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

Running title: Ecotoxicity of silver nanoparticles in *C. ripairus*

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Abstract: While 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 this 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 and ionic silver concentrations were 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 genes 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 amount 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 AgNPs-induced gene expression without sediments, we show the presence of sediment appears to mitigate the toxicity of AgNPs.

Keywords: nanoecotoxicology, sediment toxicity, invertebrate toxicology, genotoxicity

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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 alkyl chain), polymers (*e.g.*, polyvinylpyrrolidone, polyacrylate, poly (vinylalcohol), polyacrylamide, and thiol-modified oligonucleotides), polysaccharides (e.g., gum arabic, 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 [10,11,12]. In addition to being the dominant fate of many contaminants, sediments also 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 nanoparticles 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 fresh water sediment [15]. In this study, the ecotoxicity of AgNPs with various organic coatings and sizes was examined in *C. riparius*. Specifically, the toxicity of bare AgNPs was compared to particles coated with either: polyvinylpyrrolidone (PVP); gum arabic (GA); or citrate. To look for size dependent effects both 8 and 38 nm PVP-AgNPs were compared. The toxicity of $AgNO_3$ was also tested to determine, if the observed toxicity was due to dissolution or was particle-specific, as this determination is an important characteristic for determining Ag nanotoxicity [16, 17, 18, 19]. The endpoints in this study 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 silver concentrations were also measured to monitor the behavior of AgNPs in water and sediment, and to determine 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 (Daejon, Korea) and have been reared in our laboratory for more than 10 years. The larvae were reared on an artificial diet of fish food flakes (Tetramin, Tetrawerke, Melle, Germany) 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 for maintaining cultures of *C. riparius*. Acid washed sand

was used as the physical media for the larval *C. riparius*. To ensure greater inter-experimental consistency, all experiments were run in EPA moderately hard water [27]. Briefly, this standard media is characterized as having a conductivity of 340-360 µS/cm, 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 experiments [31], a blended soil from the CEINT Mesocosm Facility at Duke University was used. Briefly, three surface soils were blended to yield a final sediment texture of 64% sand, 13% silt, 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 OECD Guideline [32]. Specifically it is close to the suggested 20% clay and 4-5 % organic matter. In addition, according to trace metal analysis, background Ag in the sediment was below the detection limit (Supplementary Table 1).

Preparation of Ag exposure media and characterization of AgNPs

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

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

Acute toxicity tests

Mortality test

Mortality test was conducted using modified OECD Guideline [38]. A total of ten 4^{th} instar larvae were exposed to five concentrations (0, 0.5, 1, 2 and 5 mg/L) of Ag in all six Ag treatments (5 × AgNPs, 1 × $AgNO_3$) in 100 mL reconstituted moderately hard EPA 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 EPA water without sediment (control), or after exposure to 1 mg/L of Ag in all six Ag treatments. An alkaline comet assay was conducted, as described previously [5, 39]. Briefly, treated organisms were placed in 1 mL of Phosphate-buffered saline (PBS), containing 20 mM EDTA and 10% DMSO, and disintegrated mechanically by mincing. The cell suspension was precipitated by vortexing, and then immediately mixed with 100 µL of 1% low-melting-point (LMP) agarose. To prepare slides, 100 µL of 1% LMP agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4 °C for 5 min to allow for solidification. The 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 the electrophoresis, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place until the image analysis. Before analysis, the slides were stained with ethidium bromide (20 μg/mL), then analyzed at 400x 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. DNA damage was expressed as the tail moment (tail length × tail % DNA/100) using an automated image analysis method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

There are two potential artifacts that can arise when using the comet assay to examine genotoxicity of nanoparticles: nanoparticle-DNA association can result in modified comet length, and nanoparticles such as TiO_2 can cause photochemical DNA damage during the assay yielding overestimates of genotoxicity [40]. There are three reasons we feel these are unlikely to have influenced our findings. First, appreciable nanoparticle-DNA association seems unlikely in our 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 due to photochemistry. Third, the genotoxicity results described below in this study 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 reconstituted moderately hard EPA water to 45 g dry weight of sediment. The water column was spiked to give a concentration of 1

