Evaluation of Colloidal Stability and Ecotoxicity of Metal-based Nanoparticles in the Aquatic and Terrestrial Systems

Lok R. Pokhrel
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Evaluation of Colloidal Stability and Ecotoxicity of Metal-based Nanoparticles in the Aquatic and Terrestrial Systems

A dissertation presented to the faculty of the Department of Environmental Health East Tennessee State University

In partial fulfillment of the requirements for the degree Doctor of Philosophy in Environmental Health

by Lok Raj Pokhrel May 2013

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Keywords: aggregation, dissolution, ecotoxicity, hazard, metal nanoparticles, stability
ABSTRACT

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by

Lok Raj Pokhrel

Intrinsic to the many nano-enabled products are atomic-size multifunctional engineered nanomaterials, which upon release contaminate the environments, raising considerable health and safety concerns. This Ph.D. dissertation is designed to investigate (i) whether metals or oxide nanoparticles are more toxic than ions, and if MetPLATE™ bioassay is applicable as a rapid nanotoxicity screening tool; (ii) how variable water chemistry (dissolved organic carbon (DOC), pH, and hardness) and organic compounds (cysteine, humic acid, and trolox) modulate colloidal stability, ion release, and aquatic toxicity of silver nanoparticles (AgNP); and (iii) the developmental responses of crop plants exposed to Ag- or ZnO- (zinc oxide) nanoparticles.

Results suggest that the MetPLATE can be considered a high-throughput screening tool for rapid nanotoxicity evaluation. Detectable changes in the colloidal diameter, surface charge, and plasmonic resonance revealed modulating effects of variable water chemistry and organic ligands on the particle stability, dissolution, and toxicity of AgNPs against *Escherichia coli* or *Daphnia magna*. Silver dissolution increased as a function of DOC concentrations but decreased with increasing hardness, pH, cysteine, or trolox levels. Notably, the dissociated Ag⁺ was inadequate to explain AgNP toxicity, and that the combined effect of AgNPs and dissolved Ag⁺ under each ligand treatment was lower than of AgNO₃. Significant attenuation by trolox signifies an oxidative stress-mediated AgNP toxicity; its inability to attenuate AgNO₃ toxicity, however,
negates oxidative stress as Ag\(^+\) toxicity mechanism, and that cysteine could effectively quench free Ag\(^+\) to alleviate AgNO\(_3\) toxicity in *D. magna*. Surprisingly, DOC-AgNPs complex that apparently formed at higher DOC levels might have led daphnids filter-feed on aggregates, potentially elevating internal dose, and thus higher mortality. Maize root anatomy showed differential alterations upon exposure to AgNPs, ZnONPs, or their ions.

Overall, various metal-based nanoparticles revealed lower toxicity than their ions against multiple organisms. This study showed that particle size, surface properties, and ion release kinetics of AgNPs modify following release into aquatic environment, suggesting potential implications to ecosystem health and functions, and that caution be applied when extending one species toxicity results to another because obvious differences in organism biology—supporting species sensitivity paradigm—can significantly alter nanoparticle or ionic toxicity.
DEDICATION

To my beautiful family—Puja, Angel, and Aerin—for their immeasurable sacrifices to this effort.
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CHAPTER 1

INTRODUCTION

Background and Problem Statement

The term “nanotechnology” was first used by Taniguchi (Taniguchi, 1974); however, Feynman’s talk “There’s plenty of room at the bottom” has often been credited with the birth of nanomaterial science, offering understanding of how particle properties change in nanometer size scale (1–100 nm in at least one dimension; Feynman, 1970). An opening editorial “Small is different” by a reputable journal Nature Nanotechnology further substantiates the potential role nanoscience and nanotechnology can play in the next industrial revolution, largely due to size-related novel properties innate to nanomaterials, and the plethora of research opportunities that emanate from the challenges of understanding nanomaterial behavior and toxicity in the environment (Rodgers, Chun, Cantrill, & Thomas, 2006). Following the advent of high resolution transmission electron microscopy (HRTEM), tunneling microscopy, atomic force microscopy (AFM), x-ray diffraction (XRD), x-ray photoelectron spectroscopy (XPS), among others, new information about engineered nanomaterial characteristics, such as morphology, surface defects, core-shell structure, crystal structure, surface charge, bonding, and state of aggregation, has emerged (Maynard, Warheit, & Philbert, 2011). Nanotechnology has, thus, evolved as a multidisciplinary science geared toward manipulating, measuring, miniaturizing, and modeling matter in this diminutive (nanometer) size scale (NNI, 2006; Schmidt, 2007).

Because of the high surface reactivity inherent to smaller particle dimension, distinct characteristics that engineered nanomaterials demonstrate are absent in their bulk parent (larger size or ionic) form (Navrotsky, Mazeina, & Majzlan, 2008; NNI, 2006; Stone et al., 2009; USEPA, 2007). In addition to size-specific properties, particle shape, chemical make-up (purity,
oxidation state), surface structure (crystal structure, surface coatings/ligands), and surface energetics offer novel functionalities to engineered nanomaterials (Maynard, Warheit, & Philbert, 2011; Schmidt, 2007; USEPA, 2007). Using these novel attributes, many kinds (ca 2500) of high value nano-enabled products have been commercialized in various sectors including personal care (e.g., sunscreen lotion, cosmetics), clothing, electronics, therapeutics (as cancer drugs), and medical diagnostics (as contrast agents; Nanowerk, 2012; PEN, 2013).

Metal-based nanoparticles (e.g., nanoparticles of silver (AgNP), titanium dioxide (TiO$_2$NP), zinc oxide (ZnONP), cadmium selenide quantum dots (CdSe QD)) are among the wide variety of nanomaterials being predominantly used for their antimicrobial, opto-electrical, surface reactivity, or plasmonic properties (Adams, Lyon, & Alvarez, 2006; Elzey & Grassian, 2010; NNI, 2006; USEPA, 2007). Nanoparticles, their aggregates, and ions are released from the nano-enabled products (Benn & Westerhoff, 2008; Benn, Cavanagh, Hristovski, Posner, & Westerhoff, 2010; Impellitteri, Tolaymat, & Scheckel, 2009), which enter environments in many ways including: (i) release from the manufacturing site, (ii) accidental spill at the site of storage or en route during transportation, (iii) leaching during product use or due to weathering, (iv) end-of-life disposal and subsequent leaching from the site of disposal, (v) land application of biosolids and wastewater, and (vi) land application of residual ash generated from incineration of waste including nanowaste, (vii) land application of landfill leachate containing nanomaterials, and (viii) intentional subsurface injection of nano zero valent iron (NZVI) or associated nano-enabled products for the treatment of chlorinated hydrocarbons (e.g., trichloroethylene; Gottschalk & Nowack, 2011; Pokhrel & Dubey, 2013; Zhang, 2003). Continual application of nanomaterials in a multitude of products and their potential for leaching colloidal particles and toxic ions into the environments (Benn & Westerhoff, 2008; Benn, Cavanagh, Hristovski,
Posner, & Westerhoff, 2010; Impellitteri, Tolaymat, & Scheckel, 2009) have raised significant environmental, health and safety concerns (Nel, Xia, Madler, & Li, 2006; NNI, 2006; Oberdörster et al., 2005; Pokhrel & Dubey, 2012; Pokhrel et al., 2012; Pokhrel & Dubey, 2013; USEPA, 2009).

Published studies indicate that nanomaterials are toxic to aquatic organisms including bacteria, algae, crustacea, and fish (Kennedy et al., 2012; Laban, Nies, Turco, Bickham, & Sepulveda, 2010; Navarro et al., 2009; Yang, Zhu, Colvin, & Alvarez, 2011), with limited information available on the potential toxicity to plants (Lin & Xing, 2008; Rico, Majumdar, Duarte-Gardea, Peralta-Videa, & Gardea-Torresdrey, 2011). Mechanistic understanding of nanotoxicity has remained elusive (Xiu, Zhang, Puppala, Colvin, & Alvarez, 2012). Nonetheless, much effort has been made to understand if nanomaterial toxicity can be better explained using dose or surface area metric (Warheit, Webb, Sayes, Colvin, & Reed, 2006). Additionally, research is underway to identify factors that associate with bioactivity, to evaluate whether existing test bioassays can be appropriately applied for nanotoxicology or if modifications are necessary (Pokhrel et al., 2012), and to uncover potential molecular interactions occurring at bio-nano interface for explaining biological responses at the organism level (El Badawy et al., 2011; Pitek et al., 2012; Silva, Pokhrel, Dubey, Maier, & Tolaymat, 2013; Vecitis, Zodrow, Kang, & Elimelech, 2010).

Satisfactory characterization of multiple parameters, such as nanomaterial dose in the test matrix, particle morphology, surface functionality, chemical composition, core-shell structure, dissolution into ions, and state of aggregation, might enable identifying factors that could potentially influence nanomaterial colloidal properties and subsequently the toxicity (El Badawy et al., 2011; Jin et al., 2010; Lowry, Apte, & Lead, 2012; Nel et al., 2006; Pitek et al., 2012;
Some studies suggest that a model silver nanoparticle (AgNP) can act as a source of Ag ions that can be released for a considerable time in the environment (Liu & Hurt, 2010; Li, Lenhart, & Walker, 2010). It is suggested that AgNP toxicity can be attributed to the combined effect of monovalent Ag$^0$ and the released free Ag$^+$ (Navarro et al., 2008). An alternate hypothesis is that AgNP exposure can lead to structural modification of the cell surface, thereby causing pits on the cell wall and membrane leakage (Fabrega, Renshaw, & Lead, 2011). Potent antimicrobial activity of AgNPs has been associated with cell uptake leading to DNA damage, while potential release of dissolved Ag$^+$ (in the cell interior) originating from AgNPs inhibits ion-exchange and thus cellular respiration (Ratte, 1999). Physical interaction of AgNPs with bacterial cell surface has recently been implicated as an important factor explaining antimicrobial activity (El Badawy et al., 2011). Generation of reactive oxygen species (ROS) leading to oxidative damage is another potential mechanism of AgNP toxicity (Choi & Hu, 2008). It is, however, important to note that the environmental conditions (e.g., natural organic matters such as humic acids, pH, surface ligands, background electrolyte), and the media used for bioassay (Lee, Kwak, & An, 2012; Stampoulis, Sinha, & White, 2009) can modify nanomaterial characteristics including the particle size, particle shape, and surface charge (El Badawy et al., 2011; George et al., 2012), and thereby the toxicity (El Badawy et al., 2011, El Badawy, Schekel, Suidan, & Tolaymat, 2012; George et al., 2012; Huynh & Chen, 2011; Wirth et al., 2012).

Understanding the potential human health and environmental effects of engineered nanomaterials would only allow continual economic success of nano-industry, which otherwise might be plagued by growing apprehension and uncertainty surrounding nanotoxicity (Stern & McNeil, 2008). Despite more than a decade of efforts to elucidate the factors influencing and the
proximate mechanisms explaining nanotoxicity, the nano-research community is struggling to answer one important question “Are nanoparticles hazard?” (Xiu et al., 2012). Hence, public debate is emerging on whether nanoparticles should be evaluated for their potential environmental and human health effects on a case-by-case basis (European Commission, 2008; El Badawy et al., 2011, 2012; FIFRA, 2011). With novel properties, nanomaterials can exhibit enhanced mobility, dispersion, and bioactivity at the cellular and subcellular levels should exposure occur (El Badawy et al., 2011, 2012; George et al., 2012; Huyanh & Chen, 2011; Oberdöster, 2010; Weir et al., 2008). Contamination of aquatic systems with engineered nanomaterials will likely impact the ecosystems therein (Bone et al., 2012; Gao et al., 2009; Pokhrel et al., 2012; Silva et al., 2013), while soil contaminated with nanomaterials may harm terrestrial crop plants (Lin & Xing, 2008; Rico et al., 2011). The need to address these safety concerns calls for a better understanding of the potential toxicity of metal-based nanoparticles on the aquatic and terrestrial life forms, which enables informing risk managers of the potential risk that nanomaterials might pose to the environment and human health.

Research Objectives

To address the aforementioned knowledge gaps in nanotoxicology, this Ph.D. dissertation is designed to investigate the following research objectives:

Objective 1: To evaluate if metals or oxide nanoparticles are more toxic than their ions;

Objective 2: To assess if the MetPLATE™ Escherichia coli bioassay is applicable as a rapid screening tool for the toxicity evaluation of multiple metal-based nanoparticles;

Objective 3: To investigate the potential influence of natural water chemistry (dissolved organic carbon, pH, and hardness) on the colloidal stability, dissolution rate, and antibacterial activity of silver nanoparticles against E. coli;
Objective 4: To investigate the potential role of humic acid, cysteine, and trolox on the colloidal stability, dissolution rate, and aquatic toxicity of silver nanoparticles against *Daphnia magna*; and

Objective 5: To evaluate the developmental responses of two crop plants (*Zea mays*, and *Brassica oleracea* var. capitata) exposed to silver and zinc oxide nanoparticles.

**Dissertation Outline**

This Ph.D. dissertation consists of six chapters. Chapter 1 offers the background information, the problem statement, the research objectives, and this dissertation outline. Chapter 2 is an evaluation of the MetPLATE *E. coli* bioassay as a rapid screening tool for the toxicity assessment of multiple metal-based nanoparticles. Chapter 3 is an investigation of the potential influence of natural water chemistry (dissolved organic carbon, pH, and hardness) on the colloidal stability, ion release rate, and aquatic toxicity of silver nanoparticles in *E. coli*. Chapter 4 is an assessment of the potential role of organic ligands – humic acid, cysteine, and trolox – on the colloidal stability, ion release rate, and aquatic toxicity of silver nanoparticles against *Daphnia magna*. Potential developmental responses of two agriculturally significant crop plants, maize (*Zea mays*) and cabbage (*Brassica oleracea* var. capitata), upon exposure to silver and zinc oxide nanoparticles are evaluated in Chapter 5. Whether metals or oxide nanoparticles are more toxic than their ions is also evaluated as a common objective for Chapters 2–5. Chapter 6 presents a succinct summary of the major findings of this dissertation research and outlines the directions for future research. Chapters 2 and 5 are structured as manuscripts per the authors’ instructions of the Elsevier journal *Science of the Total Environment*, while Chapters 3 and 4 are structured as manuscripts for the ACS journal *Environmental Science & Technology* in accordance to its guide for authors. Supplementary materials for Chapters 2–5 are presented at
the end of each chapter in the form of Appendix and their cited references are available at the end of each chapter. A comprehensive list of references precedes the Vita.
CHAPTER 2

Rapid Screening of Aquatic Toxicity of Several Metal-based Nanoparticles using the

MetPLATE™ Bioassay

Lok R. Pokhrel, Thilini Silva, Brajesh Dubey, Amro M. El Badawy, Thabet M. Tolaymat,
Phillip R. Scheuerman

ABSTRACT

Current understanding of potential toxicity of engineered nanomaterials to aquatic microorganisms limits the risk assessment and management of nanomaterials. Here we evaluate if the MetPLATE™ test can be used as an effective and rapid screening tool to test for potential aquatic toxicity of various metal-based nanoparticles (NP). The MetPLATE bioassay is a heavy metal sensitive test based on β-galactosidase activity in Escherichia coli. Five different types of metal-based NPs were screened for toxicity: (1) citrate coated silver nanoparticles (Citrate–nAg); (2) polyvinylpyrrolidone coated nAg (PVP–nAg); (3) uncoated zinc oxide nanoparticles (nZnO); (4) uncoated titanium dioxide nanoparticles (nTiO₂); and (5) 1-octadecylamine coated cadmium selenide quantum dots (CdSe QDs); and compared with corresponding ionic salt toxicity. Citrate–nAg was fractionated into clean Citrate–nAg, unclean Citrate–nAg and permeate using a tangential flow filtration (TFF) system to eliminate residual ions and impurities from the stock Citrate–nAg suspension and also to differentiate between ionic- versus nano-specific toxicity. Our results showed nAg, nZnO and CdSe QDs were less toxic than their corresponding ionic salts, while the nano- or ionic form of TiO₂ was not toxic at concentrations as high as 2.5 g L⁻¹ to MetPLATE™ bacteria. Although coating-dependent toxicity was noticeable between two types of Ag NPs evaluated, particle size and surface charge were not adequate to explain the observed...
toxicity; hence, the toxicity appeared to be material-specific. Overall, the toxicity followed the trend: CdCl$_2$ > AgNO$_3$ > PVP–nAg > unclean Citrate–nAg > clean Citrate–nAg > ZnSO$_4$ > nZnO > CdSe QDs > nTiO$_2$/TiO$_2$. These results indicate that an evaluation of β-galactosidase inhibition in MetPLATE™ E. coli can be an important consideration for rapid screening of metal-based NP toxicity, and should facilitate ecological risk assessment of these emerging contaminants.

1. Introduction

Risk assessment and toxicity studies of engineered nanomaterials (ENM) are receiving heightened attention because of the following reasons: (i) their increasing use and end-of-life disposal into the environment (European Commission, 2004; USEPA, 2007; Gao et al., 2008); (ii) the challenge to fractionate toxicity resulting from ENMs alone to that of ionic forms of the same chemical including the residual impurities of the precursor compounds used during ENM synthesis (Gao et al., 2009; Kennedy et al., 2010); and (iii) limited understanding of the mechanisms by which ENMs may cause harm to biologic receptors (El Badawy et al., 2011). As materials can be tailored at nanoscale according to the need to acquire desired characteristics for different product formulations (e.g., biomarkers, biosensors, catalysts, personal care products; Santra et al., 2005a, 2005b; Schmidt, 2007; www.nanotechproject.org), such novel properties may, on the other hand, render nanomaterials hazardous to health and the environment (European Commission, 2004; Gao et al., 2009; Kennedy et al., 2010). Because ENMs could potentially leach metals and ions from the associated products (Benn et al., 2008; Benn et al., 2010) which upon release could contaminate the aquatic systems, an assessment of their potential aquatic toxicity then becomes a priority (Gao et al., 2009; Benn et al., 2010; Kennedy et al., 2010). Although size has remained central to impart toxicity, other factors such as surface
functionalization, complexation with different elements, solvent chemistry and environmental variables are also known to potentiate or abate nanotoxicity (Hardman, 2006; Baalousha et al., 2008; Fabrega et al., 2009; Gao et al., 2009).

Recent review of the nanomaterial databases, such as the Project on Emerging Nanotechnologies (www.nanotechproject.org) and the Nanowerk Nanomaterial database (www.nanowerk.com), indicated that oxides and elemental nanomaterials, mostly constituted of metals, are among the most commercialized ENMs accounting for greater fraction of materials listed in these databases (Musee, 2011). This study aims to investigate the potential toxicity of various commercially important metal-based nanoparticles (NPs; e.g., nAg, nTiO$_2$, nZnO, and CdSe Quantum Dots) against *Escherichia coli* enzyme activity. Colloidal nanoscale Ag is commercially used as a coating in solar cells (Cole and Halas, 2006), as an antimicrobial in health and personal care products and in medical imaging (Tolaymat et al., 2010). As an opacifier, TiO$_2$ (both nano and bulk) is widely used in personal care products, paints, plastics and papers; while ZnO is known for its applications in semiconductor and pigment industries (Adams et al., 2006). Due to strong fluorescence properties quantum dots (QDs) are utilized in biomedical imaging (Alivisatos, 2004; Santra et al., 2005b) and in the semiconductor industry due to their prominent opto-electrical features (Bruchez et al., 1998). Because of the greater surface area and smaller particle dimensions, significantly lower quantity of ENMs can provide equivalent or enhanced performance in such established applications as compared to their micron-sized counterparts (Adams et al., 2006).

