a potential biomarker of ecotoxicity. *Comp Biochem Physiol C Toxicol Pharmacol* 156:187–194.
27. US Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 4th ed. EPA/600/4-90-027F. Washington, DC.
28. Bone AJ, Colman BP, Gondikas AP, Newton KM, Harrold KH, Cory RM, Unrine JM, Klaine SJ, Matson CW, Di Giulio RT. 2012. Biotic and abiotic interactions in aquatic microcosms determine fate and toxicity of Ag nanoparticles. Part 2—Toxicity and Ag speciation. *Environ Sci Technol* 46:6925–6933.
29. Unrine JM, Colman BP, Bone AJ, Gondikas AP, Matson CW. 2012. Biotic and abiotic interactions in aquatic microcosms determine fate and toxicity of Ag nanoparticles. Part 1. Aggregation and dissolution. *Environ Sci Technol* 46:6915–6924.
30. Bone AJ, Matson CW, Colman BP, Yang X, Meyer JN, Di Giulio RT. 2015. Silver nanoparticle toxicity to Atlantic killifish (*Fundulus heteroclitus*) and *Caenorhabditis elegans*: A comparison of mesocosm, microcosm, and conventional laboratory studies. *Environ Toxicol Chem* 34:275–282.
31. Colman BP, Espinasse B, Richardson C, Matson CW, Lowry GV, Hunt DE, Wiesner MR, Bernhardt ES. 2014. Emerging contaminant or an old toxin in disguise? Silver nanoparticle impacts on ecosystems. *Environ Sci Technol* 48:5229–5236.
32. Organization for Economic Co-operation and Development. 2004. Test No. 219: Sediment-water chironomid toxicity using spiked water. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
33. Nair PM, Park SY, Choi J. 2013. Evaluation of the effect of silver nanoparticles and silver ions using stress responsive gene expression in *Chironomus riparius*. *Chemosphere* 92:592–599.
34. Roh JY, Sim SJ, Yi J, Park K, Chung KH, Ryu DY, Choi J. 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. *Environ Sci Technol* 43:3933–3940.
35. Zhang W, Yao Y, Sullivan N, Chen Y. 2011. Modeling the primary size effects of citrate-coated silver nanoparticles on their ion release kinetics. *Environ Sci Technol* 45:4422–4428.
36. Cheng Y, Yin L, Lin S, Wiesner M, Bernhardt E, Liu J. 2011. Toxicity reduction of polymer-stabilized silver nanoparticles by sunlight. *J Phys Chem C* 115:4425–4432.
37. Yin L, Cheng Y, Espinasse B, Colman BP, Auffan M, Wiesner M, Rose J, Liu J, Bernhardt ES. 2011. More than the ions: The effects of silver nanoparticles on *Lolium multiflorum*. *Environ Sci Technol* 45:2360–2367.
38. Organization for Economic Co-operation and Development. 2004. Test No. 235: *Chironomus* sp., acute immobilisation test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
39. Park SY, Choi J. 2007. Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure. *Environ Int* 33:817–822.
40. Petersen EJ, Henry TB, Zhao J, MacCuspie RI, Kirschling TL, Dobrovolskaia MA, Hackley V, Xing B, White JC. 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ Sci Technol* 48:4226–4246.
41. de Lima R, Seabra AB, Durán N. 2012. Silver nanoparticles: A brief review of cytotoxicity and genotoxicity of chemically and biogenically synthesized nanoparticles. *J Appl Toxicol* 32:867–879.

42. Kim S, Ryu DY. 2013. Silver nanoparticle-induced oxidative stress, genotoxicity and apoptosis in cultured cells and animal tissues. *J Appl Toxicol* 33:78–89.
43. Eom HJ, Choi J. 2010. p38 MAPK activation, DNA damage, cell cycle arrest and apoptosis as mechanisms of toxicity of silver nanoparticles in Jurkat T cells. *Environ Sci Technol* 44:8337–8342.
44. Piao MJ, Kang KA, Lee IK, Kim HS, Kim S, Choi JY, Choi J, Hyun JW. 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. *Toxicol Lett* 201:92–100.
45. Lee S, Lee J, Kim K, Sim SJ, Gu MB, Yi J, Lee J. 2009. Eco-toxicity of commercial silver nanopowders to bacterial and yeast strains. *Biotechnol Bioprocess Eng* 14:490–495.
46. Quik JT, Stuart MC, Wouterse M, Peijnenburg W, Hendriks AJ, van de Meent D. 2012. Natural colloids are the dominant factor in the sedimentation of nanoparticles. *Environ Toxicol Chem* 31:1019–1022.
47. Colman BP, Wang SY, Auffan M, Wiesner MR, Bernhardt ES. 2012. Antimicrobial effects of commercial silver nanoparticles are attenuated in natural streamwater and sediment. *Ecotoxicology* 21:1867–1877.
48. Snell TW, Brogdon SE, Morgan MB. 2003. Gene expression profiling in ecotoxicology. *Ecotoxicology* 12:475–483.
49. Snape JR, Maund SJ, Pickford DB, Hutchinson TH. 2004. Ecotoxicogenomics: The challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquat Toxicol* 67:143–154.
50. Fabrega J, Luoma SN, Tyler CR, Galloway TS, Lead JR. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environ Int* 37:517–531.
51. Navarro E, Piccapietra F, Wagner B, Marconi F, Kaegi R, Odzak N, Sigg L, Behra R. 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environ Sci Technol* 42:8959–8964.