mg/L for all six Ag treatments. After equilibration for 1 h, ten 4th 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, Carlsbad, CA, USA), following the manufacturer's instructions. The cDNA was synthesized by reverse transcribing 1 µg of total RNA using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). In order 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, Hercules, CA, USA) using 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 real time PCR detection system (Bio-Rad, Hercules, CA, USA). The expression level of each gene was calculated after exposure to the different forms of Ag. The mRNA level of each gene was normalized to that of the constitutively expressed *C. riparius* gene, GAPDH (Genbank Accession number: EU99991). The Cycle threshold (Ct) values were converted to relative gene expression levels using the CT ($2^{-\Delta Ct}$) method and analysis software provided with the CFX-96 real time PCR machine. A list of the tested genes and a description of their function is in Supplementary Table 2.

Measurement of ionic silver in water

Ionic silver dissolved from all tested AgNPs was measured. To do this, EPA 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 Beverly, MA, USA) equipped with a silver/sulfide ion-selective electrode (Orion model#9616BNWP; Thermo Scientific, Detection limit : 10 µg/L) [45]. Calibration was done against a dilution series of $AgNO_3$ solutions.

Chronic toxicity tests

Reproduction and development tests. For the chronic toxicity test, modified OECD Guideline was used [32]. Exposure aquariums were prepared by adding 400 mL of EPA water to 50 g of sediment. The EPA 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 were monitored for 25 d until all treated and control organisms were died. The emerging adults were retained with steel-wire mesh until the emergence was complete in all treatments.

In order to investigate developmental effects, the number of emergent adults from each vessel was counted. For reproduction, the numbers of 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. The test solutions were not renewed. All the 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, 12, and 24 h, and 7 and 25 d of exposure. 10 mL 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, USA) with the extraction solution at 200 °C. The composition of extraction solutions were 10 mL of a mixture of HNO_3 and HF with a 4:1 ratio for mixed acid digestion. After complete digestion, the remaining acids were evaporated until the HF acid had been completely eliminated from the solution. The residue was then diluted with 20 mL of deionized water with 1~5 % 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 (ICP-MS, Elan DRC II, Perkin Elmer, USA, Detection limit; 0.1 ng/L).

Given that the goal of this 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 three separate experiments with one lab replicate of each. We then fit the resulting data using nonlinear regression using a modified version of the formula described in Quik et al. [46] Specifically we used the equation 1

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

Where % Ag_t is the percent silver in the water column at time t, Ag_{res} is the residual silver in the water column after aggregation and/or precipitation, 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 V.11 (SAS Institute Inc., Cary, NC, USA)

Statistical analysis

Statistical differences between the control and treated samples were examined with one-way ANOVAs using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA). All data were reported as means ± standard error of the mean (SE). 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 Turkey's HSD test.

RESULTS AND DISCUSSION

Through a series of experiments using either acute or chronic exposures, we examined the

impacts of 35 nm bare particles (bare-AgNPs); 6 nm gum arabic coated particles (GA-AgNPs), 40 nm citrate coated particles (citrate-AgNPs); and 8 or 38 nm polyvinylpyrrolidone coated particles (PVP8-AgNPs and PVP38-AgNPs, respectively) on *C. riparius*. Exposures were conducted in EPA 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 ionic silver 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 sub-lethal 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 4th instar larvae of *C. riparius* was compared for $AgNO_3$ and AgNPs with different surface coatings. Mortality tests were conducted for 24 hours without sediment and no mortality was observed for controls. Larvae treated with the AgNPs and $AgNO_3$ showed less than 10% mortality even at the highest concentrations tested (5 mg/L). Across concentrations, coated AgNPs on average caused less mortality (less than 5 %) than bare-AgNPs and $AgNO_3$, however mortality rate was not significant compared to control ($p > 0.05$) (Supplementary Table 3).