Increasing concern among toxicologists is that the existing test methods available for screening conventional chemical toxicity may not be appropriately applied for assessing ENM toxicity, and may, therefore, require some modifications in light of the understanding that ENMs...
express novel physicochemical properties than their larger-sized counterparts (Hartung and Sabbioni, 2011). Moreover, most traditional toxicity tests which rely on cultured organisms (e.g., *Daphnia*, shrimps, earthworms, etc.) are not only time consuming but are also expensive and complex in nature. As an alternative, many recent studies have utilized a microbial bioassay, called MetPLATE™, to screen for toxicity of a variety of contaminants including landfill leachate, leachate from CCA (chromated copper arsenate) treated woods (Dubey et al., 2007), electronic waste (Dagan et al., 2007), and mine tailings and mine waters (Blumenstein et al., 2005; El Hamiani et al., 2010). The MetPLATE bioassay was developed specifically to screen for potential toxicity of biolabile metals using a strain of *E. coli* (Bitton et al., 1994). This organism synthesizes and uses β-galactosidase, an enzyme that catalyzes the conversion of dissachharides, including lactose, to monosaccharides such as galactose and glucose, which are important energy sources for young mammals, including humans (Hansen and Gitzelmann, 1975). Driven by the realization that the MetPLATE bioassay is less responsive to organics (Bitton et al., 1994), which are mostly applied as surface coatings on metal NPs for acquiring desired characteristics and stability in aqueous matrix (Ma et al., 2012), this study attempts to establish its effectiveness as a rapid method for toxicity screening of several metal-based NPs.

This study evaluates the potential toxicity of five different metal-based NPs and comparing toxicity with corresponding ionic salts utilizing the MetPLATE *E. coli* bioassay as a rapid screening method. The selected NPs are among the most commercialized NPs used in various consumer applications (Musee, 2011; www.nanotechproject.org). Using a Tangential flow filtration system (TFF), we fractionated Citrate–nAg into clean Citrate–nAg, unclean Citrate–nAg (as-synthesized), permeate (obtained as a filtrate) and ionic Ag⁺ (as AgNO₃) to enable differentiating nano- versus ion-specific toxicity.
2. Materials and methods

2.1. Materials

Various metal-based NPs and their corresponding ionic salts were tested for their potential toxicity using the MetPLATE™ *E. coli* bioassay. These NPs were (1) citrate coated nanosilver (Citrate–nAg), (2) polyvinylpyrrolidone coated nAg (PVP–nAg), (3) uncoated nZnO, (4) uncoated nTiO₂ (anatase), and (5) 1-octadecylamine coated CdSe core quantum dots (CdSe QDs); their corresponding ionic counterparts were AgNO₃, ZnSO₄, TiO₂ (anatase), and CdCl₂, respectively. Citrate–nAg and PVP–nAg were synthesized and stabilized following the procedures previously described (El Badawy et al., 2010, 2011). Citrate–nAg was used as unclean Citrate–nAg (as-synthesized) or clean Citrate–nAg (purified sample). Purification of Citrate-nAg was carried out using a Tangential flow filtration (TFF) system (detailed in the following section). Permeate obtained as a result of purifying Citrate–nAg was also tested for its potential toxicity. CdSe QDs were purchased from Ocean Nano Tech, Arkansas, USA (Cat. # QCO-60-0050); nTiO₂ (anatase) was procured from Creative Nanotech, NY, USA (purity: 99.9%); and nZnO was supplied by Meliorum Technologies, Inc., NY, USA (purity: 99.9%). nZnO and nTiO₂ were obtained from the manufacturers as aqueous suspensions, without any surface functionalization. CdSe QDs were surface functionalized with 1-Octadecylamine (an organic ligand) and were received as a stable suspension in toluene. We also tested the toxicity of 1-octadecylamine purchased from Fisher Scientific, Inc., USA (purity - 98%, Cat # 50-701-5343). All chemicals used as the sources of ions were purchased from Fisher Scientific, Inc., USA and were either USP or ACS grade (AgNO₃, Cat # S486-100; CdCl₂, Cat # C10-100; ZnSO₄, Cat # 19145280; TiO₂, Cat # AC21358-1000).
2.2. Purification of Citrate–nAg

Purification of Citrate–nAg (as-synthesized) was carried out using polysulfone (PS) 10 kD hollow fiber diafiltration membranes (P/N: X31S-300-02P, surface area = 145 cm$^2$) connected to Kros Flo Research II/ TFF system (Spectrum Laboratories, CA, USA), and was controlled with KF COMM data collection software (Spectrum Laboratories, CA, USA). Purification involved buffer exchange of the residual impurities and ions in NPs suspension with nanopure water (electrical conductivity = 2 µS cm$^{-1}$; Appendix Table 2.2). Applying a suitable shear pressure and maintaining tangential peristaltic flow of the suspension through the hollow fiber membranes allowed sub-nanometer particles, dissolved ions and impurities to flow across the hollow membranes and forming the permeate, while allowing greater than 1 nm size particles to pass through the hollow lumen of the membranes; hence, forming the retentate (El Badawy et al., 2011; Rinzler et al., 1998; Kanel and Al-Abed, 2011). The permeate and clean Citrate–nAg were characterized for their basic properties following purification. The samples were digested using standard USEPA method 3050B, and the concentrations were determined using Inductively coupled plasma-mass spectrometry (Bruker 820-ICP-MS).

2.3. Characterization of nanoparticles

All types of NP suspensions were diluted in moderately hard water (MHW) and were tested for their volume weighted hydrodynamic diameters (HDD) and zeta ($\zeta$) potential measurements using dynamic light scattering (DLS) method. Average $\zeta$ potential was approximated from the electrophoretic mobility of the particles using NICOMP 380 ZLS Particle sizer/zeta potential unit (PSS NICOMP Particle Sizing Systems, CA, USA). The unit was calibrated at 23 °C using Duke 500 (491 nm) NIST 3490A standard (PSS Nicomp, FL, USA) prior to the analysis of HDDs and $\zeta$ potential values for the NPs. Typically, the $\zeta$ potential value provides information
about the strength of repulsive force between particles with the same charge in the colloidal solution. In practice, higher (±) \( \zeta \) value indicates higher stability, i.e., greater resistance to particles aggregation. The surface Plasmon resonance (SPR) spectra of the NPs were recorded using HACH DR 5000 UV/Vis spectrophotometer (HACH Company, CO, USA). Transmission electron microscopy (TEM) was used to visualize the morphology (i.e., size and shape) of the nanoparticles. An aliquot of each NP sample was sonicated for about 10 minutes, after which few drops of each sample were pipetted onto a support carbon coated copper formvar, allowing it to air dry before recording the images using a Philips EM 420 operated at 120 kV in the brightfield mode. ImageJ 1.44 software (http://rsb.info.nih.gov/ij/) was used to estimate particle size distributions (PSD) from a representative TEM imagery. A Multiskan microplate reader equipped with 570 nm filter and Ascent software (ver 2.6) was used to record the absorbance obtained as a result of differential microbial enzymatic activity at varying concentrations of the materials tested.

2.4. Toxicity bioassay

As previously mentioned, the MetPLATE™ bioassay is a \( \beta \)-galactosidase based assay in which chlorophenol-red \( \beta \)-galactopyranoside (CPRG) is used as a chromogenic substrate that is cleaved by the enzyme, forming galactopyranose and chlorophenol red as the by-products. During this event yellow-colored CPRG is transformed into magenta-colored chlorophenol red, the concentration of which is proportional to the activity of \( \beta \)-galactosidase, which is quantified at 570 nm using a microplate reader (Fig. 2.1; Bitton et al., 1994). MHW was used as a negative control, while 1 mg Cu\(^{2+} \) L\(^{-1} \) (as CuSO\(_4\)) was used as a positive control with each set of the analysis. At least triplicate samples were analyzed for each dilution. The detailed procedure of the MetPLATE™ test can be found elsewhere (Bitton et al., 1994). The MetPLATE™ kit was
procured from M2B Research & Innovative Technologies, LLC, Gainesville, FL.

To determine if NPs also caused mortality of the MetPLATE bacteria, a growth assay was performed for unclean Citrate–nAg used as a model NP. For this, a 50 ml of sterile nutrient broth

![Mechanistic basis of β-galactosidase mediated conversion of chlorophenol red galactopyranoside (CPRG used as a substrate; yellow color) into chlorophenol red (magenta color) with negative control (moderately hard water). Note the dose-dependent inhibition of enzyme activity with all evaluated nanoparticles (except for CdSe quantum dots dispersed in MHW) or their corresponding ionic salt treatments (see Figs. 2.4 & 2.5 for their toxicity profiles).](image)

(Difco™, autoclaved at 121 °C for 20 mins) prepared in MHW was inoculated with the MetPLATE bacteria (5 mL as recommended by the supplier) in a 250 mL flask in the sterile working environment. This represented a negative control. An aliquot of unclean Citrate–nAg was added to another set of similar inoculation representing a treatment (resultant concentration = 6.78 mg Ag L⁻¹). From each of these flasks, an aliquot of 50 µL was transferred to a test tube containing 5 mL of the broth at successive time periods of 0, 1, 2, and 3 h. These samples were incubated for the next 5 h at 35 °C, following which its optical density was measured using a UV/Vis spectrophotometer (Hach DR 5000) at 600 nm. Five mL of nutrient broth incubated at the same temperature and time represented a blank.
2.5. Statistical analysis

Based on the measured absorbance readings using the microplate reader, the percent inhibitions were calculated with the assumption that the negative control indicates null inhibition (Bitton et al., 1994). The EC$_{50}$ (i.e., effective concentrations for 50% enzyme activity inhibition) values were estimated using the slope and intercept of the linear regression line obtained by plotting percent inhibition against the test chemical concentration in a logarithmic scale (Bitton et al., 1994). The Mann-Whitney U test was used to test if the mean EC$_{50}$ values were significantly different between the two treatments at the $p \leq 0.05$ level. Statistical analysis was performed using SPSS version 18.0 (SPSS, 2010).

3. Results and Discussion

3.1. Characteristics of nanoparticles

The characteristics of different types of metal-based NPs used in this study are summarized in Table 2.1. Our measurements of average HDDs of all NPs were in good agreement with the manufacturers’ reported average diameters, except for nTiO$_2$ that showed a higher average HDD with bimodal PSD (Table 2.1, Appendix Fig. 2.8E). This likely indicated an occurrence of sedimentation, possibly due to aggregation of TiO$_2$ nanoparticles when suspended in MHW, which was confirmed by TEM (Fig. 2.2D). Likewise, CdSe QDs obtained as a suspension in toluene showed HDD consistent with the manufacturer’s particle dimension (Fig. 2.2E; Appendix Fig. 2.8F); however, when suspended in MHW its average HDD of 913.3 nm (SD = ± 79.8 nm) revealed by DLS suggests an aggregation of QD particles (Table 2.1, Appendix Fig. 2.8F). Previous studies have shown that QDs are incompatible (insoluble) in aqueous suspensions (Feng et al., 2005; Karabanovas, 2008), the results consistent with our observation.
Both clean and unclean Citrate–nAg suspensions showed the same HDD in MHW, supporting the earlier measurements of El Badawy et al. (2010).

Table 2.1.
Characteristics of evaluated metal-based nanoparticles.

<table>
<thead>
<tr>
<th>material^a</th>
<th>pH</th>
<th>particle size distribution</th>
<th>average zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>manufacturer reported</td>
<td>hydrodynamic diameter^b</td>
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<td></td>
<td></td>
<td>primary particle size</td>
<td>(Mean ± SD) nm</td>
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<tr>
<td></td>
<td></td>
<td>(nm)</td>
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<tr>
<td>Unclean Citrate–nAg</td>
<td>7.45</td>
<td>na</td>
<td>10.9 ± 0.8</td>
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<td>Clean Citrate–nAg</td>
<td>7.24</td>
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<td>11.0 ± 0.7</td>
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<tr>
<td>PVP–Ag</td>
<td>7.02</td>
<td>na</td>
<td>10.9 ± 0.8</td>
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<tr>
<td>nZnO^c</td>
<td>7.03</td>
<td>10</td>
<td>11.0 ± 0.7</td>
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<tr>
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<td></td>
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<td></td>
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<tr>
<td>nTiO^c</td>
<td>7.09</td>
<td>10</td>
<td>184.5 ± 13.3; 895.6 ± 57.2</td>
</tr>
<tr>
<td>CdSe QD^c</td>
<td>6.98</td>
<td>&lt;10</td>
<td>11.0 ± 0.7; 4.3 ± 0.5^t</td>
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</tbody>
</table>

^aMaterial suspended in moderately hard water (MHW); ^bVolume weighted hydrodynamic diameter measured using DLS method; ^cNanomaterials procured from manufacturers; na, Data not available; na, size not estimated due to aggregation of particles; ^tmeasured in toluene; ^wmeasured in MHW; PVP-nAg, polyvinylpyrrolidone coated Ag nanoparticles; QD, quantum dots functionalized with 1-Octadecylamine; n = number of particles analyzed for estimating particle diameter from Transmission Electron Microscopy (TEM) imagery using ImageJ 1.44 program; n = number of particles measured for size estimation.

Both types of Citrate-nAg showed net negative charge with higher ζ potential values.
indicating stable particle dispersion in the suspensions (Table 2.1), which was confirmed by TEM (Fig. 2.2A; Appendix Fig. 2.8A, B). The plausible explanation for stabilization of Citrate–nAg is that the ionic citrate carboxyl groups may have capped the Ag⁰/Ag⁺, thereby stabilizing particles electrostatically (Kimling et al., 2006; El Badawy et al., 2010). On the other hand, PVP–nAg was sterically stabilized due to adsorption of PVP on the surface of nAg (El Badawy et al., 2010). Despite lower ζ potential, TEM characterization reflected higher stability of PVP–nAg as particles were well spaced apart (Fig. 2.2B). For other types of NPs evaluated average ζ potential values were comparatively lower (Table 2.1).

The representative SPR spectra of all NPs evaluated are shown in Fig. 2.2F. A slight blue-shift of the Plasmon peak coupled with substantial reduction in electrical conductivity (EC = 5 µS cm⁻¹) for clean Citrate–nAg (obtained as a retentate following TFF) compared to unclean Citrate–nAg (EC = 1095 µS cm⁻¹) indicated significant reduction in its impurities fraction (El Badawy et al., 2011; Kanel and Al-Abed, 2011). The measured parameters for Citrate–nAg before and after cleaning remained unaltered (Table 2.1), and this suggests that the cleaning process did not impact Citrate–nAg characteristics. As expected, complete resemblance of SPR spectrum of permeate (obtained as a waste following TFF) to that of Ag⁺ suggests the presence of ionic Ag and possibly an absence of NPs of Ag in the permeate (Fig. 2.2F). For the remaining NPs, SPR spectra were consistent with the manufacturers’ certificate of analysis (COA) or the literature reviewed (Fig. 2.2F; Liu et al., 2006; Wang et al., 2009). The potential impact of dilution on the stability of NPs when suspended in the test medium (MHW) and during incubation at 35 °C for 3 h were also investigated for Citrate–nAg, PVP–nAg and nZnO. Results showed that the incubation time and temperature required for MetPLATE test, including dilution in MHW, had no impact on the tested NPs characteristics, and therefore remained fairly stable.
Fig. 2.2. Representative Transmission electron microscopy (operated at 120 kV in a brightfield mode) images of: (A) Citrate–nAg (scale bar = 50 nm), (B) PVP–nAg (scale bar = 20 nm), (C) nZnO (aqueous; scale bar = 20 nm), (D) nTiO$_2$ (aqueous; scale bar = 100 nm) and (E).CdSe Quantum Dots coated with 1-Octadecylamine (in toluene; scale bar = 50 nm). (F) Representative UV-Vis spectra of different nanoparticles evaluated. The particle size distributions of the nanoparticles are presented in Table 2.1.
3.1. Toxicity of metal-based nanoparticles to MetPLATE™ bioassay

Few previous studies had used MetPLATE to study potential aquatic toxicity of only a few types of NPs (Gao et al., 2008; Gao et al., 2009), but whether MetPLATE is sensitive to a wide variety of nanomaterials including oxides (i.e., nTiO$_2$ and nZnO), metallic (i.e., nAg), and bimetallic (i.e., CdSe QDs) NPs had not been studied until now. Our results showed that the MetPLATE test can be used as an effective and rapid screening tool for toxicity assessment of the wide variety of NPs (Figs. 2.3 – 2.5). MHW that was used as a carrier medium or a negative control had acceptable water quality parameters (pH = 7.1, electrical conductivity = 599 µS cm$^{-1}$, total dissolved solids = 299 mg L$^{-1}$) required by the test protocol (Bitton et al., 1994). The pH values of all the test chemicals, including that of all evaluated NPs, were also within the range (pH = 6 – 7.5) suitable for the test and therefore required no pH adjustment (Table 2.1).

Comparison of the EC$_{50}$ values revealed that PVP–nAg was the most toxic among all the tested NP types, while clean Citrate–nAg showed similar toxicity to unclean Citrate–nAg (Fig. 2.3A). Sodium citrate dihydrate (used as reducing/stabilizing agent for Citrate–nAg) or PVP (used as coating/stabilizing agent for PVP–nAg) was not toxic to the bacteria at the highest concentration which was theoretically expected to be in the respective NP suspensions (sodium citrate dihydrate = 10 mM; PVP = 1.5 g L$^{-1}$). Nanoscale TiO$_2$ was not toxic even at a concentration as high as 2.5 g L$^{-1}$ (Fig. 2.3B; Appendix Table 2.5), a result consistent with the earlier findings for similar test organisms (Heinlaan et al., 2008; Jiang et al., 2009; Kasemets et al., 2009). Lower ζ potential value (-10.14 mV) coupled with bimodal PSDs for nTiO$_2$ indicated potential aggregation of the particles (Appendix Fig. 2.8), which might have rendered lower surface area, little to no dissolution, and little to no bioavailability. These characteristics could be
Fig. 2.3. Toxic impacts of various metal-based nanoparticles and their corresponding ionic salts to MetPLATE™ microbial assay. Note that lower EC₅₀ values represent higher toxicity. NT, not toxic at the entire range of concentrations tested (see Appendix Table 1.4). For CdSe Quantum Dots (QDs), % inhibition is shown because EC₅₀ was not estimated due to lower inhibition in the entire range of concentrations tested (0.01 – 100 mg/L). Error bars represent standard deviation of the triplicate test runs.
attributed to an absence of inhibition of β-galactosidase activity by nTiO₂ in *E. coli*. CdSe QDs suspension in MHW showed, on average, 34.42% inhibition of β-galactosidase activity in the entire range of concentrations tested (0.1 µg L⁻¹-100 mg L⁻¹; Fig. 2.3C). Lower inhibition of the microbial activity on exposure to CdSe QDs indicates that aggregation of the CdSe QD particles in MHW, as revealed by DLS (Appendix Fig. 2.8F) and TEM (Fig. 2.2E), might have resulted into lower interaction/bioavailability of NPs to the bacterial populations.