Genotoxicity test

The lack of differences in mortality in silver treatments compared to 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 AgNPs-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 also in *C. riparius* [33] using the same bare-AgNPs that were used here, as well as, the PVP-AgNPs also used here (*C. elegans*; [5]). Here 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), while the GA-AgNPs were not significantly different from controls ($p = 0.159$). The citrate-AgNPs were the most genotoxic NPs, increasing DNA damage roughly three-fold compared to controls. While there is a trend towards the smaller PVP-AgNPs having a higher genotoxic potential than larger ones—the tail moment measured in the PVP8-AgNPs-exposed larvae was slightly higher than that of the PVP38-AgNPs-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 oxidative stress is involved in AgNPs 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 bare-type (Supplementary Figure 1), higher genotoxicity was observed in citrate and PVP-AgNPs than even $AgNO_3$. These results showed that AgNPs toxicity could be linked not only to ion effects but also particle specific effects while coating agent could change 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 genes 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 $AgNO_3$ [25]. In this 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 current experiment was conducted using more environmentally realistic methods (i.e. in presence of sediment). Ten genes involved in the oxidative stress response were selected: *SODs* (superoxide dismutases: *Cu-ZnSOD, MnSOD*), *CAT* (catalase), *GSTs* (glutathione S transferase: *GSTd3, GSTs4, GSTe1*), *PHGPx* (phospholipid glutathione peroxidase), *TrxR1* (thioredoxin reductase1), *HO-1* (heme oxyganase-1), and *TF* (transferrin). 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 also analyzed. A brief description of these genes and previous publications using these selected oxidative stress-related genes are presented in Supplementary Table 2.

Six of the ten stress genes showed changes in expression for at least one silver 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 four of the six forms of silver tested, with all four notably being nanoparticle forms (bare-AgNPs, PVP38-AgNPs, citrate-AgNPs, and GA-AgNPs) (Figure 2). Of all the forms of silver investigated, citrate-AgNPs had the most widespread effects on gene expression, leading to significant increases in the expression of five of the genes ($p < 0.05$) (Figure 2). All significant changes relative to controls were increases with the exception of those for *CAT* (bare-AgNP and $AgNO_3$ treatments) and *TF* ($AgNO_3$ and PVP8-AgNP treatments), which had significantly decreased

expression (Figure 2).

Of all silver forms examined, only GA-AgNPs induced *Cu-ZnSOD* gene expression, whereas bare-AgNPs, PVP38-AgNPs, citrate-AgNPs and GA-AgNPs induced *MnSOD* gene expression (Figure 2). The mitochondria are often considered to be particularly susceptible to AgNPs, which lead 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 AgNP-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, while exposure to bare-AgNPs led to a significant decrease in *CAT* gene expression, rather than the expected increase. This might be due to 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 current experiment with sediment was observed in *GST* mRNA expression. In our previous study, the expression of three types of *GST* mRNA (i.e. *GST d1*, *s1* and *e1*) increased dramatically after exposure to bare-AgNPs and $AgNO_3$ [21, 26], whereas such a tendency was not observed in this study. The only case in which there was significantly increased *GST* expression was observed with *GSTe1* and was caused by citrate-AgNPs (Figure 2, Supplementary Figure 2). Much like *GSTe1*, the expression of *HO-1* was increased only after citrate AgNPs exposure (Figure 2). When taken together, these results may suggest citrate-AgNPs 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* gene through unknown mechanisms (Figure 2). In our previous study, *PHGPx1* and *TrxR1* mRNA expression in *C. riparius* were reported to be up-regulated as a consequence of oxidative stress [21, 24, 25]. However, in the present study, none of the silver forms tested affected the expression of these two antioxidant enzyme genes (Supplementary Figure 2). The increased expression of the steroidogenesis related gene *START1* by citrate AgNPs suggests 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 significant 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 current study showed evidence of a less sensitive response in the presence of sediment (Table 2). In aquatic system with sediment, the low oxidation reduction potential of high organic matter present in sediment could decrease AgNPs toxicity [47]. According to our acute effect 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 mechanism. 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, due to the various potential confounding factors.

Extent of dissolution of AgNPs in water

Given that our results suggest that the presence or absence of a surface coating may affect the toxicity of AgNPs to *C. riparius*, we looked at the amount of Ag ions released by coated AgNPs relative to the bare-AgNPs after spiking 1 mg/L of the bare and coated-AgNPs into EPA water (Supplementary Figure 1). The results clearly document reduced dissolution for the coated AgNPs, indicating that the reduced toxicity of the coated-AgNPs compared to the bare-AgNPs is likely at least in part related to ion release. However, we did not find 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 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 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 while 4th 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 significant changes in gene expression and DNA damage. $AgNO_3$ was also examined 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]. $AgNO_3$ exposure also led to a decreased emergence, but the magnitude of the decrease was smaller than 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 one of our four coated AgNPs, the results with citrate-AgNPs suggest that bare-AgNPs were more toxic than citrate-AgNPs and $AgNO_3$. It is also of note that, while citrate-AgNPs caused strong short-term molecular level responses in our acute toxicity tests, they had much more modest chronic effects, suggesting a potential

disconnect between the two 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], less than 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 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 (Supplementary Figure 3).

Though we cannot provide the exact explanation for this disconnect in toxicity between acute and chronic exposures, we hypothesize that it is due both to: the interplay of particle and larval behavior leading to different exposures to the three forms of silver 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 (Supplementary Table 4). The behavior of citrate-AgNPs showed them to be more stable in the EPA 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, Supplementary Figure 3).

Therefore, we might conclude that short-term toxicity of citrate-AgNPs was induced by relatively high particle concentration in the water column and the presence of larvae in the water column, while low chronic toxicity was due to lower precipitation to the sediment and thus low exposure to the larvae which were in the sediment (Figure 4). Beyond exposure, as described earlier 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* (Supplementary Table 5). The most significant results 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 PVP-AgNPs, whereas 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].

Here, we found coated AgNPs had different effects compared to bare-AgNPs in both acute (gene expression, Figure 2) and chronic toxicity (emergence and reproduction, Figure 3), and different fates as reflected in dissolution (Supplementary Figure 1) 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 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 (Supplementary Table 6). In this study, relative to bare-AgNPs, coated AgNPs had reduced dissolution (Supplementary Figure 1) and similarly reduced toxicity (Figures 1-3).

The particle size of the PVP-AgNPs had an effect on *HSP70* gene expression (Figure 2) with 38 nm particles having higher expression. Moreover, although the difference was not statistically significant, there was a trend suggesting that smaller PVP-AgNPs were 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-OHdG adducts, an oxidative DNA damage indicator, while 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 AgNPs exposure, suggesting 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 our study [29], which was correlated to reduced Ag concentrations in the water.

Other studies also suggest that sediment altered transformation and biological activity of AgNPs in aquatic environments, which would likely affect their toxicity [10, 48].

CONCLUSION

This 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. The coated and bare-AgNPs 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 organismal level. We found significant chronic toxicity at a concentration for which no acute toxicity (mortality) was observed. Gene expression can be a sensitive indicator of organismal stress, modes of toxicity, and may align with toxicity. However, changes in gene expression do not necessarily beget changes in toxicity, as changes in gene expression may occur with low acute toxicity and they also do not necessarily align with chronic toxicity. For example, in this study citrate-AgNPs did not have stronger toxic potential than other particles but induced significant changes in expression of various genes, which did not translate to higher-level effects.

SUPPLEMENTAL DATA

Tables S1–S6. (185 KB DOC).

Figures S1–S3. (93 KB PDF).

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

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Figure 1. DNA damage as measured by comet assay in bare and coated AgNPs and $AgNO_3$ exposed *C. riparius*. The results were expressed as tail moment (n = 3, mean ± SE). Asterisks indicate significant difference (*P < 0.05*, P < 0.01***) as compared to the control group using ANOVA test.

Figure 2. Stress response gene expression after treatment with 1 mg/L of bare and coated AgNPs and $AgNO_3$ for 24 h in presence of the sediment. Gene expression levels (*Cu-MnSOD*, *MnSOD*, *CAT*, *GSTe1*, *HO-1*, *TF*, *START-1*, *HSP70*) were calculated relative to GAPDH expression and shown as mean ± SE (control=1, n =5). The *Chironomus* GAPDH gene was used as a reference gene. Asterisks indicate significant difference (*P < 0.05*, P < 0.01***) as compared to the control group using ANOVA test.

Figure 3. Development (Adult emergence) and reproduction (Eggmass) parameters in $AgNO_3$ and bare and citrate-AgNPs exposed 4^{th} instar larvae of *C. riparius.* The results are expressed as relative values compared to control (control=1, n = 3, mean ± SE, *P < 0.05*, P < 0.01***).