A concentration-dependent inhibitory effect was observed for all NPs and ionic salts tested, except for CdSe QDs and 1-Octadecylamine which showed similar toxicity at all tested concentrations (Figs. 2.4 and 2.5; Appendix Table 2.5). Toxicity comparison among all evaluated NPs and their corresponding ionic forms showed Cd²⁺ (as CdCl₂) as the most inhibitory to the enzyme activity followed by Ag⁺ (as AgNO₃; Figs. 1.3 – 1.5). nZnO showed significantly lower toxicity than Zn²⁺ (as ZnSO₄; p = 0.05; Fig. 2.3). Ionic- or nano-form of TiO₂ was not toxic at a concentration as high as 2.5 g L⁻¹ (Appendix Table 2.5). Despite having lower ζ potential (~ -10 mV) and apparent particle settling (Table 2.1; Fig. 2.6) nZnO and nTiO₂ did not show a similar toxicity pattern. nZnO was toxic at mg L⁻¹ level, while nTiO₂ was not toxic even at g L⁻¹ level (Appendix Table 2.5). A recent study has also shown higher toxicity of nZnO to both gram negative and gram positive bacteria than its ionic salt (Jiang et al., 2009). These researchers attributed the toxicity to the interactions between NPs and the bacterial cell wall (Jiang et al., 2009). While Cd²⁺ (as CdCl₂) was more toxic than CdSe QDs, the toxicity profile of 1-Octadecylamine alone was similar to that exhibited by CdSe QDs surface functionalized with 1-Octadecylamine (p > 0.5, based on % inhibition; Appendix Table 12.5). It, therefore, remained unclear whether the resulting inhibition of the bacterial enzyme activity was due to the capping agent alone or due to the CdSe core of the QDs with potential to release ions of Cd and Se.
(Mahendra et al., 2008). At similar EC$_{50}$ values for Cd$^{2+}$, earlier studies have documented 50% lethality to rainbow trout in the laboratory experiments (Khangarot and Ray, 1987; Munkittrick et al., 1991). Interestingly, a study has shown that CdSe alone as weathered QDs (with no surface

![Fig. 2.4. Concentration-dependent inhibition of β-galactosidase by different nanosilver particles, including AgNO$_3$ used as an ionic source of Ag. PVP–nAg was more toxic at the comparative concentrations among the tested nanoparticles types. Error bars represent ± 1 standard deviation of the triplicate test runs.](image)

![Fig. 2.5. Concentration-dependent inhibition of β-galactosidase on exposure to nanoZnO, ZnSO$_4$, and CdCl$_2$, but not with CdSe quantum dots (QDs) or 1-Octadecylamine (used as coating for quantum dots) treatment. Error bars represent ± 1 standard deviation of the triplicate test runs.](image)
functionalization) was toxic to E. coli (Mahendra et al., 2008), and other reports suggest that 1-Octadecylamine (as an organic surfactant) can also be toxic to a wide variety of aquatic organisms (Noack, 1984; Hoechst, 1988). A recent study by Hoshino et al. (2004) indicates that surface coatings (e.g., cysteamine, mercaptoundecanoic acid, etc.) could potentially impart higher cellular toxicity than the QDs core (Hosino et al., 2004).

Fig. 2.6. Photograph showing suspensions of nZnO (left) and nTiO$_2$ (right) tested for toxicity against the MetPLATE™ bioassay. Note the insets showing sedimentation in both stock nano-suspensions.

3.2. Fractionating silver to differentiate toxicity

Fractionating Citrate–nAg into clean Citrate–nAg, unclean Citrate–nAg and permeate (containing residual Ag ions and impurities), and testing ionic Ag toxicity for comparative understanding is an important consideration in distinguishing nano- versus ionic-toxicity (Kennedy et al., 2010; El Badawy et al., 2011). In this study, purification of unclean Citrate–nAg via well-established TFF procedure likely removed impurities (e.g., Na$^+$, citrate ions), including the dissolved Ag ions, such that they were collected as permeate while retaining clean Citrate–
nAg as retentate (El Badawy et al., 2011; Kanel and Al-Abed, 2011). Differential enzyme activity of *E. coli* revealed ionic Ag more acutely inhibitory than both types of Citrate–nAg (unclean and clean Citrate–nAg; Figs. 2.3 and 2.4). These results are consistent with the previous work that demonstrated higher toxicity of Ag ions to *Bacillus* species using the 5-day Biochemical Oxygen Demand (BOD$_5$) and live/dead tests as compared to Citrate–nAg synthesized using the same method (El Badawy et al., 2011). By removing Ag ions and residual impurities from the unclean Citrate–nAg suspension, both types of Citrate–nAg exhibited similar toxicity (Fig. 2.3A; p > 0.5). Although permeate at the highest concentration of 65 µg Ag L$^{-1}$ (as total Ag) remained nontoxic to the MetPLATE bioassay (Fig. 2.3A), it appeared that having the same amount of Ag$^+$ ions in unclean Citrate–nAg suspension slightly enhanced the toxicity of the latter, although this was not statistically significant (Fig. 2.3; p > 0.5).

Ag$^+$ (as AgNO$_3$) was more than an order of magnitude (16 times) more toxic than clean Citrate–nAg. However, Ag$^+$ was only 2.25 times more toxic than PVP–nAg (Fig. 2.3A). This substantiates the earlier finding that surface coating (Citrate versus PVP) of nAg could alter the toxic response of the bacterial populations (El Badawy et al. 2011). However, this was not true with other types of tested NPs. Moreover, characteristics such as particle size and surface charge (as $\zeta$ potential) were not adequate to explain the nanotoxicity observed; hence, the toxicity rather appeared to be material-specific. Nonetheless, these results clearly indicate lower nano-specific toxicity than ionic toxicity to the MetPLATE bioassay.

Because the MetPLATE bioassay is based on the colorimetric quantification of the enzyme-substrate reaction (Fig. 2.1), it becomes important to understand if exposure to NPs also inhibited growth of the test population. As previously mentioned, a growth assay was performed using the same MetPLATE bacteria, which upon exposure to unclean Citrate–nAg resulted into complete
growth inhibition of the microbial population following 2 h of exposure. In contrast, the bacterial population nearly doubled in the negative control (MHW) by the end of 3 h test period (Fig. 2.7), which corresponds to the time required for conducting the MetPLATE test. Taken together, these findings suggest that not only could Ag nanoparticles inhibit the enzymatic activity of the tested bacteria, they could also be detrimental to the growth and survival of the bacterial population.

![Bacterial Growth Curves](image)

**Fig. 2.7.** Growth curves showing significant mortality of MetPLATE *E. coli* population when exposed to unclean Citrate-nAg (6.78 mg Ag L<sup>-1</sup>). The absorbance readings were obtained at 600 nm using HACH DR 5000 UV-Vis Spectrophotometer. MHW, moderately hard water was used as the negative control. Note significant bacterial growth in MHW, but complete growth inhibition occurred with unclean Citrate-nAg treatment. X-axis represents the time during which the bacteria were exposed to unclean Citrate-nAg, after which they were allowed to grow for next five hours.

Recently, Lu et al. (2010) reported several fold higher toxicity of ionic Ag (at 10 mg L<sup>-1</sup>) than nAg suspension (at 100 mg L<sup>-1</sup>) as determined by HaCaT keratinocytes viability and reactive oxygen species (ROS) measurements. Further, ionic Ag elicited higher genotoxicity to the bacteria and HaCaT keratinocytes, unlike nAg that did not cause DNA damage (Hwang et al., 2008) even at 10 times higher concentration to that of ionic Ag (Lu et al., 2010). Ag nanoparticles in contact with the algal cell have been shown to promote Ag ions release, which
subsequently inhibited the photosynthetic activity of the algal cells (Navarro et al., 2008). In contrast, a few recent studies have also indicated nano-specific toxicity of Ag NPs (Hoshino et al., 2004; Fabrega et al., 2009; Kennedy et al., 2010). When advanced analytical methods, such as the use of biotic ligand model (De Schamphelaere and Janssen, 2004), TFF (El Badawy et al., 2011; Kanel and Al-Abed, 2011) or field flow fractionation for NPs purification (Kennedy et al., 2010), are beginning to be used to discriminate nano-specific toxicity from ionic toxicity, we envisage that better understanding of nanotoxicology could be achieved in the foreseeable future.

Toxic impacts of Ag as ions are well recognized and mechanisms better understood. Cellular interactions of Ag ions with sulphydryl (−SH) group containing important enzymes and amino acids (such as cysteine and glutathione) are known to impair cellular respiration and ion-exchange across the membranes, and subsequently leading to cell death (Ratte, 1999; Luoma, 2008). When understanding of mechanisms of nanotoxicity is limited, our study indicates that inhibition of β-galactosidase activity in the MetPLATE E coli can be an important consideration for rapid screening of metal-based nanomaterials since it can be accomplished within a short span of time.

Increasing applications of wide variety of ENMs and their subsequent disposition into the environment call for better understanding of their potential risks to human health and the environment. Natural environment consists of diversity of bacteria—some catalyzing nutrient cycles (e.g., Streptomyces thermoautotrophicus, Clostridium pasteurianum, Azotobacter, etc.) (Bishop et al., 1985; Wang et al., 1988; Ribbe et al., 1997), some are pathogenic (e.g., E. coli 0157:H7) while others are commensals (e.g., E. coli MG1655; Blattner et al., 1997). With increasing applications of nAg and nZnO in hundreds of commercial products (Adams et al., 2006; www.nanotechproject.org), consistent with previous studies (Gao et al., 2009; Jiang et al.,
2009) these results demonstrate potential toxicity of nAg and nZnO, causing risk to the aquatic receptors such as environmentally significant bacterial communities (Choi and Hu, 2008). On the other hand, unlike the experimental conditions, in the natural waters where excess of sulfide and chloride ions including the naturally occurring organic matters (NOMs such as humic and fulvic acids), extracellular polymeric substances, and biopolymers (e.g., polypeptides, polynucleotides, polysaccharides) exist, surface modifications of ENMs can be envisaged, thereby potentially abating their toxicity on the exposed aquatic organisms (Baalousha et al., 2008; Fabrega et al., 2009; Gao et al., 2009). Moreover, surface functionalized CdSe QDs that showed apparently very little inhibition to the enzymatic activity of the E. coli may cause higher toxicity provided its surface coating gets eroded when present in acidic (pH ≤ 4) or basic (pH ≥10) waters as shown by Mahendra et al. (2008). In contrast, as mentioned earlier, when the receiving waters contain NOMs, proteins and other organic ligands the toxicity of CdSe QDs may be reduced due to low metal bioavailability (Mahendra et al., 2008).

Overall, the MetPLATE™ toxicity followed the trend: CdCl₂ > AgNO₃ > PVP–nAg > unclean Citrate–nAg > clean Citrate–nAg > ZnSO₄ > nZnO > CdSe > nTiO₂/TiO₂. These results showed that nAg, nZnO, and CdSe QDs were less toxic than their corresponding ionic salts tested, and NPs or ions of TiO₂ were not toxic at as high as 2.5 g L⁻¹ to the tested bioassay. As previously recommended, the results of this study also highlight the importance of fractionating metal NPs suspension to eliminate potential impurities, ions and any other remnant precursor molecules used during NPs synthesis; thus, allowing us to better understand potential toxicity resulting from the NPs exposure to the biotic receptors (Gao et al., 2009; Kennedy et al., 20010; El Badawy et al., 2011).
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APPENDIX

Synthesis of Citrate–nAg: A solution of 1 mM AgNO₃ was added to 10 mM Sodium citrate dihydrate in a volume ratio of 2:1, and heated for four hours at 70 °C in a water bath (El Badawy et al., 2010). Citrate capped Ag nanoparticles (Citrate–nAg) thus formed were characterized as described in the manuscript. The particles are known to be electrostatically stabilized by ionic citrate carboxyl groups (El Badawy et al., 2010, 2011, Kimling et al., 2006).

Synthesis of PVP–nAg: 5 mM AgNO₃ (50 mL) solution was mixed in a drop-wise fashion with 2 mM NaBH₄ containing 1% PVP solution. The NaBH₄ solution was ice-cold and was vigorously stirred during the reaction. The ratio of AgNO₃ to NaBH₄ was 1:3 (v:v) (El Badawy et al., 2011). PVP–nAg thus synthesized was characterized as explained in the manuscript.

Figure 2.8. Particle size distributions (PSD) of different types of metal-based nanoparticles suspended in moderately hard water (MHW; shown in pink) obtained using dynamic light scattering (DLS) method: (A) unclean Citrate-nAg, (B) clean Citrate–nAg, (C) PVP–nAg, (D) nZnO, (E) nTiO₂, and (F) nCdSe Quantum Dots suspended in toluene (shown in blue).
Table 2.2. Cleaning protocol applied for the purification of unclean Citrate–nAg using Tangential Flow Filtration (TFF) system.

<table>
<thead>
<tr>
<th>Purification of unclean Citrate–nAg</th>
<th>Electrical Conductivity (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Started Volume = 500 ml</td>
<td>1095</td>
</tr>
<tr>
<td>Ended Volume = 70 ml</td>
<td>1162</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>185</td>
</tr>
<tr>
<td>Ended Volume = 100 ml</td>
<td>283</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>36</td>
</tr>
<tr>
<td>Ended Volume = 75 ml</td>
<td>68</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>11</td>
</tr>
<tr>
<td>Ended Volume = 150 ml</td>
<td>20</td>
</tr>
<tr>
<td>Volume increased to 500 ml</td>
<td>5*</td>
</tr>
</tbody>
</table>

* obtained as clean citrate-nAg suspension with electrical conductivity of 5 µS/cm and was used for MetPLATE toxicity studies.
Table 2.3. Impact of nanoparticles dilution in moderately hard water (MHW) evaluated by measuring average hydrodynamic diameters (HDD). Data showed that dilution did not impact the characteristic of nanoparticles in the test matrix (MHW) as HDD remained mostly unchanged with dilution. SD, Standard deviation of the sample; x, dilution factor.

<table>
<thead>
<tr>
<th>Dilution factor (v:v)</th>
<th>Clean Citrate–nAg</th>
<th>PVP–nAg</th>
<th>nZnO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDD ± SD (nm)</td>
<td>% Volume</td>
<td>HDD ± SD (nm)</td>
</tr>
<tr>
<td>1x</td>
<td>11.0 ± 0.7</td>
<td>100</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>2x</td>
<td>10.9 ± 0.7</td>
<td>99.9</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>233 ± 30.5</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>5x</td>
<td>11 ± 0.7</td>
<td>99.8</td>
<td>17 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>249 ± 19</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>10x</td>
<td>11 ± 0.7</td>
<td>99.5</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>134.8 ± 2.1</td>
<td>0.5</td>
<td>83.4 ± 12.8</td>
</tr>
<tr>
<td>20x</td>
<td>10.9 ± 0.7</td>
<td>99.9</td>
<td>20.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>313.2 ± 33</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

* Volume weighted hydrodynamic diameter measured using DLS method.

Table 2.4. Impact of incubation time (3 h) and temperature (35 °C) on the stability of nanoparticles in the carrier medium, i.e., moderately hard water (MHW), evaluated by measuring average hydrodynamic diameter (HDD) and zeta potential.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Clean Citrate–nAg</th>
<th>PVP–nAg</th>
<th>nZnO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before incubation</td>
<td>After incubation</td>
<td>Before incubation</td>
</tr>
<tr>
<td>HDD ± SD (nm)*</td>
<td>11.0 ± 0.7</td>
<td>11.2 ± 1.3</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-25.13</td>
<td>-18.41</td>
<td>-10.67</td>
</tr>
</tbody>
</table>

* Volume weighted hydrodynamic diameter measured using DLS method, and all size measurements were 100% by volume.
Table 2.5. Exposure concentrations of different types of nanoparticles and their ionic counterparts to MetPLATE bacteria and their respective EC$_{50}$ values showing variation in the toxicity levels.

<table>
<thead>
<tr>
<th>Materials tested</th>
<th>Exposed concentrations (mg/L)</th>
<th>EC$_{50}$ ± S.D. (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean Citrate–nAg</td>
<td>92.05, 46.03, 4.6, 0.52, 0.05</td>
<td>5.79 ± 2.87</td>
</tr>
<tr>
<td>Unclean Citrate–nAg</td>
<td>41.0, 20.5, 2, 0.2, 0.02</td>
<td>4.17 ± 0.22</td>
</tr>
<tr>
<td>PVP–nAg</td>
<td>69.38, 34.69, 17.35, 1, 0.1, 0.01</td>
<td>0.80 ± 0.15</td>
</tr>
<tr>
<td>AgNO$_3$</td>
<td>5, 2.5, 1, 0.1, 0.05, 0.01</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>Permeate</td>
<td>65, 32.5, 16.25, 8.12, 4.06, 1, 0.1, 0.01 (µg/L)</td>
<td>Not Toxic</td>
</tr>
<tr>
<td>nZnO</td>
<td>10, 1, 0.5, 0.1, 0.05, 0.01</td>
<td>57.7 ± 5.84</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>10, 1, 0.5, 0.1, 0.05, 0.01</td>
<td>22.3 ± 14.8</td>
</tr>
<tr>
<td>nTiO$_2$</td>
<td>2500, 1250, 625, 312, 156, 78, 39, 10, 5, 1, 0.1, 0.05, 0.01</td>
<td>Not Toxic</td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>2500, 1250, 625, 312, 156, 78, 39, 10, 5, 1, 0.1, 0.05, 0.01</td>
<td>Not Toxic</td>
</tr>
<tr>
<td>CdSe QDs</td>
<td>100, 50, 10, 1, 0.1, 0.01</td>
<td>34.42%§</td>
</tr>
<tr>
<td>CdCl$_2$</td>
<td>100, 50, 10, 1, 0.1, 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>1-Octadecylamine</td>
<td>20000, 10000, 1000, 100, 10, 1, 0.1, 0.01</td>
<td>31.27%§</td>
</tr>
<tr>
<td>Polyvinylpyridolone</td>
<td>1.5 g/L</td>
<td>Not Toxic</td>
</tr>
<tr>
<td>Na citrate dihydrate</td>
<td>10 mM</td>
<td>Not Toxic</td>
</tr>
</tbody>
</table>

§ Toxicity was tested by dispersing Quantum Dots (QD) in moderately hard water (MHW) because toluene was found to be incompatible with 96-well plate material when used as a solvent for QDs dispersion, so the data reported are average inhibition of MetPLATE bioassay on exposure to QDs suspended in MHW; S.D., standard deviation of the triplicate runs.
Figure 2.9. Schematic of MetPLATE protocol (Adapted from Bitton et al., 1994).