Figure 4. ICP-MS analysis of silver content in water and sediment after spiking 1 mg/L of bare-AgNPs, $AgNO_3$ and citrate-AgNPs to water. Analysis was conducted on 4, 12, and 24 h, and 7 and 25 d exposed samples. The data were fit to 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.

Tables

Table 1. Characterization of bare-, coated AgNPs. The particle shape was determined using a transmission electron microscope (TEM) and hydrodynamic size distribution (HDD) and zeta potential were evaluated using a Photal dynamic light scattering (DLS) spectrometer. [a] and [b] indicate the results from Yang et al. [16] and Yin et al. [37], respectively

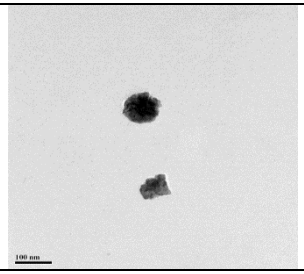
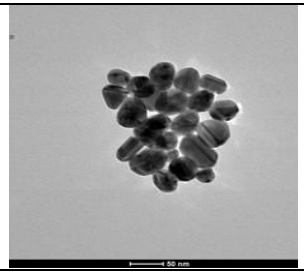
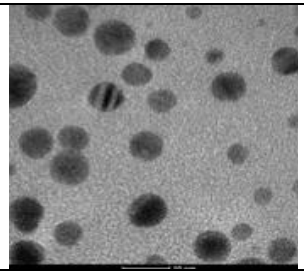
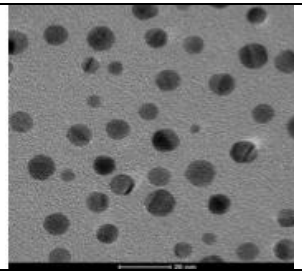
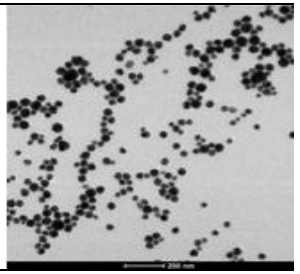
	Bare-AgNPs	**Citrate-AgNPs** [a]	**GA-AgNPs** [b]	**PVP8-AgNPs** [a]	**PVP38-AgNPs** [a]
TEM image					
Zeta potential (mV)	-26.3±1.2	-30±3 mV	-46 ± 2.5 mV	-5.0 ± 0.2 mV	-10.9± 0.4 mV
HDD (nm)	31.6±6.7	7 ± 11 nm	6 ± 1.7	8 ± 2 nm	38 ± 8 nm

Table 2. Comparison of stress response genes expression results between previous (water only condition) and current studies (in presence of the sediment). Gene expression levels were measured in the 4th instar larvae exposed to 1mg/L of AgNPs and $AgNO_3$ for 24h and were calculated relative to each control group (control=1) and shown as mean ± SE (n=3(W), 5(S)). Expression levels of selected genes were compared between water only and sediment conditions. The *Chironomus* GAPDH gene was used as a reference gene. [a] indicates the results from Nair et al. [30]. *p* values showed difference between water only condition and sediment condition using t-test (< 0.05: significant)

	AgNPs			$AgNO_3$		
Exposure condition	water only condition	sediment condition	*p* value	water only condition	sediment condition	*p* value
CuZnSOD	0.93 ± 0.09 [a]	1.03 ± 0.16	0.67	1.48 ± 0.06 [a]	0.76 ± 0.08	8.3 E-04
MnSOD	0.67 ± 0.03 [a]	1.48 ± 0.22	0.030	0.87 ± 0.05 [a]	1.07 ± 0.08	0.13
CAT	1.15 ± 0.15 [a]	0.34 ± 0.14	8.7 E-03	0.65 ± 0.03 [a]	0.44 ± 0.14	0.32
PHGPx	3.56 ± 0.03 [a]	1.18 ± 0.18	3.7E-05	1.47 ± 0.21 [a]	1.27 ± 0.19	0.55
GST d3	11.64 ± 0.29 [a]	1.05 ± 0.01	1.2E-07	2.79 ± 0.13 [a]	1.22 ± 0.12	1.3 E-04
GST s4	11.37 ± 0.35 [a]	1.4 ± 0.16	9.5E-08	2.67 ± 0.21 [a]	0.82 ± 0.18	6.4E-04
GST e1	9.15 ± 0.32 [a]	1.13 ± 0.39	7.8E-06	2.93 ± 0.20 [a]	0.55 ± 0.08	1.1E-05
1.1TrxR1	1.55 ± 0.04 [a]	1.17 ± 0.2	0.19	1.16 ± 0.12 [a]	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