References Cited in Appendix


Natural Water Chemistry (Dissolved Organic Carbon, pH, and Hardness) Modulates Citrate-coated Silver Nanoparticle Stability, Dissolution Rate, and Antibacterial Activity

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ABSTRACT: Understanding of how natural water chemistry influences silver nanoparticles’ (AgNP) fate, dissolution rate, and toxicity should allow making valued judgment about the potential ecological risk of AgNPs. Herein we investigate and report the results of modulating effects of multiple water chemistry (i.e., dissolved organic carbon (DOC), pH, and hardness) on the colloidal stability, ion release rate, and antibacterial activity of citrate-coated silver nanoparticles (Citrate–AgNP) against Escherichia coli. Concomitant assessment of AgNO₃ toxicity under variable water chemistry allowed comparison with Citrate–AgNPs, and in a representative surface water collected across three seasons. Detectable changes in the hydrodynamic diameter, zeta potential, and surface resonance revealed modulating effects of water chemistry on the colloidal stability of Citrate–AgNPs. Although, overall Ag release rate was low (0.33–3.62%), it increased with increasing DOC concentrations (0–20 mg/L) but decreased with increasing hardness (150–280 mg/L as CaCO₃) and pH (5–7.5). While Ag dissociated from Citrate–AgNPs was inadequate to fully account for nanotoxicity, Citrate–AgNPs showed ca 3 – 44 times lower toxicity than AgNO₃ (total Ag basis). Notably, higher DOC or pH conferred protection to E. coli against Citrate–AgNPs and Ag⁺; increasing solution hardness tended to enhance toxicity, however. Citrate–AgNPs or AgNO₃ toxicity in the river water matrix revealed no seasonality and was comparable to the control. Generalized linear models developed, by parameterizing particle properties, could fairly predict experimentally
derived nanotoxicity. As an indirect evidence of silver internalization causing intracellular β-Galactosidase inhibition in *E. coli* under prevailing natural water conditions, this study reaffirms the usefulness of this high-throughput bioassay for probing nanoparticle toxicity in aquatic system. Our data show that particle size, surface characteristics, and ion release kinetics of AgNPs can be modified upon release into aquatic environment, suggesting potential implications to ecosystem health and functions.

**INTRODUCTION**

As nanotoxicology is yet to mature as much as nanotechnology has, research efforts to better understand whether engineered nanomaterials (ENM) pose hazards upon exposure are ongoing. Some recent studies employing *in vitro* or *in vivo* models, however, indicate potential environmental, health, and safety (EHS) effects of ENMs. Because ENMs are integral elements of hundreds of consumer and commercial products, including the toothpaste, soap, sunscreen lotion, clothing, medical masks, plastic wares, electronics, cement, paint, etc., the likelihood of ecological exposure to these nanoproducts vis-à-vis their leachable by-products (i.e., as nanoparticles, their aggregates, or ions) is inevitable.

Because the wet chemical processes are commonly used for ENM synthesis, precursor chemical molecules, capping agents, and left-over reducing agents are likely present in the nanosuspension. Whether the observed toxicity is due to the combined effects of impurities, released ions, and/or colloidal particles in the exposure medium, if it is due to the change in particle size and/or surface characteristics, or if interactive effects of particle size, surface charge and the amount of released ions occur during the experimental period are premises generally less well explored or understood.
Elucidating factors playing a significant role in ENM stability and potential toxicity have offered a challenging opportunity for nanoresearch community.\textsuperscript{10,14} Interactions with the natural colloids vis-à-vis with a multitude of environmental factors such as pH, monovalent and divalent cations, background electrolyte types,\textsuperscript{16,17} and natural organic matters (e.g., humic acid/fulvic acid)\textsuperscript{18,19} could modify colloidal stability of nanoparticles following their entry into aquatic systems by modulating particle size, surface characteristics, and mobility,\textsuperscript{16,20-23} which could subsequently influence the toxicity.\textsuperscript{9,10,19,24} Only a few studies have assessed the potential fate and toxicity of silver nanoparticles (AgNP), one of the widely sought ENMs for broad spectrum antatimicrobial and plasmonic properties,\textsuperscript{8,11} under multiple water chemistry conditions such as variable pH, monovalent and divalent cations, background electrolyte types,\textsuperscript{16,17,20} or natural organic matter (e.g., humic acid/fulvic acid).\textsuperscript{18,19} Often results are confounded by multiple parameters such as the particle size, surface coating, or the toxicity media used\textsuperscript{8,25} perhaps due to the less routine use of statistical methods which could parameterize multiple variables to appropriately model their effects and allow us in explaining the potential contribution of each variable to nanotoxicity.\textsuperscript{14,26}

In this study, we aim to understand the potential modulating effects of multiple water chemistry conditions on the colloidal stability, dissolution rate, and antibacterial activity of the model citrate-coated silver nanoparticles (Citrate–AgNP) against Gram-negative \textit{Escherichia coli} in the aquatic media. To this end, potential changes in particle stability are investigated by measuring the hydrodynamic diameter (HDD), zeta (\(\zeta\)) potential, and the surface Plasmon resonance of Citrate–AgNPs as a function of dissolved organic carbon (DOC), pH, and hardness; two (of the three) natural water parameters were held constant while evaluating the effect of the third. Rate of Ag released under variable DOC, pH, and hardness conditions are evaluated to
discern potential contribution of the released Ag$^+$ on the toxicity under the experimental conditions. Finally, the effects of variable DOC, pH, and hardness conditions on the stability and Ag dissociation rates are used to explain the toxicity of Citrate–AgNPs using the previously reported high-throughput β-Galactosidase (β-Gal, hereafter) E. coli bioassay. The toxicity of AgNO$_3$, as an ionic source of free Ag$^+$, is assessed concurrently under variable DOC, pH, and hardness conditions, which allowed for mass-based comparison of toxicity between Citrate–AgNP versus free Ag$^+$ (as AgNO$_3$). The toxicity of Citrate–AgNPs and AgNO$_3$ is also assessed in the representative river water samples collected across three seasons (for seven months). Employing the Generalized linear model, we also probe and quantify the effects of particle properties to explain nanotoxicity. Herein, we systematically show that organic carbon, pH, and hardness in the natural waters could significantly influence AgNP characteristics and alter its toxicity against E. coli.

**MATERIALS AND METHODS**

**Nanoparticle Synthesis and Purification.** Citrate-coated silver nanoparticles (Citrate–AgNP) were synthesized following the previously established procedure (details in Appendix B).$^{16}$ Purification of Citrate–AgNP was performed using polysulfone (PS) 10 kD hollow fiber membranes (P/N: X31S-300-02P, surface area = 145 cm$^2$) connected to Kros Flo Research IIi tangential flow filtration (TFF) system, and controlled by KF COMM data collection software (Spectrum Laboratories, CA, USA). The details of TFF procedure have been described previously (Appendix Figure 3.9, Table 3.1).$^7,8$ The purified sample was digested using the standard USEPA method 3050B, and Ag concentration in stock nanosuspension was determined (as total Ag) using flame-atomic absorption spectroscopy (AAS).
Nanoparticle Characterization. Citrate–AgNPs were tested for the volume-weighted hydrodynamic diameter (HDD) and zeta ($\zeta$) potential using the dynamic light scattering (DLS) method prior to and post purification. Calibration of the particle sizer/zeta potential unit (PSS NICOMP Particle Sizing Systems, CA) at 23 °C using Duke 500 (491 nm) NIST 3490A standard (PSS Nicomp, FL, USA) preceded sample measurements for particle size and surface charge. The SPR (surface Plasmon resonance) spectra were recorded for Citrate–AgNPs using UV/vis spectrophotometer (Shimadzu PharmaSpec UV-1700). A transmission electron microscope (Philips EM 420) was operated at 120 kV in the bright-field mode to visualize particle morphology. ImageJ 1.44 software\textsuperscript{27} was used to estimate particle circularity and size distributions (PSD) from the representative TEM imageries.

Toxicity Bioassay. The toxicity of biolabile Citrate–AgNPs or Ag\textsuperscript{+} (as AgNO\textsubscript{3}) was measured as $\beta$-Gal ($\beta$-Galactosidase) activity inhibition in \textit{E. coli}.\textsuperscript{8,28} The $\beta$-Gal bioassay involves an hydrolysis of glycosidic bond of chlorophenol-red $\beta$-galactopyranoside (CPRG), a chromogenic substrate, which leads to the formation of galactopyranose and chlorophenol red as the reaction by-products. Colorimetric quantification of chlorophenol red at 570 nm in a 96 well-plate using a Multiskan microplate reader offered an estimate proportional to the activity of intracellular $\beta$-Gal.\textsuperscript{8,28} One mg Cu\textsuperscript{2+} L\textsuperscript{-1} (as CuSO\textsubscript{4}) and moderately hard water (MHW, as Evian water) represented the positive and negative controls, respectively, for each set of analysis. At least triplicate samples were analyzed for each test concentration. The incubation temperature and time required for $\beta$-Gal assay are 35 °C and 3 – 4 h, respectively; the details of $\beta$-Gal assay are previously described (schematics shown in Appendix Figures 3.11 and 3.12).\textsuperscript{8} The bacterial reagent and CPRG were procured through M2B Research & Innovative Technologies, LLC, Gainesville, FL.
Preparation of Test Solutions and Exposure Conditions. Leonardite humic acid (LHA, International Humic Substances Society (IHSS); Cat # 1S104H-5) was prepared in MHW (as Evian Water) stirring overnight, and filtered under vacuum through a 0.45 µm pore size filter (Millipore). Following the method SM 5310C coupled with persulfate-UV oxidation procedure, the filtrate was analyzed to determine dissolved organic carbon (DOC) concentration. Concurrently, multiple concentrations of DOC were verified by developing a calibration curve using UV-vis absorbance at 280 nm (Appendix Figure 3.10), which offers meaningful information on the aromaticity and molecular weight of the humic acid molecules. The solution was then stored at 4 °C in dark until use. Detailed chemical characteristics of this LHA are documented by Thorn et al.31

Potential intracellular inhibition of β-Gal activity in E. coli upon exposure to Citrate–AgNPs or free Ag⁺ (as AgNO₃) was investigated under various water chemistry conditions, i.e., under different solution pH, hardness, and DOC concentrations. Because the narrow pH range of 5 – 7.5 is recommended for the bioassay, four different pH values (i.e., 5, 6, 7, and 7.5) within the specified pH range were applied to test the effects of pH on Citrate–AgNP stability, rate of ion release, and subsequently on toxicity. The test solution hardness (280 mg/L as CaCO₃) and DOC concentrations (2 mg/L) were held constant while testing for the effects of pH. Solution pH was adjusted using a dilute solution of HNO₃ or NaOH, and measured using a Hanna instruments multiparameter meter 9828 (Hanna Instruments, Michigan). Using nanopure water, the hardness of Evian water (280 mg/L as CaCO₃) was adjusted to obtain the desired hardness of 280, 250, 200, and 150 mg/L, which was analytically confirmed by the standard EDTA titration method (Hach method 10247). Potential changes in colloidal stability, rate of ion release, and toxicity of Citrate–AgNPs were evaluated using this differentially hard water while maintaining the pH.
(neutral) and DOC (2 mg/L) constant; 2 mg/L DOC is representative of the natural water samples collected from the Watauga River, near Elizabethton, TN (36.3339 °N, -82.2704 °W). Toxicity was also evaluated using the Watauga River water matrix sampled four times over the period of seven months for comparison. To assess the impacts of DOC concentration on Citrate–AgNP stability and antimicrobial activity, five different DOC concentrations (0, 2, 5, 10, and 20 mg/L) were applied while maintaining pH 7 and hardness 280 mg/L (as CaCO₃). A minimum of triplicate samples were tested for particle stability and enzyme activity tests. Potential changes in HDD and ζ potential measured under the multiple water chemistry conditions using the DLS method were used to characterize particle stability in the test media,¹⁷,²⁰,³² which was further compared with the likely changes observed in the corresponding SPR spectra.³² For this, Citrate–AgNPs were incubated at 35 °C for 4 h (conditions required for β-Gal bioassay) under variable DOC, hardness, and pH levels before the DLS or UV-vis measurements were recorded. Triplicate samples were run for the DLS measurements and the data are reported as an average ± 1 standard deviation.

**Ion Release Rate under Variable Water Chemistry Conditions.** Separate experiments were conducted in duplicate to determine the rates of dissolved Ag released from the colloidal Citrate–AgNP suspension under variable water chemistry conditions as stated earlier. For this, 25 mL of Citrate–AgNPs (10 mg/L) were incubated at 35 °C for 4 h under a range of DOC concentrations, hardness, and pH levels as used in toxicity tests. Each sample was then ultracentrifuged (Thermo Scientific Sorvall WX Ultra Series Centrifuge SN# N13V-427288-NV) at 45,000 rpm (205,835 g at the bottom of the bottle; 146,347 g at the middle of the bottle; and 86,858 g at the topmost part of the bottle) in a polycarbonate bottle (Thermo Scientific Cat# 314348) for an hour; this followed pipetting out of 3 mL of clear supernatant, which was soon digested with equal volume
of conc. HNO₃ (trace metal grade) before the total Ag concentration would be determined using the Graphite Furnace-AAS.

**Statistical Analysis.** The EC₅₀ (i.e., effective concentrations for 50% enzyme activity inhibition) values were estimated using the linear regression analysis. Potential toxicity of Citrate–AgNPs or free Ag⁺ are reported for different water chemistry conditions as an average EC₅₀ obtained from at least triplicate test runs. One-way analysis of variance (ANOVA) was used to test for significant difference between the sample EC₅₀s and the controls, followed by the Dunnett t-test (post-hoc) for multiple comparisons. Generalized linear models were developed to probe and quantify the main and interactive effects of particle properties under the variable DOC, pH, and hardness conditions to explain the observed toxicity. Model deviance value was compared among the models to test the goodness of fit of the final model based on the information criteria that ‘small-is-better’. Significant difference was established at the $p \leq 0.05$ using an IBM SPSS ver. 20.33

**RESULTS AND DISCUSSION**

**Characteristics of Citrate–AgNPs.** The synthesized Citrate–AgNPs (both before and after purification) had an average HDD of ~11 nm and the TEM diameter of 56.5 nm (SD = 19.2 nm; n = 208 particles; Appendix Figure 3.8A,B,D). Because average ζ potentials remained fairly similar before (~21.43 mV) and after (~25.13 mV) purification, it showed that the particles remained unchanged during purification using the TFF system and were stably dispersed in the suspension, which is in agreement with the particles visible on the TEM imagery (Appendix Figure 3.8A). As predicted by Mie theory, its characteristic SPR peak was observed at 445 nm (Appendix Figure 3.8C). Following purification, Citrate–AgNPs were found to be of high circularity of 0.88 and purity; the latter verified by the change in electrical conductivity (from
initial 1095 µS/cm to final 5 µS/cm),\textsuperscript{8} a significantly lower amount of dissolved Ag (~65 µg/L as total Ag) quantified in the permeate (wastewater),\textsuperscript{8} and the X-ray photoelectron spectroscopy and nuclear magnetic resonance analyses by El Badawy et al.\textsuperscript{9}

**Modulation of AgNP Stability by DOC, pH, and Hardness.** Inter-particle bridging of AgNPs with humic acid molecules,\textsuperscript{35} particularly at higher DOC concentrations, resulted in significantly larger particles as shown by the HDDs (Figure 3.1A) vis-à-vis a gradual decrease in ζ potentials with increasing DOC concentrations as confirmed by the DLS measurements (Figure 3.1B). This corresponds with the increase in absorbance and broadening, with slight blue-shift, of the SPR spectra of Citrate–AgNPs with increasing DOC concentrations (Figure 3.2A). These data together suggest that Citrate–AgNPs were less stable following their interactions with humic acid molecules, thus indicative of potential agglomeration or sedimentation of the particles.

Although not statistically significant, increasing solution pH (range 5 – 7.5) tended to slightly decrease particle size (Figure 3.1C), which corresponded to no significant change in ζ potential in the pH range 5 – 7 (Figure 3.1D).\textsuperscript{9} However, at pH 7.5 the surface charge of Citrate–AgNP significantly declined ($p \leq 0.0001$) and reached to circumneutral, on average (ζ potential = –0.35 mV; Figure 3.1D). Overall, the DLS data are consistent with the UV-vis data for absorbance, which revealed no change in SPR peak and an absence of blue/red shift of the Plasmon spectra (Figure 3.2B), suggesting an absence of potential agglomeration or sedimentation of the particles under the measured pH range.\textsuperscript{20,32}

With increasing solution hardness (150 – 280 mg/L as CaCO$_3$), average particle diameter (HDD) showed a decreasing, but not significantly different ($p > 0.1$), trend (Figure 3.1E). In agreement were the ζ potential values which did not change on average, but to our surprise it significantly decreased to –3.07 mV at solution hardness of 250 mg/L (as CaCO$_3$; Figure 3.1F).
Figure 3.1. Effects of variable water chemistry conditions on the (A, C, E) hydrodynamic diameter (HDD) and (B, D, F) surface charge (as zeta potential) of Citrate–AgNPs. To obtain reliable DLS measurements for the HDD and ζ potential, 9.1 mg/L citrate-AgNP was used under a range of water chemistry conditions. DOC, dissolved organic carbon. ‘*’ denotes $p \leq 0.01$; ‘**’ denotes $p \leq 0.001$; and ‘***’ denotes $p \leq 0.0001$ as compared to the baseline of 0 mg DOC/L, pH 7, and 280 mg hardness/L (as CaCO$_3$); significant difference was analyzed by ANOVA followed by Dunnett t (posthoc) test. Two (of the three) parameters were held constant while evaluating the effects of the third.
Figure 3.2. Potential changes in the surface Plasmon resonance (SPR) spectra of Citrate–AgNPs in the presence of variable (A) concentrations of dissolved organic carbon (DOC), (B) pH, and (C) hardness (mg/L as CaCO$_3$). Two (of the three) parameters were held constant while evaluating the effects of the third.
No change in SPR peak or UV-vis absorbance in the measured hardness range (Figure 3.2C) is suggestive of stable particle persistence in the test conditions.

**Modulation of Ion Release Rate by DOC, pH, and Hardness.** Figure 3.3 shows Ag ion released or dissociated from Citrate–AgNPs under different water chemistry conditions and within the incubation period of 4 h. The dissociation rate increased as a function of DOC concentrations; it increased to nearly 5 times at 20 mg/L DOC concentration compared to the one without added DOC (Figure 3.3A). Increasing solution hardness or pH, however, caused a decrease in Ag ion release (Figs. 3.3B,C). Ag ion release rate was about 3.5 times greater at solution hardness 150 mg/L (as CaCO₃) than at 280 mg/L (as CaCO₃). At pH 5, this rate was 1.5 times greater than at pH 7.5 (Figure 3.3C). Under the tested water chemistry conditions relevant to the freshwater environments, overall Ag ion release was in the narrow range 0.33 – 3.62 %

![Figure 3.3. Potential ion release rates (measured as total Ag) of Citrate-AgNP under variable water quality conditions: (A) different concentrations of dissolved organic carbon (DOC), (B) different hardness (mg/L as CaCO₃), and (C) different pH. Two (of the three) parameters were held constant while evaluating the effects of the third.](image)

(Figure 3.3). Only 0.75 % (75.1µg/L) of Ag was released from the stock Citrate-AgNP suspension. The results reported herein are consistent with the previous studies on similarly coated AgNPs.¹⁵,¹⁷,¹⁸,³⁶,³⁷ There was no detectable amount of Ag in 20 mg/L DOC when tested alone as the absorbance (using GF-AAS) for Ag was an order of magnitude lower (0.012 au)
than that of the method blank (0.10 au), confirming that the source of released Ag under various DOC concentrations was Citrate–AgNPs, not the DOC (LHA) itself. Ag recovery was in the range 104.8 – 112.4 % for GF-AAS.

**Modulation of AgNP toxicity by DOC, pH, and Hardness.** A concentration-dependent antibacterial activity (revealed by β-Gal inhibition in *E. coli*) was observed for both the Citrate–AgNPs and free Ag⁺ (as AgNO₃; Figure 3.4A,B). Potential antibacterial activity under the conditions: pH 7, hardness 280 mg/L (as CaCO₃), and without added DOC are considered baseline for comparison. The baseline EC₅₀ for Citrate–AgNPs was 5.79 mg/L (as total Ag) versus 0.36 mg/L (as total Ag) for free Ag⁺ (as AgNO₃) – an evidence that shows lower (by 16 times) toxicity of Citrate–AgNPs compared to that of free Ag⁺.

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 3.4.** Concentration-dependent inhibition of β-Galactosidase activity in *Escherichia coli* upon exposure to (A) Citrate–AgNPs or (B) dissolved Ag⁺ ions (used as AgNO₃). Error bars represent ± 1 standard deviation (SD) of at least triplicate test runs.

Toxicity of Citrate–AgNPs and free Ag⁺ (as AgNO₃) in Watauga River water matrix was also measured over the period of seven months (at four different times) and was not significantly
different among themselves (ANOVA: \( p > 0.5 \); Figure 3.5) or with the baseline control using moderately hard water (MHW, as Evian water with DOC = 2 mg/L, pH = 7, and hardness = 280 mg/L as CaCO\(_3\); ANOVA: \( p > 0.5 \)) for Citrate–AgNPs or free Ag\(^+\) (Figure 3.6). Evaluation of this natural water samples over the course of seven months showed comparable physicochemical properties (Appendix Table 3.6), which could be attributed to the observed similarity in EC\(_{50}\) values for both the Citrate–AgNPs and free Ag\(^+\) assessed at different time periods with the toxicity measured in MHW (DOC = 2 mg/L, pH = 7, and hardness = 280 mg/L as CaCO\(_3\)) considered a baseline control. It is important to note the limitation of using natural water matrix owing to the fact that its composition and other physicochemical characteristics generally vary spatially and temporally within and between the aquatic systems.\(^{38}\) Here, the purpose of using natural water matrix was to investigate how the toxicity of colloidal Ag and its free ions vary in the matrix representative of natural surface water system and how that compares with the toxicity in the laboratory water. No seasonal variation in antibacterial activity of Citrate–AgNPs or free Ag\(^+\) was observed; therefore, in this case the synthetic laboratory water (MHW) could adequately capture the toxicity of Citrate–AgNPs or free Ag\(^+\).

Increasing DOC concentrations resulted in a significant decrease in antibacterial activity (evidenced by linearly increasing EC\(_{50}\) values) for both the Citrate–AgNPs and free Ag\(^+\) (as AgNO\(_3\); \( p \leq 0.01 \); Figure 3.6A,B). On the basis of total Ag mass, comparison of the EC\(_{50}\) values under different DOC concentrations revealed Citrate–AgNPs to be 3 – 16 times less toxic than the free Ag\(^+\) (as AgNO\(_3\); Figure 3.6A,B).

A significant decrease in antibacterial activity was also observed with increasing pH values (range 5 – 7.5) for both the Citrate–AgNPs and free Ag\(^+\) (as AgNO\(_3\); Figure 3.6C,D). At pH 5, the toxicity of Citrate–AgNP was 3.2 and 13.8 times higher than at pH 7 and 7.5, respectively.
For dissolved Ag\(^+\) (as AgNO\(_3\)), the toxicity at pH 5 was 6.8 and 6.4 times higher than at pH 7 and 7.5, respectively. Comparison of EC\(_{50}\) values in the measured pH range showed about 10 – 44 times lower antimicrobial activity of Citrate–AgNPs than of the free Ag\(^+\) (as AgNO\(_3\); Figure 3.6C,D). Significant attenuation of Citrate–AgNPs or free Ag\(^+\) toxicity with increasing pH (5 – 7.5) can be attributed to increasing OH\(^-\) concentrations, which could compete for the prevalent binding sites on the cell-surface,\(^9\) thus lowering potential for Ag bioavailability and reducing the toxicity. Although a linear decline in the rate of Ag release was evident with increasing pH, the amount released in this study was not enough to cause 50% β-Gal activity inhibition.

![Figure 3.5](image.png)

**Figure 3.5.** Toxicity of Citrate–AgNPs and free Ag\(^+\) ions (as AgNO\(_3\)) to *E. coli* in Watauga River water matrix measured over the period of seven months covering three seasons. EC\(_{50}\)s over the course of seven months were not significantly different from each other (ANOVA: \(p > 0.5\)) or with the baseline control using moderately hard water (MHW, as Evian water with DOC 2 mg/L, pH7, and hardness 280 mg/L as CaCO\(_3\); ANOVA: \(p > 0.5\)) for both the Citrate–AgNPs and Ag\(^+\) ions. The sample collection dates (mm/dd/yy) are shown on X-axis.

Although hardness has been known to confer protective effects against the toxic metals in the natural waters,\(^{39-42}\) a study by Ryan et al.\(^{43}\) contradicts this showing greater toxicity of copper with increasing hardness. With increasing solution hardness, the toxicity tended to increase.
Figure 3.6. Effects of water chemistry on the toxicity of (A, C, E) Citrate–AgNPs or (B, D, F) dissolved Ag\(^+\) ions (used as AgNO\(_3\)) measured as inhibition of \(\beta\)-Galactosidase activity in Escherichia coli. Error bars represent ± 1 standard deviation (SD) of at least triplicate test runs. Note, higher EC\(_{50}\) (mg/L as total Ag) values denote lower toxicity and vice-versa. DOC, dissolved organic carbon. ‘*’ denotes \(p \leq 0.01\); ‘**’ denotes \(p \leq 0.001\); and ‘***’ denotes \(p \leq 0.0001\) as compared to the baseline of 0 mg DOC/L, pH 7, and 280 mg hardness/L (as CaCO\(_3\)); significant difference was analyzed by ANOVA followed by Dunnett t (posthoc) test. Two (of the three) parameters were held constant while evaluating the effects of the third.
(lower EC\textsubscript{50}s) for Citrate–AgNPs and Ag\textsuperscript{+} (as AgNO\textsubscript{3}; Figure 3.6E,F). At lower hardness (150 mg/L as CaCO\textsubscript{3}), Citrate–AgNP toxicity was 4.4 times lower than at higher hardness (280 mg/L as CaCO\textsubscript{3}). For Ag\textsuperscript{+}, the toxicity was not significantly different between 150 mg hardness/L and 280 mg hardness/L (as CaCO\textsubscript{3}; \( p > 0.05 \)). For zerrovalent Ag (Citrate–AgNP), an assessment of EC\textsubscript{50} values did not support the premise that the competing Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions and different carbonate (CO\textsubscript{2}, HCO\textsubscript{3}\textsuperscript{−}, CO\textsubscript{3}\textsuperscript{2−}) and noncarbonate (NCH) species present in the hard water attenuate its toxicity. Instead, the toxicity increased significantly when the hardness increased from 150 mg/L to 200 mg/L (Figure 3.6E), suggesting that an increased hardness might have promoted Ag biouptake, thereby leading to greater inhibition of intracellular \( \beta \)-Gal activity with Citrate–AgNP treatment. At higher hardness, however, the toxicity of Citrate–AgNPs leveled off (Dunnett t test, \( p > 0.05 \); Figure 3.6E); using the Kruskal-Wallis test to adjust for unequal sample size and variance did not change the statistical significance for both the Citrate–AgNPs and Ag\textsuperscript{+}.

It is possible that the small sample size might explain some of the differences observed. Mass-based (as total Ag) comparison of the EC\textsubscript{50} values under the tested hardness range showed about 10 – 43 times lower toxicity of Citrate–AgNPs than of Ag\textsuperscript{+} (as AgNO\textsubscript{3}).

A significant change in zeta potential (toward positive value) at pH 7.5 or at hardness 250 mg/L (as CaCO\textsubscript{3}) might be explained by adsorption of free Ag\textsuperscript{+} on the surface of AgNPs. Dissolution profiles of AgNPs as shown in Figure 3.3 support the earlier supposition as measurably less dissolved Ag was detected in the supernatant at pH 7.5 or at hardness 250 mg/L. The forms (species) in which Ag might be present in the dissolved form in the test suspension remained unclear and, therefore, if more free Ag\textsuperscript{+} were available at pH 7.5 or at hardness 250 mg/L (as CaCO\textsubscript{3}) awaits studies evaluating the fate and speciation of AgNPs in aqueous test matrices as applied in this study.
Generally, smaller size AgNPs are known for their lower redox potentials which facilitate higher oxidative dissolution than the larger size AgNPs or other forms of bulk Ag. Because greater dissolution occurred under higher DOC concentrations, it implies that the higher aromaticity ($f_a = 0.58$; the value determined from quantitative C-13 nuclear magnetic resonance spectra by Thorn et al., using a ratio of spectrum area from 65 – 110 ppm to total spectrum area) of the LHA molecule (compared to several other humic and fulvic acids standards and reference chemicals, including the Suwanee River humic acid [for SRHA $f_a = 0.37$], commercially sold by IHSS and well-characterized by Thorn et al.) might have offered more reactive sites promoting oxidative dissolution of Citrate–AgNPs, despite some aggregation that might have occurred as shown by the DLS measurements of the HDDs and ζ potentials (Figure 3.1A,B), including the SPR peaks that blue-shifted (Figure 3.2A). Consistent with the LHA’s higher aromaticity and reactivity as observed in this study, Fukushima et al. demonstrated enhanced reactivity of the humic acid molecules with highest aromaticity. The wide differences in the molecular characteristics of humic substances are primarily due to the nature of precursor compounds and the environmental conditions which supported their formation. A previous study by Liu and Hurt, in contrast to our results, reported a linear decrease in rates of Ag release from their Citrate-coated AgNPs (synthesized by different method than ours) upon exposure to increasing concentrations of added natural organic matter (i.e., SRHA and SR fulvic acid) in a 1-day experiment. These contrasting differences in the results can be attributed to the distinct differences in the molecular structures, offering variability in aromatic and/or aliphatic characteristics, of the LHA versus the SRHA or SRFA (for SRFA $f_a = 0.24$ as reported in ref. 31) used in the previous study.
The greatest amount of dissolved Ag was released from Citrate–AgNPs under the highest DOC (20 mg/L) concentration; the former being equal to the EC$_{50}$ value for free Ag$^+$ (as AgNO$_3$, but without DOC).$^8$ Comparison of free Ag$^+$ toxicity under added DOC concentrations (2 – 20 mg/L) showed significant attenuation of toxicity by DOC addition, which was 12.5 times lower at 20 mg/L DOC than one without added DOC (Figure 3.6B). This strongly suggests that the released Ag$^+$ under the experimental conditions did not fully account for the observed Citrate–AgNPs toxicity,$^7,48$ 16 times less toxic Citrate–AgNPs turned to have 37 times lower toxicity than free Ag$^+$ when 20 mg/L DOC was added to the nanosuspension (Figure 3.6A,B). Hence, clearly DOC conferred strong protective effects not only against exposure to Citrate–AgNPs evaluated herein, but also against the free Ag$^+$ (as AgNO$_3$) tested. The solution containing (i) 20 mg/L DOC (with pH 7 and hardness 280 mg/L as CaCO$_3$), (ii) pH 7.5 (with 2 mg/L DOC and hardness 280 mg/L as CaCO$_3$), and (iii) hardness 150 mg/L as CaCO$_3$ (with 2 mg/L DOC and pH 7) appeared to be the media that conferred highest mitigating effects against Citrate–AgNPs (Figure 3.6A). A previous study has reported that in an anaerobic environment released Ag$^+$ effects were more prominent than that of AgNPs tested.$^{10}$ Under an aerobic environment with multiple water quality characteristics investigated herein, the effect of released Ag$^+$ from Citrate–AgNP suspension was rather less prominent and, interestingly, our evidence of lower toxic potency of Citrate–AgNPs compared to dissolved Ag$^+$ (either released from AgNPs or added as free Ag$^+$, the latter source being AgNO$_3$) clearly shows protective effects of Citrate–AgNP suspension (Citrate–AgNPs combined with released Ag ions) to that of its ionic counterpart alone. Potential adsorption of DOC molecules onto Citrate–AgNP surface and subsequent modification of Citrate–AgNP size and surface properties might explain its lower antibacterial activity. It is very likely that the released Ag$^+$ and/or Citrate–AgNPs were prevented from uptake
by the *E. coli* cells due to DOC-AgNP-Ag\(^+\) complex formation, likely resulting into reduced biouptake and evidently lower toxic outcome.

Comparison of Citrate–AgNPs’ surface charge at pH 7.5 (\(\zeta\) potential = \(-0.35 \pm 1.14\) mV) to that of *E. coli* cells (in Evian water, \(\zeta\) potential = \(-11\) mV) suggests an occurrence of dominant attraction force between the Citrate–AgNPs and the bacterial cell-surface as compared to under other (lower) pH values tested. According to a previous study,\(^4\) the greater cell-surface interactions that might occur at nano-bio interface at pH 7.5 should have led to higher toxicity due to potential physical contact; which, to our surprise, the results do not support the premise as lowest toxicity was observed at pH 7.5 than at other pH environments (Figure 3.6C). Alternately, increased OH\(^-\) ions at pH 7.5 might have favored competitive binding of, and subsequently low internalization of, Citrate–AgNPs and/or released Ag\(^+\) and therefore lower toxicity. At hardness 250 mg/L (as CaCO\(_3\)), unlike other degree of hardness investigated, circumneutral surface charge (\(\zeta\) potential = \(-3.07 \pm 2.58\) mV) of Citrate–AgNPs also corresponded to slightly lower toxicity compared to the hard water with 200 or 280 mg/L as CaCO\(_3\) (Figure 3.6E). A schematic summarizing variable exposure conditions, surface modifications, ion release, and toxicity of Citrate–AgNPs in *E. coli* is illustrated in Figure 3.7.

Using the Generalized linear model (GLM), we probed and quantified the main and interactive effects of particle properties under variable test conditions to explain the observed toxicity of Citrate–AgNPs. Modeling the changeable particle properties data synthesized under a range of DOC concentrations, as presented in equation (i), our GLM predicted significant main and interactive effects of HDD, \(\zeta\) potential, and Ag release rate % (Ag\(_{\text{diss}}\)) explaining the nanotoxicity (as EC\(_{50}\)(DOC)). Under variable pH environments, however, only the HDD showed a significant main effect, while the two-way interactive effects of HDD and \(\zeta\) potential, and HDD
and $\text{Ag}_{\text{diss}}$, including the three-way interactions among HDD, $\zeta$ potential and $\text{Ag}_{\text{diss}}$ could significantly explain the observed nanotoxicity (as $\text{EC}_{50}(\text{pH})$, equation ii). Interestingly, for the variable hardness conditions both HDD and $\text{Ag}_{\text{diss}}$ showed significant main effects, including the significant interactive effects of HDD, $\zeta$ potential, and $\text{Ag}_{\text{diss}}$, to predict Citrate–AgNP toxicity (as $\text{EC}_{50}(\text{Hardness})$, equation iii). $\varepsilon_{i}$, $\varepsilon_{ii}$ and $\varepsilon_{iii}$ are the respective error terms of the models representing any variance unaccounted for by each model. The detailed model effects and their parameter estimates are presented in Appendix Tables 3.2 – 3.4.

$$\text{EC}_{50}(\text{DOC}) = 0.157 \times \text{HDD} - 0.25 \times \zeta \text{ potential} + 2.815 \times \text{Ag}_{\text{diss}} + 0.005 (\text{HDD} \times \zeta \text{ potential} \times \text{Ag}_{\text{diss}}) + \varepsilon_{i}$$. \hspace{1cm} (i) 

$$\text{EC}_{50}(\text{pH}) = 10.995 \times \text{HDD} + 0.819 (\text{HDD} \times \zeta \text{ potential}) - 8.992 (\text{HDD} \times \text{Ag}_{\text{diss}}) - 0.691 (\text{HDD} \times \zeta \text{ potential} \times \text{Ag}_{\text{diss}}) + \varepsilon_{ii}$. \hspace{1cm} (ii) 

$$\text{EC}_{50}(\text{Hardness}) = -0.621 \times \text{HDD} + 32.049 \times \text{Ag}_{\text{diss}} - 0.057 (\text{HDD} \times \zeta \text{ potential} \times \text{Ag}_{\text{diss}}) + \varepsilon_{iii}$$. \hspace{1cm} (iii) 

Figure 3.7. Schematic depicting variable exposure scenarios, surface modifications, ion release rate, and toxicity of Citrate–AgNPs in *E. coli*. 

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Generally, the interaction term’s (HDD x ζ potential x Ag\textsubscript{diss}) contribution to the models (equations i - iii) was relatively smaller, although statistically significant, as depicted by their smaller coefficient values (Appendix Tables 3.2 – 3.4). The precision of the models to correctly predict Citrate-AgNP toxicity under variable test conditions was calculated using the equation:\textsuperscript{14}

\[
% \text{Precision} = 100 \left( \frac{\text{GLM-predicted } EC_{50}}{\text{Experimental } EC_{50}} \right)
\]

and the results are presented in Appendix Table 2.5. Under variable DOC concentrations, our model precision was in the range 88.3 – 105.4 %, suggesting the model’s ability to effectively predict AgNP toxicity. Under different pH conditions, however, the model precision ranged from 75.8 – 112.4 %, and for variable hardness the model was less precise to predict the observed toxicity of Citrate–AgNPs (Appendix Table 3.5), which could possibly be due to (i) larger standard error of the means associated to Ag dissociation rate % (Appendix Table 3.4), and (ii) the inherent complexity and heterogeneity regarding the water quality encountered in natural systems, as evaluated in this study particularly under the range of pH and hardness conditions, which cannot be adequately captured even by the robust algorithm such as the GLM.

Studies suggest that AgNPs can act as a reserve of Ag ions with potential for continual environmental release.\textsuperscript{18,19,21} Environmental exposures to AgNPs include effects such as: (i) reactive oxygen species (ROS) generation leading to oxidative stress in nitrifying bacteria,\textsuperscript{49} (ii) modification of phospholipid bilayer, causing pits on the cell wall and altering the membrane permeability,\textsuperscript{48} (iii) inhibition of β-galactosidase activity in \textit{E. coil} could result into cell death,\textsuperscript{8} (iv) potential internalization of AgNPs into the cell could cause DNA damage, and potential ionic Ag release from internalized AgNPs could affect ion-exchange and cellular respiration,\textsuperscript{50} (v) direct physical interactions of AgNPs due to charge difference leading to cell death,\textsuperscript{9} and (vi) potential disruption of sodium ion regulation by Citrate-coated AgNPs in the gills of young
rainbow trout (*Oncorhynchus mykiss*). This study provides indirect evidence of cell internalization of Ag, though unclear if internalization occurred as Citrate–AgNPs or dissolved Ag ions or both, leading to inhibition of intracellular β-Gal activity in *E. coli* under various conditions that prevail in the aquatic environments and reaffirms the usefulness of β-Galactosidase bioassay as a high-throughput screening tool for evaluation of metal nanoparticle toxicology in aqueous media.