Figure 1

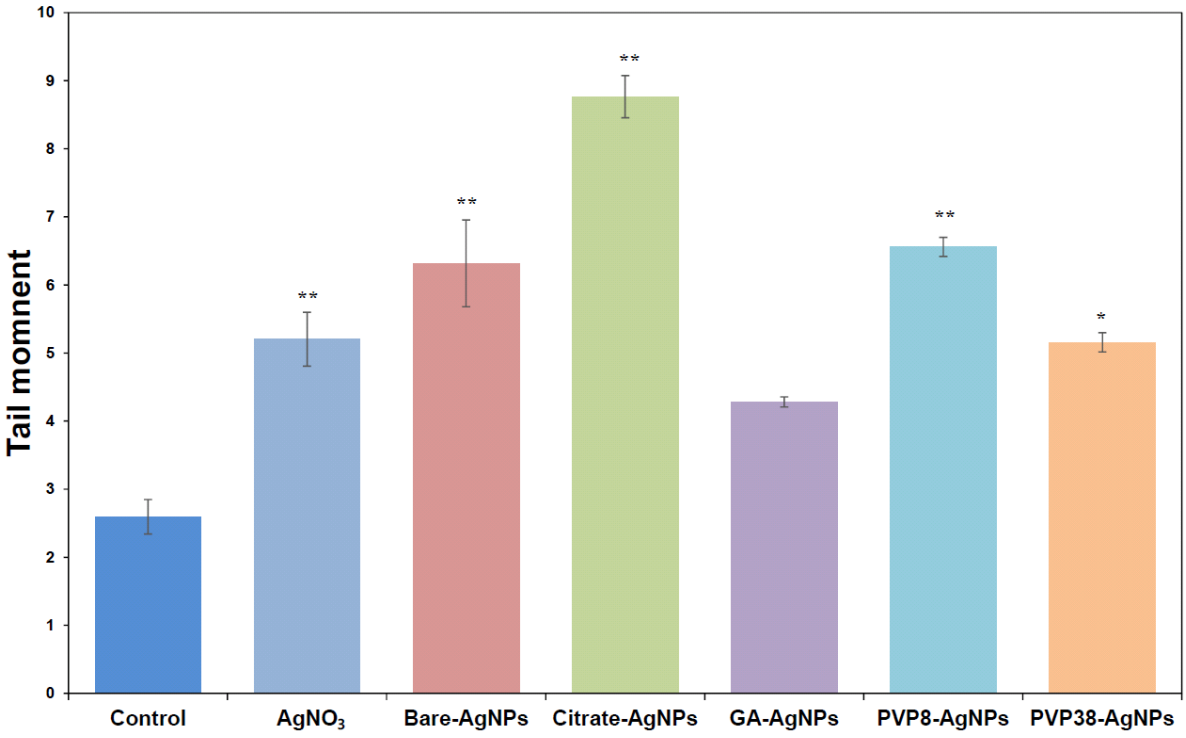


Figure 2

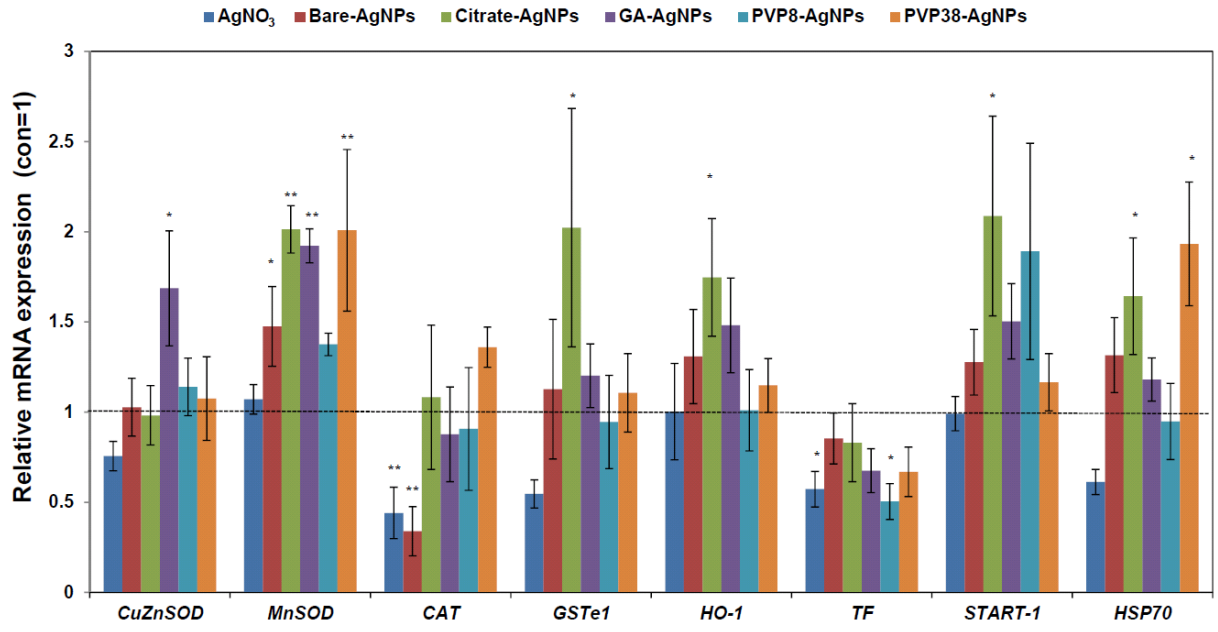