**Environmental Implications.** Understanding the potential toxic effects of AgNPs under prevailing environmental conditions using the previously established high throughput *E. coli* bioassay is limited to a few studies. In this study, we systematically assessed the toxicity of Citrate–AgNPs under multiple natural water chemistry conditions (DOC, pH, and hardness), and concurrently evaluated the potential changes in particle surface properties and size, including the rates of Ag⁺ released under those conditions. Our results show water chemistry conditions such as variable pH, DOC, and hardness could significantly modulate Citrate–AgNPs or AgNO₃ (as source of ionic Ag⁺) toxicity toward Gram negative *E. coli*. Clearly, the lower release rate of dissolved Ag⁺ emanating from Citrate–AgNP surface layer(s) under different water quality conditions was inadequate to fully account for Citrate–AgNP toxicity. Overall, Citrate–AgNPs were ca 3 – 44 times less toxic than AgNO₃ on total Ag mass basis under the evaluated environmental conditions. While added Leonardite humic acid (as a source of DOC) and increasing pH were able to confer protective effects, increase in the degree of hardness however tended to promote antibacterial effects in *E. coli*, which was counterintuitive though. Detectable changes in hydrodynamic diameter, zeta potential, and surface Plasmon resonance are indicative of modulating effects of the tested water chemistry conditions on the colloidal stability of Citrate–AgNPs in the aqueous environments. The data presented herein are of ecological
significance as they suggest that engineered or incidental AgNPs following release into the aquatic system could modulate their size, surface characteristics, and ion release kinetics, which could subsequently alter their toxicity. This study underscores the need to evaluate aquatic toxicity of AgNPs, owing to its growing use as a broad-spectrum antimicrobial agent in the myriad of consumer and biomedical products,\textsuperscript{11} under additional water chemistry conditions (e.g., other natural organic matter such as fulvic acid, polysaccharides, variable mono-/divalent cations, etc.) to better understand the dynamics underlying their fate, mobility, and persistence (as discrete particles, agglomerates, and/or source of ion release) in the aqueous environments as they might have significant implications to ecosystem health and functions.\textsuperscript{6-10,12,14-20,23-25,48,53,54}

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Figure 3.8. Representative TEM imagery (A), particle size distribution (B), surface Plasmon resonance spectrum (C), and stable colloidal suspension (D) of stock Citrate-AgNPs. Scale bar = 100 nm.
Citrate–AgNP Synthesis: 1 mM AgNO₃ and 10 mM Sodium citrate dihydrate solutions were mixed together in a volume ratio of 2:1, respectively and the mixture was heated for 4 h at 70 °C using a water bath as previously described by El Badawy et al.¹

Table 3.1. Purification protocol applied for cleaning as-synthesize Citrate-AgNPs using Tangential Flow Filtration (TFF) system.²

<table>
<thead>
<tr>
<th>Purification of unclean Citrate-nAg</th>
<th>Electrical Conductivity (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Started Volume = 500 ml</td>
<td>1095</td>
</tr>
<tr>
<td>Ended Volume = 70 ml</td>
<td>1162</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>185</td>
</tr>
<tr>
<td>Ended Volume = 100 ml</td>
<td>283</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>36</td>
</tr>
<tr>
<td>Ended Volume = 75 ml</td>
<td>68</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>11</td>
</tr>
<tr>
<td>Ended Volume = 150 ml</td>
<td>20</td>
</tr>
<tr>
<td>Volume increased to 500 ml</td>
<td>5*</td>
</tr>
</tbody>
</table>

*obtained as clean Citrate-AgNP suspension with electrical conductivity 5 µS/cm.
Figure 3.9. Photograph showing Kros Flo Research IIi Tangential Flow Filtration (TFF) System (right panel) equipped with 10 kD polysulfone hollow fiber diafiltration membranes (left panel) used for the purification of Citrate-AgNPs. Adapted from www.spectrumlabs.com; NP, nanoparticle suspension.

Figure 3.10. DOC calibration curve produced by UV-vis absorbance measurement at 280 nm and the concentrations were verified by persulfate-UV oxidation procedure.
Figure 3.11. Mechanism of intracellular β-Galactosidase mediated conversion of chlorophenol red galactopyranoside (CPRG used as a substrate; yellow color) into chlorophenol red (magenta color) which is quantified at 570 nm using a microplate reader.²

Figure 3.12. Schematic showing protocol for conducting β-Galactosidase bioassay using *E. coli* (Adapted from ref. 3). CPRG, Chlorophenol red galactopyranoside used as a substrate.
Table 3.2. Generalized Linear Model and parameter estimates showing main effects of HDD, zeta potential and Ag dissociation rate (%) under variable dissolved organic carbon (DOC) concentrations and their interactive effects on the toxicity of Citrate-AgNPs (used as EC$_{50}$ values for β-Gal bioassay under a range of DOC concentrations, a dependent variable in the model). Model deviance value was compared with the other models to test the goodness of fit of the model presented based on information criteria that small-is-better.

<table>
<thead>
<tr>
<th>Dependent variable: EC$_{50}$(DOC)</th>
<th>Likelihood Ratio</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Chi-Square</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>59.137</td>
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</tr>
<tr>
<td>HDD</td>
<td>8.487</td>
<td>1</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Zeta Potential</td>
<td>6.311</td>
<td>1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ag Dissociation Rate %</td>
<td>16.102</td>
<td>1</td>
<td>&lt; 10E-4</td>
</tr>
<tr>
<td>HDD x Zeta Potential x Ag Dissociation Rate %</td>
<td>6.498</td>
<td>1</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coefficient B</th>
<th>Std Error</th>
<th>Wald Chi-Square (df)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDD</td>
<td>0.157</td>
<td>0.0464</td>
<td>11.413 (1)</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Zeta Potential</td>
<td>-0.25</td>
<td>0.0894</td>
<td>7.846 (1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ag Dissociation Rate %</td>
<td>2.815</td>
<td>0.5237</td>
<td>28.883 (1)</td>
<td>&lt;10E-6</td>
</tr>
<tr>
<td>HDD x Zeta Potential x Ag</td>
<td>0.005</td>
<td>0.0017</td>
<td>8.132 (1)</td>
<td>&lt; 0.005</td>
</tr>
</tbody>
</table>

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Table 3.3. Generalized Linear Model and parameter estimates showing main effects of HDD under variable pH and the interactive effects of HDD, zeta potential and Ag dissociation rate (%) on the toxicity of Citrate-AgNPs (used as EC$_{50}$ (pH) values for β-Gal bioassay under variable pH, a dependent variable in the model). Model deviance value was compared with the other models to test the goodness of fit of the final model based on the information criteria that small-is-better.

<table>
<thead>
<tr>
<th>Source</th>
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<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>42.479</td>
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<td>&lt; 10E-6</td>
</tr>
<tr>
<td>HDD</td>
<td>9.355</td>
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<td>&lt; 0.005</td>
</tr>
<tr>
<td>HDD x Zeta Potential</td>
<td>7.393</td>
<td>1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>HDD x Ag Dissociation Rate %</td>
<td>5.718</td>
<td>1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>HDD x Zeta Potential x Ag</td>
<td>4.672</td>
<td>1</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Dependent variable: EC$_{50}$ (pH)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coefficient B</th>
<th>Std Error</th>
<th>Wald Chi-Square (df)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDD</td>
<td>10.995</td>
<td>2.9214</td>
<td>14.166 (1)</td>
<td>&lt; 10E-6</td>
</tr>
<tr>
<td>HDD x Zeta Potential</td>
<td>0.819</td>
<td>0.2562</td>
<td>10.220 (1)</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>HDD x Ag Dissociation Rate %</td>
<td>-8.992</td>
<td>3.3225</td>
<td>7.324 (1)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>HDD x Zeta Potential x Ag</td>
<td>-0.691</td>
<td>0.2892</td>
<td>5.713 (1)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Dissociation Rate %
Table 3.4. Generalized Linear Model and parameter estimates showing main effects of Ag dissociation rate (%) under variable hardness conditions on the toxicity of Citrate-AgNPs (used as EC$_{50}$ (Hardness) values for β-Gal bioassay under variable hardness, a dependent variable in the model). Model deviance value was compared with the other models to test the goodness of fit of the model presented based on information criteria that small-is-better.

<table>
<thead>
<tr>
<th>Dependent variable:</th>
<th>Likelihood Ratio</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$ (Hardness)</td>
<td>Chi-Square</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>39.604</td>
<td>3</td>
<td>&lt; 10E-6</td>
</tr>
<tr>
<td>HDD</td>
<td>6.003</td>
<td>1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ag Dissociation Rate %</td>
<td>18.567</td>
<td>1</td>
<td>&lt; 10E-4</td>
</tr>
<tr>
<td>HDD x Zeta Potential x Ag Dissociation Rate %</td>
<td>5.711</td>
<td>1</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coefficient B</th>
<th>Std Error</th>
<th>Wald Chi-Square (df)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDD</td>
<td>-0.621</td>
<td>0.2223</td>
<td>7.789 (1)</td>
<td>0.005</td>
</tr>
<tr>
<td>Ag Dissociation Rate %</td>
<td>32.049</td>
<td>4.8108</td>
<td>44.380 (1)</td>
<td>&lt; 10E-4</td>
</tr>
<tr>
<td>HDD x Zeta Potential x Ag</td>
<td>-0.057</td>
<td>0.0210</td>
<td>7.313 (1)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Dissociation Rate %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.5. Comparison of the Generalized Linear Model (GLM)-predicted toxicity (EC\text{50}) versus experimentally-derived toxicity of Citrate-coated AgNPs in *Escherichia coli*.

<table>
<thead>
<tr>
<th>Citrate-AgNP</th>
<th>β-Galactosidase activity in <em>Escherichia coli</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental EC\text{50}±S.D. (mg/L)</td>
<td>GLM-Predicted EC\text{50} (mg/L)</td>
</tr>
<tr>
<td><strong>DOC (mg/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.79±2.87</td>
<td>5.68</td>
</tr>
<tr>
<td>2</td>
<td>8.56±0.24</td>
<td>8.66</td>
</tr>
<tr>
<td>5</td>
<td>11.55±0.35</td>
<td>11.71</td>
</tr>
<tr>
<td>10</td>
<td>13.28±0.50</td>
<td>11.72</td>
</tr>
<tr>
<td>20</td>
<td>13.38±0.62</td>
<td>14.10</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.65±0.53</td>
<td>2.01</td>
</tr>
<tr>
<td>6</td>
<td>3.33±0.28</td>
<td>3.50</td>
</tr>
<tr>
<td>7</td>
<td>8.56±0.24</td>
<td>9.62</td>
</tr>
<tr>
<td>7.5</td>
<td>36.7±3.33</td>
<td>35.46</td>
</tr>
<tr>
<td><strong>Hardness (mg/L as CaCO\text{3})</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>37.6±3.03</td>
<td>34.98</td>
</tr>
<tr>
<td>200</td>
<td>11.78±0.5</td>
<td>17.52</td>
</tr>
<tr>
<td>250</td>
<td>11.4±1.49</td>
<td>11.61</td>
</tr>
<tr>
<td>280</td>
<td>8.56±0.24</td>
<td>5.24</td>
</tr>
</tbody>
</table>

GLM, Generalized Linear Model; % Precision = 100(GLM Predicted EC\text{50}/Experimental EC\text{50}). Citrate–AgNP, Citrate-coated AgNP.
Table 3.6. Physicochemical characteristics of the water samples collected from the Watauga River, Elizabethton, TN (36.3339 °N, -82.2704 °W).

<table>
<thead>
<tr>
<th>Date Sampled</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Electrical Conductivity (µS/cm)</th>
<th>Dissolved Oxygen (mg/L)</th>
<th>Hardness (mg/L as CaCO₃)</th>
<th>Alkalinity (mg/L as CaCO₃)</th>
<th>NH₃-N₂ (mg/L)</th>
<th>Dissolved Organic Carbon (mg/L)</th>
<th>Total Ag (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/30/3012</td>
<td>7.3</td>
<td>6.7</td>
<td>98.5</td>
<td>8.1</td>
<td>316.5</td>
<td>39</td>
<td>0.02</td>
<td>1.87</td>
<td>bdl</td>
</tr>
<tr>
<td>5/30/3012</td>
<td>6.6</td>
<td>13</td>
<td>96</td>
<td>8.0</td>
<td>318.5</td>
<td>31</td>
<td>0.02</td>
<td>1.96</td>
<td>bdl</td>
</tr>
<tr>
<td>7/27/2012</td>
<td>7.1</td>
<td>17</td>
<td>112.5</td>
<td>8.0</td>
<td>306</td>
<td>44</td>
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<td>2.07</td>
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<td>7.0</td>
<td>19</td>
<td>119</td>
<td>8.3</td>
<td>311</td>
<td>45.5</td>
<td>0.02</td>
<td>2.08</td>
<td>bdl</td>
</tr>
</tbody>
</table>

Reported values are the means of the duplicate samples; bdl denotes below detection limit of the Graphite Furnace-AAS; detection limit for Ag was 0.54 µg/L; pH, temperature, electrical conductivity, and dissolved oxygen were measured by Hanna Instruments multiparameter meter 9828 (Hanna Instruments, Michigan); hardness (method 10247), alkalinity (method 8203) and NH₃-N₂ (method 8038) were measured using the standard Hach methods; dissolved organic carbon was verified following the method SM 5310C coupled with persulfate-UV oxidation procedure.
QA/QC. All containers used for this study were soaked in 5% HNO₃ overnight, cleaned several times using nanopure water (resistance = 18.3 MΩ-cm), and air dried before use. Typical metal analysis using Atomic absorption spectroscopy (AAS)-Flame/furnace comprised of the method blank, digested samples, sample duplicate, spiked sample, and appropriate internal standards. The rinse blank consisting of 2% HNO₃ made in nanopure water was used to clean the system following analysis of every ten samples. Maintenance of AAS is routinely performed through permanent maintenance contract with the manufacturer. Five-point calibration curves were typically developed for Ag analysis with AAS. Typical Ag recovery was in the range 104.8 – 112.4 % for the GF-AAS.

References Cited in Appendix


Humic Acid-, Cysteine-, and Trolox-induced Changes in Colloidal Stability, Dissolution Rate, and Aquatic Toxicity of Silver Nanoparticles

Lok R. Pokhrel, Brajesh Dubey, Phillip R. Scheuerman

ABSTRACT: Naturally ubiquitous organic ligands such as thioles (Cysteine) and dissolved organic carbon (DOC; humic acid) in aquatic systems may chemisorb onto nanoparticle surface and modify its colloidal properties. Specific information about key transformations that can occur once silver nanoparticles (AgNP) are released into aquatic systems is limited. This study reports on how Cysteine (as Ag\(^+\) quencher), DOC (that complexes with both AgNP and Ag\(^+\)), and Trolox (as antioxidant) could alter colloidal stability, Ag release rate, and aquatic toxicity of Citrate–AgNPs in a keystone crustacean, Daphnia magna. We show that the applied organic ligands could modify Citrate–AgNP colloidal stability by altering its size, surface properties (surface charge and plasmonic spectra), and Ag\(^+\) release rate, thereby attenuating (with Cysteine and Trolox) or promoting (with DOC) nanotoxicity. Notably, however the combined effect of AgNPs and released Ag\(^+\) under each ligand treatment was lower than that of AgNO\(_3\) alone. While Trolox ability to attenuate Citrate–AgNP toxicity can be attributed to oxidative stress, its inability to attenuate free Ag\(^+\) toxicity however negates oxidative stress as a driver of Ag\(^+\) toxicity and that Cysteine could effectively quench free Ag\(^+\) to alleviate AgNO\(_3\) toxicity in D. magna. Surprisingly, DOC-AgNP aggregates that apparently formed under higher DOC concentrations might have led daphnids filter-feed on aggregates, potentially causing higher exposure and therefore higher mortality, unlike at lower DOC levels with no apparent aggregation and lack of mortality. Taken together, this and our previous study (ref. 1) suggest
applying caution to extending one species toxicity results to another as the obvious differences in organism biology could significantly modify AgNPs or Ag\(^+\) toxicity in aquatic systems.

INTRODUCTION

As we witness regulatory undersight,\(^1\) nanotechnology continues to grow with the introduction of new engineered nanoparticles (NP) and associated consumer products in the commercial market.\(^2\) As a model nanoparticle, silver nanoparticle (AgNP) is among the most widely used engineered NPs in consumer and medical applications for its strong catalytic, antimicrobial, and plasmonic characteristics,\(^2,3\) and therefore has been the subject of much scrutiny from nanochemists and nanotoxicologists to elucidate its underlying toxicity mechanism.\(^1,4-8\)

Identifying the toxicity mechanism of AgNPs would enable the risk managers find ways for mitigating risk (if any).

Surface modification of incidental or engineered NPs has generally emerged as an accepted premise in environmental nanochemistry.\(^1,5,9-11\) An inherent problem with this tenet, however, is that not all NPs show similar surface modifications following their release into aquatic or terrestrial environments, where various organic and inorganic ligands exist.\(^5,9-13\) Therefore, a need to evaluate potential changes that might occur on NP surface and how such changes would modulate NP toxicity may arise on an individual NP basis.\(^5,14\)

Natural organic ligands that are ubiquitous in aquatic systems include reduced –SH (a functional group in Cysteine), –COOH (a functional group in organic acids and amino acids/vitamins), humic and fulvic substances (with various aliphatic and aromatic group ratios), carbohydrates, among others, which are the products of metabolic and decomposition processes emanating of both the aquatic and terrestrial systems.\(^13\) As environmental release of engineered NPs from associated products is inevitable,\(^15\) specific information about key transformations that
might occur once model AgNPs are released into aquatic medium\textsuperscript{5,9-11} and how such modifications would change its toxicity is limited to few studies;\textsuperscript{1,16,17} therefore, more research is needed to explain if/how particles are surface modified and to elucidate underlying toxicity mechanisms. Previous studies have reported mixed results; some show particle-specific toxicity,\textsuperscript{7,16,18,19} others predominantly show free Ag\textsuperscript{+} toxicity,\textsuperscript{6} while the reports revealing combined effects of particles and ions to be lower than that of free Ag\textsuperscript{+} alone are rare.\textsuperscript{1} Assay-dependent toxicity\textsuperscript{21} or species-specific sensitivity to NPs is also emerging as a viable explanation – one that implicates differences in species biology (Gram positive/Gram negative bacteria, feeding behavior in higher organisms, predator-prey interactions, etc.), in explaining NP toxicity.\textsuperscript{1,7,12,22-24}

Herein, we investigate the potential effects of L-Cysteine, dissolved organic carbon (DOC), and Trolox on the colloidal stability, Ag\textsuperscript{+} release rate, and aquatic toxicity of citrate-coated AgNPs (Citrate–AgNP) against the keystone species, \textit{Daphnia magna}. We measure changes in hydrodynamic diameter (HDD), zeta (\(\zeta\)) potential, and the surface Plasmon resonance to assess colloidal stability of Citrate–AgNPs in the presence of three types of organic ligands/compounds. Rate of Ag\textsuperscript{+} released in the presence of each ligand type is determined to understand whether the released Ag\textsuperscript{+} has any effect on the toxicity of Citrate–AgNPs under the conditions analyzed. Our strategic utilization of natural organic ligands (cysteine and humic acid) including a well-known antioxidant (Trolox) offered complementary ways to investigate a model AgNP toxicity and enabled us to associate changes in colloidal stability and ion release rate with the observed toxicity in \textit{D. magna}. As a free Ag\textsuperscript{+} source, AgNO\textsubscript{3} toxicity is assessed concomitantly in the presence of variable organic ligands; this allowed us to differentiate between zerovalent Ag\textsuperscript{0} toxicity from that of monovalent Ag\textsuperscript{+} alone.
MATERIALS AND METHODS

Nanoparticle Synthesis, Purification and Characterization. Citrate-coated silver nanoparticles (Citrate–AgNP) were synthesized and purified using a tangential flow filtration (TFF) system following the previously reported methods (detailed in Appendix Figures 3.6 & 3.7; Tables 3.1 & 3.2). To determine Ag concentration (as total Ag) in nanosuspension, flame-atomic absorption spectroscopy (AAS, Varian 220Z/220FS) was used, prior to which the purified sample was digested using ultrapure HNO₃ following the standard method 3050B.

The hydrodynamic diameter (HDD, volume-weighted) and zeta (ζ) potential of the purified Citrate–AgNP suspension were recorded using the dynamic light scattering (DLS) method (PSS NICOMP Particle Sizing Systems, CA). The DLS unit was calibrated using Duke 500 (491 nm) NIST 3490A standard (PSS Nicomp, FL, USA) before the samples were measured for HDD and ζ potential. The surface plasmonic spectra were obtained using the UV/vis spectrophotometer (Shimadzu PharmaSpec UV-1700). Carbon-coated copper formvar grid (Ted Pella, Cat # 01813-F) was used as a support onto which an aliquot of sample was dropped and air dried before a transmission electron microscope (Philips EM 420; operated at 120 kV in the bright-field mode) would visualize AgNP morphology; the images captured were used to estimate particle size distribution (PSD) and circularity using ImageJ 1.44.

Toxicity Bioassay. Daphnia magna 48 h acute toxicity test was conducted following the standard USEPA guidelines. This static, non-renewable test followed a randomized block design. Ten, less than 24 h old, D. magna neonates were introduced randomly into each 50 mL test beaker under variable test conditions (detailed in the following section). Each block of treatment or controls was randomized for spatial placement during the test period. A range-finding test preceded the definitive tests. Tests were performed in a minimum of triplicates (total
30 daphnids for each concentration) in moderately hard water (MHW)\textsuperscript{26} used as a test matrix.

Animals were unfed during the test period, but were fed during the culture and maintenance, per the guideline.\textsuperscript{26} A 16:8 h light:dark photoperiod was maintained using 2-feet long wide-spectrum fluorescent tubes. Following 48 h, total dead daphnids in each test chamber were counted to assess mortality under various treatments.

To assess the potential influence (attenuate or enhance) of cysteine (CYS), humic acid (DOC, dissolved organic carbon), and trolox (TRX) on the toxicity of Citrate–AgNPs in \textit{Daphnia magna}, toxicity tests were performed using the freshly prepared multiple concentrations of each organic ligand: 0, 2, 5, 10, 15, 20 and 25 mg/L DOC; 0, 0.01, 0.05, 0.25, 0.5, 1, 2, 5, 10 and 20 mg/L CYS; and 0, 0.5, 1, 10, 25, 50, 100 and 200 mg/L TRX. Concurrently, AgNO\textsubscript{3} was used to test for free Ag\textsuperscript{+}-specific toxicity under the same treatment conditions as stated above for Citrate–AgNPs. Cu\textsuperscript{2+} (as CuSO\textsubscript{4}) that was used as a positive control showed that \textit{D. magna} neonates were appropriately sensitive to be used as a bioassay for evaluating Citrate–AgNPs and Ag\textsuperscript{+} toxicity. MHW (without NP) was used as a negative control.

\textbf{Preparation of Test Solutions and Exposure Conditions.} MHW was used to prepare DOC by stirring a known quantity of Leonardite humic acid (International Humic Substances Society; Cat # 1S104H-5) overnight, and was vacuum filtered using a 0.45 µm pore size Millipore filter. The concentration of DOC thus prepared was determined using the UV-vis absorbance at 280 nm (Appendix Figure 4.9), and verified following the method SM 5310C coupled with persulfate-UV oxidation procedure.\textsuperscript{1} Before use, we stored the solution in dark at 4 °C. The characteristics of this humic acid have been published in detail by Thorn et al.\textsuperscript{27}

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 97% purity, Cat#
218940250) and L-cysteine (C₃H₇NO₂S; 99+% purity, Cat# 173601000) were procured from Acros Organics through Fisher Scientific, USA. Both trolox and cysteine solutions were prepared in MHW by stirring (with magnetic stirrer) over night at 1000 rpm and used as-prepared; nominal concentrations are reported for multiple dilutions of TRX and CYS. Unlike CYS and DOC, the purpose of using TRX was to indirectly assess if oxidative stress was the cause of AgNP toxicity as was previously reported.²⁸-³⁰ Because ~85% mortality of daphnid occurred at 1 mg/L Citrate–AgNPs or at 7.5 µg/L Ag⁺ (used as AgNO₃), these concentrations were further considered for the detailed toxicity experiments with an aim to observe measurable mitigating effects in the presence of organic ligands as indicated by the scarce body of literature.⁴,⁷,¹⁶ Each organic ligand was added to Citrate–AgNP suspension or AgNO₃ solution, and stirred (using magnetic stirrer) for 5 min at 350 rpm to gently allow interactions to occur between each ligand and AgNP/Ag⁺ before the neonates were exposed to the test chemical. To obtain reliable/reproducible measurements for assessing AgNP stability in the evaluated test conditions, Citrate–AgNP concentration was necessary to be doubled (2 mg Ag/L) for the DLS and UV-vis measurements. All experiments (stability, ion release, and toxicity) were also concomitantly conducted with each organic ligand alone as a control.

**Ion Release Rate under Variable Ligands.** Duplicate samples were run with each ligand to assess for Ag⁺ dissociation rate from the Citrate–AgNP suspension. 25 mL of each sample type was incubated in a plastic centrifuge tube placed on the same shelf where the toxicity assays were performed, and a 16:8 h light:dark photoperiod was maintained at 20 ± 2 °C for 48 h. Each sample was ultracentrifuged (Thermo Scientific Sorvall WX Ultra Series Centrifuge SN# N13V-427288-NV) at 45,000 rpm (205,835 g at the bottom, 146,347 g in the middle, and 86,858 g at the topmost part of the bottle) in a polycarbonate bottle (Thermo Scientific Cat# 314348) for an
hour, following which 3 mL of supernatant was pipeted; the samples were digested using same volume of ultrapure HNO₃ and total Ag concentration was determined using a graphite furnace-AAS. Data are reported as an average of duplicate runs.¹

**Statistical Analysis.** Linear regression analysis was used to calculate EC₅₀ (i.e., effective concentrations that kill 50% of the test population) values and are reported as an average of at least three replicates. One-way analysis of variance (ANOVA) was used to test for significant difference between the sample EC₅₀s and the controls, followed by the Dunnett t (posthoc) test for multiple comparisons. Significant difference was established at the p ≤ 0.05 using IBM SPSS ver. 20.

**RESULTS AND DISCUSSION**

**Characteristics of Citrate–AgNPs.** The characteristics of Citrate–AgNPs are previously documented in our publication (detailed in Appendix Figures 4.7 & 4.8; Tables 4.1 and 4.2).³,²² Briefly, Citrate–AgNPs showed 14.8 ± 0.9 nm in HDD and 56.5 ± 19.2 nm as TEM diameter, with ζ potential of -22.5 ± 1.8 mV (Appendix Figure 4.7, Table 4.2). Comparable ζ potentials before and after cleaning (Appendix Table 4.2) and the particles as revealed by the TEM imagery strongly suggest stable colloidal dispersion and that the TFF did not change Citrate–AgNP properties.³ Observed plasmonic peak was as characterized by the Mie theory (Appendix Figure 4.7C).³¹ The Citrate–AgNPs used had higher circularity (0.88) and were of high purity as previously reported.¹,³,¹⁶

**Modulation of AgNP Stability by Trolox, DOC and Cysteine.** The measures of HDD, ζ potential, and SPR spectra have routinely offered information to characterize the colloidal stability of nanoparticles in aqueous media.³²,³³ Using the DLS and UV–vis methods, the data
collected for HDD and ζ potential are then compared with the measurable modifications in the SPR spectra to assess for potential changes in particle stability in MHW containing various

Figure 4.1. Effects of variable organic compounds on the (A, C, E) hydrodynamic diameter (HDD) and (B, D, F) surface charge (as zeta potential) of Citrate–AgNPs. To obtain reliable DLS measurements for the HDD and ζ potential, 2 mg/L citrate-AgNP was used with each ligand type. DOC, dissolved organic carbon. ‘*’ denotes \( p \leq 0.01 \); ‘***’ denotes \( p \leq 0.001 \); and ‘****’ denotes \( p \leq 0.0001 \) as compared to the baseline of 0 mg DOC/L, pH 7, and 280 mg hardness/L (as CaCO\(_3\)); significant difference was analyzed by ANOVA followed by Dunnett t (posthoc) test.
organic ligands. On average, DLS measurements revealed slight decrement in HDD with increasing TRX concentrations, which however abruptly increased by 1.75 fold (from ~15 nm to ~ 26 nm) at 200 mg TRX/L. Steep decline in ζ potential occurred with increasing TRX concentrations, suggesting potential agglomeration of Citrate–AgNPs that might have occurred at greater TRX concentrations. At 200 mg TRX/L, average ζ potential decreased by more than 3 fold compared to Citrate–AgNPs alone (2 mg Ag/L, with no added TRX; Figure 4.1B) – the result corroborated observed increase in HDD (Figure 4.1A). Increasing TRX concentrations reduced absorbance and blue-shifted the SPR peak for Citrate–AgNPs. It was surprising that the complete masking of SPR peak was observed at ≥ 100 mg TRX/L, with an emergence of new peak centered at λ of 350 nm (Figure 4.2A).

Inter-particle bridging of AgNPs at higher DOC concentrations apparently resulted in larger particles (Figure 4.1C), which corresponded to significant decline in ζ potentials (Figure 4.1D; \( p \leq 0.001 \)). This is consistent with the change in absorbance and broadening, with no (blue/red) shift, of SPR peaks with increasing DOC concentrations (Figure 4.2B). CYS-coating of AgNP surface confers stability forming Ag-SH (thiolate bond between amino acid and Citrate–AgNP surface) and H-bonding among the CYS-AgNPs (Appendix Figure 4.10). Our observation of slight blue-shift (shift to the left) of SPR peaks is indicative of surface modification of Citrate–AgNPs with increasing CYS concentrations. CYS could significantly mask Citrate–AgNP absorbance in a concentration-dependent manner (Figure 4.2C). Relatively lower absorbance of Citrate–AgNPs at higher CYS concentrations is suggestive of adequate CYS-coating of AgNP surface as reported by Liu et al. (Appendix Figure 4.10). Despite consistent ζ potential fluctuation, apparently no significant change in HDD under a wide range of CYS concentrations – the result consistent with a previous report by Gondikas et al. – suggests that Citrate–AgNPs might have fairly remained stable in the test matrix (Figure 4.1E,F).
Figure 4.2. Changes in surface Plasmon resonance (SPR) spectra of Citrate–AgNPs in the presence of variable concentrations of (A) Trolox, (B) dissolved organic carbon (DOC), and (C) L-Cysteine.
Modulation of Ion Release Rate by Trolox, DOC and Cysteine. Incubation of Citrate–AgNPs with different organic ligands for 48 h under conditions similar to the *D. magna* toxicity bioassay revealed remarkable differences in the rates of Ag⁺ dissociation from Citrate–AgNPs (Figure 4.3). Ag⁺ release rate (%) steeply declined as a function of TRX concentrations, with greatest amount (4.65 %) being released at 0.5 mg TRX/L. At 10-200 mg TRX/L, % Ag⁺ released was lower (1.38-0.95 %) than in stock Citrate–AgNP suspension (1.65% Ag released of initial concentration 1 mg Ag/L) with no added TRX. This suggests that higher TRX concentrations (10-200 mg TRX/L) could, in fact, inhibit Ag⁺ release, verifying the former role as an antioxidant, i.e., TRX scavenges the oxygen species present or otherwise formed in the test environment, thus providing a less conducive condition for oxidative dissolution of Ag⁺ to coour from Citrate–AgNP surface layer(s).

As previously reported,¹ Ag⁺ release rate steeply increased as a function of DOC concentrations. At 25 mg/L DOC, the rate of Ag⁺ release was 29.5 fold higher than at 2 mg/L DOC, but this was 17.7 fold higher than that with no added DOC (Figure 4.3B). In a 4 h experimental period, we had observed only 3.6 % Ag⁺ released from the same Citrate–AgNP suspension with 20 mg/L DOC and with an order of magnitude higher initial Citrate–AgNP
concentration.\textsuperscript{1} Nearly 30\% of the Ag\textsuperscript{+} dissociation that occurred in this study from Citrate–AgNPs can be attributed to the disparate test conditions between the two studies (4 h test conducted in dark in the previous study\textsuperscript{1} vs. 48 h test period with 16:8 light:dark cycle in this study). Hence, these data suggest that Ag\textsuperscript{+} dissociation from Citrate–AgNPs is not dependent on initial concentration of Citrate–AgNPs, rather a dependency on time and exposure condition (favored by light) emerged. The lower overall Ag\textsuperscript{+} release (0.15 – 1.41\%) upon CYS treatment (0.1 – 10 mg CYS/L) is indicative of a less favorable dissolution environment for the Citrate–AgNPs to dissociate into dissolved ionic form (Figure 4.3C). As previously stated,\textsuperscript{34} the thiolate and H-bonding among CYS-AgNPs might be responsible for the low amount of Ag\textsuperscript{+} release from Citrate–AgNP surface. Total Ag recovery was in the range 104.8 – 112.4\% for GF-AAS. Because no measurable amount of Ag was detected in the highest concentration of different organic compounds used in this study, this justifies that the source of released Ag\textsuperscript{+} under the evaluated conditions was the Citrate–AgNPs, not the TRX, DOC (Leonardite humic acid), or CYS itself.

**Modulation of AgNP toxicity by Trolox, DOC and Cysteine.** A concentration-dependent mortality of *D. magna* neonates was observed for both the Citrate–AgNPs and free Ag\textsuperscript{+} (as added AgNO\textsubscript{3}; Figure 3.4A,B). Comparison of EC\textsubscript{50}s revealed Citrate–AgNPs (EC\textsubscript{50} = 798 ± 156 µg Ag/L) 185 times less toxic than free Ag\textsuperscript{+} (EC\textsubscript{50} = 4.3 ± 0.4 µg Ag/L).

Figure 4.5 provides insights into how different organic ligands can modulate aquatic toxicity in *D. magna*. In this study, survival of *D. magna* neonates was improved with increasing TRX concentrations; at ≥100 mg TRX/L neonate survival reached 100\%, while at 0.5 mg TRX/L no protection was conferred upon exposure to 1 mg/L Citrate–AgNPs (Figure 4.5A). On the other
hand, TRX was unable to confer a protective effect even as high as 800 mg TRX/L upon exposure to free Ag⁺ (as added AgNO₃; 7.5 µg Ag/L; Figure 4.5B). The complete masking and blue-shifting of the SPR peak at 100 or 200 mg TRX/L, suggesting surface interactions between TRX and Citrate–AgNPs, coupled with low amount of Ag⁺ released at higher TRX concentrations can be directly associated with 100% daphnid survival.

![Graph](image)

**Figure 4.4.** Concentration-dependent effects on survival of *Daphnia magna* neonates in moderately hard water (without organic ligands) upon exposure to (A) Citrate–AgNPs or (B) dissolved Ag⁺ ions (used as AgNO₃). Error bars represent ± 1 standard deviation (SD) of at least triplicate test runs.

Known for its cell-permeability owing to its hydrophilicity, Trolox is a derivative of Vitamin E with inherent antioxidant and membrane-protective characteristics, which are justified by its routine use as a standard/positive control in many antioxidant assays, including in the studies evaluating aging and neuronal death under oxidative stress. Adequate protection conferred by TRX at higher concentrations against Citrate–AgNPs might be associated with its (the former) potent antioxidant (as it quenches reactive oxygen radicals) and membrane-protective properties, thus providing an indirect evidence of oxidative stress emanating of Citrate–AgNPs in the polar medium. It has been reported that Citrate–AgNPs could lead to the generation of strong
oxidant such as H$_2$O$_2$ in the aqueous media, unlike with free Ag$^+$ (used as AgClO$_4$) that barely led to the formation of the oxidant.$^{35}$ Contrasting toxicity results obtained here for Citrate–AgNPs and free Ag$^+$ strongly suggest distinct mechanisms underlying the toxicity of zero-valent Ag$^0$ versus the monovalent Ag$^+$, with oxidative stress being the apparent cause of Citrate–AgNP toxicity$^{28-30}$ versus the direct ionic effects (disruption of ionic balance across the membrane) of free Ag$^+$,$^{41,42}$ and this indicates that oxidative stress was not the mechanism responsible for free Ag$^+$ toxicity.$^{36}$ Daphnid survival was not compromised at lower DOC concentrations for Citrate–AgNPs or free Ag$^+$ (up to 10 mg/L DOC with Citrate–AgNPs, and up to 5 mg/L DOC with free Ag$^+$; Figure 4.5C,D). Interestingly, however, daphnid mortality increased significantly at higher DOC concentrations: at ≥15 mg/L DOC with Citrate–AgNPs, and ≥10 mg/L DOC with free Ag$^+$, which appeared to be counterintuitive. Humic acid, which binds to the surface of NPs and ions and also eliminates some reactive oxygen radicals present or otherwise formed in an aqueous suspension,$^{43,44}$ was expected to quench the potential oxygen radicals present or formed during the experiment and reduce bioavailability of Citrate–AgNPs and free Ag$^+$ ions in the test suspension, hence minimal toxicity was expected. Because this was clearly not the case, alternately, it seems plausible that larger aggregates of DOC-Citrate–AgNPs complex that were apparently formed under higher DOC concentrations (Figure 4.1C) might have been filter-fed by daphnids, exposing them to a higher internal dose then when lower DOC concentrations were present in the nanosuspension, hence resulting in higher observed mortality. Because protective effects declined with increasing DOC concentrations under free Ag$^+$ treatment, it can again be attributed to the daphnids’ feeding behavior that might have led to greater exposure to free Ag$^+$ and thus higher toxicity (Figure 4.5D). An addition of –SH ligand conferred protection to daphnids upon exposure to Citrate–AgNPs or free Ag$^+$ (Figure 4.5E,F). At 2 mg CYS/L or
Figure 4.5. Influence of variable organic ligands on the toxicity of (A, C, E) Citrate–AgNPs or (B, D, F) dissolved Ag\(^+\) ions (used as AgNO\(_3\)) measured as change in \textit{D. magna} survival. Error bars represent ± 1 standard deviation (SD) of at least triplicate test runs. ‘*’ denotes \(p \leq 0.01\); ‘**’ denotes \(p \leq 0.001\); and ‘***’ denotes \(p \leq 0.00001\); significant difference was analyzed by ANOVA followed by Dunnett t test for multiple comparisons. DOC,
dissolved organic carbon; C, moderately hard water (MHW) control; AgNP, only AgNP in MHW; Ag⁺, only Ag⁺ in MHW; Trolox only, only Trolox used in MHW at both its highest and lowest concentrations evaluated; DOC only, only DOC used in MHW at both its highest and lowest concentrations evaluated; Cysteine only, only Cysteine used in MHW at both its highest and lowest concentrations evaluated. A fixed concentration of Citrate–AgNPs (1 mg/L) or Ag⁺ (7.5 µg/L) was used to assess the effects of a wide range of Trolox, DOC, and Cysteine on the survival of D. magna neonates.