Figure 3

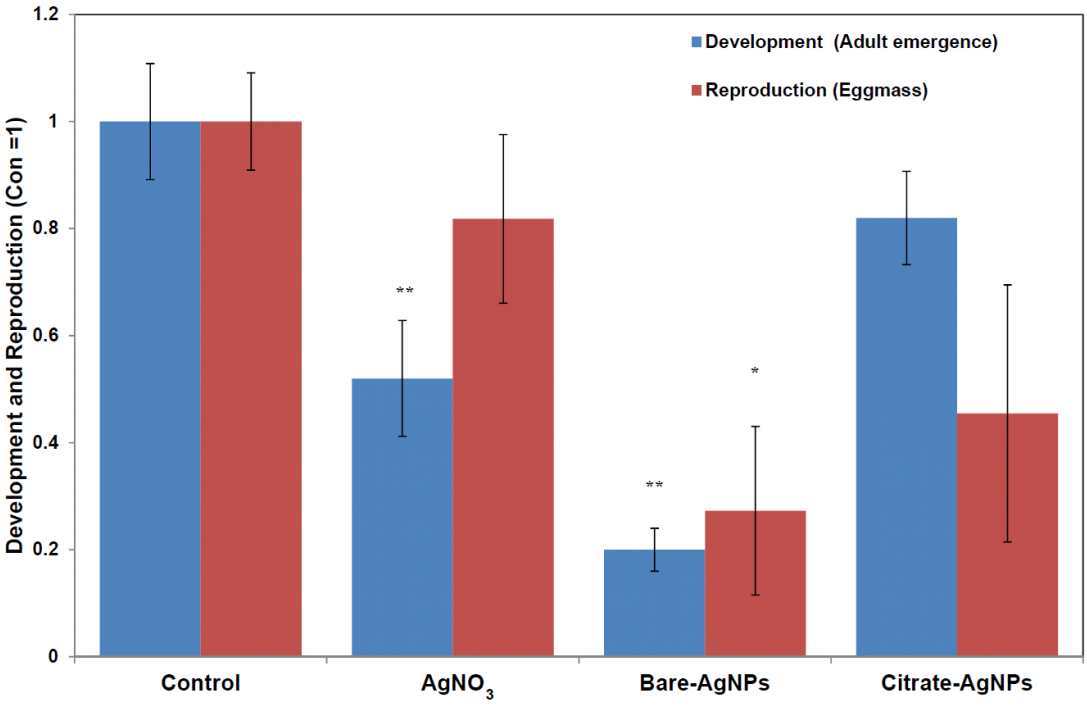


Figure 4