Higher, survival of the neonates reached 100% with either Citrate–AgNPs or free Ag⁺ treatment, suggesting that the applied CYS concentration was optimal to confer adequate protection against Ag stress. As CYS is known for its higher affinity to complex with free Ag⁺, it is expected to scavenge free Ag⁺ that might be present or otherwise formed in the Citrate–AgNP suspension during the experimental period. Our results imply that CYS could chelate monovalent Ag⁺ in AgNO₃ suspension and therefore could protect the daphnids from toxic free Ag⁺; while similar results observed for Citrate–AgNPs might explain enough CYS-coating of AgNP surface, as verified by lower absorbance (Figure 4.2C; and previously reported by Liu et al.), and scavenging of free Ag⁺ released during the experiment.

Toxicity evaluation for various ligands (without Citrate–AgNPs or free Ag⁺) used in this study at both the lowest and highest concentrations evaluated showed no effects on daphnid survival (100% survived; Fig. 4.5A-F), confirming that the daphnid mortality that occurred under lower or higher concentrations of the tested organics was the effects of Citrate–AgNPs and/or the free Ag⁺. The amount of dissolved Ag released from Citrate–AgNPs is greater than the calculated EC₅₀ value for Ag⁺ (as AgNO₃). Because Citrate–AgNPs were less toxic even with greater amount of Ag⁺ being released in the test suspensions, it suggests that the released Ag may not be in the highly toxic monovalent form (Ag⁺) but rather could likely be in the form of less harmful Ag species/Ag-complexes, perhaps due to the chemical make-up of the organic compounds used vis-à-vis that of the MHW (NaHCO₃ = 96 mg/L, CaSO₄.2H₂O = 60 mg/L,
MgSO$_4$ = 60 mg/L, KCl = 4 mg/L).\textsuperscript{26} This also suggests that the combined effects of AgNPs and released Ag were lower than that of free Ag$^+$ (as AgNO$_3$).

The MHW used as a matrix for $D. magna$ bioassay is considered suitable for TRX solubility owing to the presence of its two functional groups, –COOH and –OH, with dissociation constants (pK) 3.89 and 11.92, respectively (per TRX molecule), thus offering higher solubility in aqueous media at circumneutral pH.\textsuperscript{47,48} A schematic illustrating multiple exposure conditions, subsequent modifications of AgNP surface, dissolution rate, and toxicity in $D. magna$ is summarized in Figure 3.6. An application of TRX (i) modified AgNP surface properties (verified by changes in $\zeta$ potential and SPR spectra), (ii) inhibited Ag dissociation, possibly due to quenching of reactive oxygen present/formed during the experiment which otherwise would have promoted oxidative dissolution of Ag, and (iii) consequently provided protection against the widely used model AgNPs to the daphnids due to its antioxidative effects. The presence of DOC in the aqueous medium (i) altered particle size (HDD) and surface properties (revealed by changes in $\zeta$ potential and SPR spectra), (ii) which enabled formation of larger DOC-AgNP aggregates (revealed by HDD), (iii) followed by filter-feeding of DOC-AgNP aggregates by daphnids which might have contributed to greater exposure to AgNPs, and (iv) consequently, higher toxicity leading to daphnid mortality. When tested for the effects of comparable Citrate–AgNPs (different batch but with similar physicochemical properties) under analogous DOC concentrations in $E. coli$, unlike for $D. magna$ as observed in this study, toxicity was rather attenuated in our previous study, thus supporting variability in species sensitivity due to distinct biology of the organism employed in toxicity testing.\textsuperscript{1,49} This finding strongly suggests the role of species biology (e.g., feeding behavior here) as an important factor to be considered when
investigating nanoparticle toxicity in aquatic systems.\cite{6,49} An addition of thiol ligand into Citrate–AgNP suspension (i) modified surface properties forming CYS-AgNP complex (revealed by slight blue-shift and broadening of SPR peaks, and lower UV-vis absorbance), (ii) inhibited Ag$^+$ release from nanosuspension, (iii) scavenged released Ag$^+$, and (iv) promoted daphnids survival perhaps due to decreased presence of biolabile Ag as a result of CYS-AgNP complex formation and quenching of free Ag$^+$ by CYS molecules.

**Environmental Significance.** The tenet that “AgNPs are environmentally transformed” is generally well-received and has been \textit{a priori} concept, \textit{albeit} it has been less well-explored and understood.\cite{1,5,9-11} In this study, we systematically evaluated how different organic ligands such as –SH (Cysteine), –COOH and –OH (Trolox), and DOC (Leonardite humic acid with high aromaticity, \(f_a = 0.58\), as previously determined by Thorn et al.\cite{27}) could influence (enhance or attenuate) colloidal stability, Ag$^+$ release rate, and aquatic toxicity of Citrate–AgNPs. Our results show that ligands that are ubiquitous in the environment (such as –SH, –COOH and –OH, and

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\textbf{Figure 4.6.} Schematic showing influence of variable organic ligands on the surface modifications, dissolution rate, and toxicity of Citrate–AgNPs in \textit{Daphnia magna}. 

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humic acid) could surface modify Citrate–AgNPs, alter their colloidal persistence and rate of oxidative dissolution, and subsequently could attenuate or enhance toxicity, the latter likely dependent on species biology (as revealed by comparing results for E. coli versus D. magna). These results suggest applying caution to extending toxicity results obtained for one species to another as the obvious differences in the organism biology could significantly change zero-valent Citrate–AgNP or monovalent Ag⁺ toxicity in aqueous media. While the ability of Trolox to attenuate Citrate–AgNP toxicity can be ascribed to oxidative stress, its inability to attenuate Ag⁺ toxicity however negates oxidative stress as a driver of free Ag⁺ toxicity in D. magna. Notably, the combined effects of AgNPs and released Ag⁺ were lower than that of AgNO₃ alone under the evaluated conditions is unique to the literature. Whether low level (ng/L) of naturally occurring Cysteine in sediment porewater and surface water would confer protection against naturally-modified engineered AgNPs at lower (ng/L) doses (should they be toxic) as predicted for aquatic systems could be an important thesis for future research. “How would various humic substances (e.g., Suwanee River humic/fulvic acids, Leonardite himic acid, soil humic/fulvic acids, peat humic acid, other bulk natural organic matters, etc.) alone or in combination transform (size, aggregation/sedimentation, coating density, surface charge/reactivity) AgNPs and consequently the toxicity in aquatic receptors?” is a question not only heuristically interesting but one that carries ecological significance as the answers obtained could offer meaningful and novel insights into the nanochemistry potentially occurring in aqueous systems enriched with natural organic matters.

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Figure 4.7. Representative TEM imagery (A), particle size distribution (B), surface Plasmon resonance spectrum (C), and stable colloidal suspension (D) of stock Citrate-AgNPs. Scale bar = 100 nm (taken from our previous publication1).

Citrate–AgNP Synthesis: 1 mM AgNO₃ and 10 mM Sodium citrate dihydrate solutions were mixed together in a volume ratio of 2:1, respectively and the mixture was heated for 4 h at 70 °C using a water bath as previously described by El Badawy et al.²
Figure 4.8. Photograph showing Kros Flo Research IIi Tangential Flow Filtration (TFF) System (right panel) equipped with 10 kD polysulfone hollow fiber diafiltration membranes (left panel) used for the purification of Citrate-AgNPs. Adapted from www.spectrumlabs.com; NP, nanoparticle suspension.

QA/QC. All containers used for this study were soaked in 5% HNO₃ overnight, cleaned several times using nanopure water (resistance = 18.3 MΩ-cm), and air dried before use. Typical metal analysis, using an Atomic absorption spectroscopy (AAS)-Flame/Furnace, comprised of the method blank, digested samples, sample duplicate, spiked sample, and appropriate internal standards. The rinse blank consisting of 2% HNO₃ made in nanopure water was used to clean the system following analysis of every ten samples. Maintenance of AAS is routinely performed through permanent maintenance contract with the manufacturer. Five-point calibration curves were typically developed for Ag analysis using an AAS.
Table 4.1. Purification protocol applied for cleaning as-synthesized Citrate-AgNPs using Tangential Flow Filtration (TFF) system.

<table>
<thead>
<tr>
<th>Purification of unclean Citrate-nAg</th>
<th>Electrical Conductivity (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Started Volume = 500 ml</td>
<td>1095</td>
</tr>
<tr>
<td>Ended Volume = 70 ml</td>
<td>1162</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>185</td>
</tr>
<tr>
<td>Ended Volume = 100 ml</td>
<td>283</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>36</td>
</tr>
<tr>
<td>Ended Volume = 75 ml</td>
<td>68</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>11</td>
</tr>
<tr>
<td>Ended Volume = 150 ml</td>
<td>20</td>
</tr>
<tr>
<td>Volume increased to 500 ml</td>
<td>5*</td>
</tr>
</tbody>
</table>

*obtained as clean Citrate-AgNP suspension with electrical conductivity 5 µS/cm.
Figure 4.9. DOC calibration curve produced by UV-vis absorbance measurement at 280 nm and the concentrations were verified by persulfate-UV oxidation procedure.

Figure 4.10. Molecular interaction of L-Cysteine with silver nanoparticle surface showing Ag-SH (thiolate bond between amino acid and Citrate–AgNP surface) and H-bonding among the CYS-AgNPs (adapted from Liu et al.\textsuperscript{4}).
Table 4.2. Characteristics of Citrate-AgNPs.

<table>
<thead>
<tr>
<th>material</th>
<th>pH</th>
<th>particle size distribution</th>
<th>average zeta potential (mV)</th>
<th>plasmon resonance spectra</th>
<th>average circularity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>hydrodynamic diameter (Mean ± S.D.) (nm)</td>
<td>TEM diameter (Mean ± S.D.) (nm)</td>
<td>λ(_{\text{max}}) (nm)</td>
<td>absorbance (au)</td>
</tr>
<tr>
<td>Citrate-AgNP</td>
<td>7.2</td>
<td>14.8 ± 0.9</td>
<td>56.5 ± 19.2 (n = 208)</td>
<td>-22.5 ± 1.8</td>
<td>445</td>
</tr>
</tbody>
</table>

\(a\) all measurements were taken at 2 mg/L Citrate-AgNPs; SD, Standard deviation of the sample; Smoluchowski equation estimated mean ζ potential from the electrophoretic mobility of the particles; mV, millivolt. \(λ_{\text{max}}\) represents maximum wavelength at which the peak was observed; n = number of particles analyzed for estimating particle diameter from Transmission Electron Microscopy (TEM) imagery; n = number of particles measured for estimating size from a TEM image using ImageJ 1.44. For stock nanosuspension, ζ potentials remained fairly similar before (-21.43 mV) and after (-25.13 mV) purification.

Table 4.3. Water quality parameters of moderately hard reconstituted water used as the test media for *Daphnia magna* bioassay.

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp (°C)</th>
<th>Conductivity (µS/cm)</th>
<th>DO (mg/L)</th>
<th>Alkalinity (mg/L as CaCO(_3))</th>
<th>Hardness (mg/L as CaCO(_3))</th>
<th>NH(_3)-N (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>20.5</td>
<td>379</td>
<td>8.42</td>
<td>45</td>
<td>85</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Analysis of Water Quality Parameters. Water quality parameters such as pH, temperature, dissolved oxygen (DO), conductivity, hardness, alkalinity, and ammonia-nitrogen were assessed in the test media, and were found to have acceptable quality as per the USEPA guidelines for *D. magna* culture.\(^{38}\) Total hardness, total alkalinity, and ammonia-nitrogen were measured using standard colorimetric HACH methods as described by the manufacturer (HACH Company, Loveland, CO). Water conductivity, pH, dissolved oxygen, and temperature were determined using Hanna Instrument HI9828 multiparameter meter (Transcat Inc., Rochester, NY).
References Cited in Appendix


CHAPTER 5

Evaluation of Developmental Responses of Two Crop Plants Exposed to Silver and Zinc Oxide Nanoparticles*

Lok R. Pokhrel, Brajesh Dubey

ABSTRACT

The increasing applications of engineered nanomaterials in the myriad of nano-enabled products and their potential for leaching have raised considerable environmental, health and safety (EHS) concerns. As systematic studies investigating potential anomalies in the morphology and anatomy of crop plants are scarce, herein we report on the developmental responses of two agriculturally significant crop plants, maize (*Zea mays* L.) and cabbage (*Brassica oleracea* var. *capitata* L.), upon *in vitro* exposure to nanoparticles of citrate-coated silver (Citrate–nAg) and zinc oxide (nZnO). Analyses involve histology of the primary root morphology and anatomy using light microscopy, metal biouptake, moisture content, rate of germination, and root elongation. Comparative toxicity profiles of the ionic salts (AgNO$_3$ and ZnSO$_4$) are developed. Notably, we uncover structural changes in maize primary root cells upon exposure to Citrate–nAg, nZnO, AgNO$_3$, and ZnSO$_4$, possibly due to metal biouptake, suggesting potential for functional impairments in the plant growth and development. Citrate–nAg exposure results in lower Ag biouptake compared to AgNO$_3$ treatment in maize. Microscopic evidence reveals ‘tunneling-like effect’ with nZnO treatment, while exposure to AgNO$_3$ leads to cell erosion in maize root apical meristem. In maize, a significant change in metaxylem count is evident with Citrate–nAg, AgNO$_3$, and ZnSO$_4$ treatment, but not with nZnO treatment (p > 0.1). In both maize and cabbage, measures of germination and root elongation reveal relatively lower

nanoparticle toxicity compared to free ions. As moisture data do not support osmotically-induced water stress hypothesis for explaining toxicity, we discuss other proximate mechanisms including the potential role of growth hormones and transcription factors. These findings highlight previously overlooked, anatomically significant effects of metal nanoparticles, and recommend considering detailed anatomical investigations in tandem with the standard developmental phytotoxicity assays (germination and root elongation) as the latter ones appear less sensitive for screening plant responses to nanomaterial insults.

1. Introduction

Novel functionalities (e.g., catalytic, electrical, mechanical, optical, and electromagnetic) that emerge at nanoscale (1–100 nm in at least one dimension; NNI, 2006; Maynard, 2011) have allowed for manufacturing nano-enabled products, which are being commercialized in the myriad of sectors including electronics, therapeutics, medical diagnostics, clothing, and personal care (www.nanotechproject.org). Among the many types of nanomaterials are the silver- and zinc-based nanoparticles (NP) that are increasingly used (www.nanowerk.com) for their prominent antibacterial, plasmonic, and opto-electrical properties (Pokhrel et al., 2012). The same particle-intrinsic novel properties that enabled wide applications of nanomaterials have, on the other hand, raised considerable environmental, health and safety (EHS) concerns (Poland et al., 2008; Auffan et al., 2009; FIFRA, 2009; Pokhrel et al., 2012; Pokhrel and Dubey, 2012). Despite particle size, surface charge/ligands, and surface area are generally identified as factors for NPs toxicity (El Badawy et al., 2011; Maynard, 2011; Silva et al., 2013), it has remained unclear how NPs impart toxicity to the biotic receptors (European Commission, 2008), including to the terrestrial plant species (NNI, 2006; Yin et al., 2011).

Terrestrial plants can be exposed to a multitude of nanomaterials through soil in many ways:
potential leaching from nano-enabled products, intentional sub-surface release for environmental remediation (e.g., nano zero valent iron for trichloroethylene removal; Zhang et al., 2003), surface run-off, irrigation using contaminated surface water, land applications of contaminated biosolids, or wastewater effluent discharge. Although some progress toward understanding nanotoxicology has been achieved studying microbial populations, algae, animal models, or mammalian cell lines (Navarro et al., 2008; Yu et al., 2009; El Badawy et al., 2011; Pokhrel et al., 2012; Pokhrel and Dubey, 2012, 2013; Silva et al., 2013), yet understanding of nanotoxicity in higher plants is limited to few studies, which evaluated biouptake/bioaccumulation (Gardea-Torresdey et al., 2003; Lin and Xing, 2007, 2008; Khodakovskaya et al., 2009; Judy et al., 2011; Rico et al., 2011; Yin et al., 2011), phenotypic changes (e.g., root/shoot length, biomass; Lin and Xing, 2007; Khodakovskaya et al., 2009; Rico et al., 2011), biodistribution (Lin and Xing, 2008; Khodakovskaya et al., 2009), or the DNA damage (Atha et al., 2012). More recently, measuring the seedling growth in *Phaseolus radiatus* and *Sorghum bicolor*, Lee et al. (2012) reported particle-mediated toxicity of citrate-coated silver nanoparticles (Citrate–nAg) in soil, while free Ag⁺ toxicity was found to be more pronounced when tested in agar medium. A concentration-dependent inhibition of two different sized (20 nm vs. 100 nm diameter) AgNPs on the biomass growth and frond number, including the greater free Ag⁺ effects compared to AgNPs, were observed in *Lemna minor* (Gubbins et al., 2011). However, the toxicity of gum Arabic-coated AgNPs was higher compared to the same concentration of dissolved Ag⁺ in *Lolium multiflorum*, with greater bioaccumulation occurred with AgNP treatment than with Ag⁺ (Yin et al., 2011). Studying *Zea mays*, Zhao et al. (2012) analyzed multiple variables related to stress upon exposure to CeO₂ (cerium dioxide) NPs. Interestingly, the authors found no lipid peroxidation (despite higher H₂O₂ in leaves) or ion leakage, and that the physiological measures
(transpiration, photosynthesis, and stomatal conductance) also remained unchanged. In the same study, the authors, however, found increased enzyme activity (catalase and ascorbate peroxidase) and upregulation of heat shock protein (Hsp70), suggestive of protective effects of CeO$_2$ NPs in maize (Zhao et al., 2012). In *Lolium perenne*, considerable effects of ZnO NPs in the root epidermis and cortex, with internalization of NPs in the endodermal and vascular tissues were observed (Lin and Xing, 2008). Studies have also shown plant potential to biotransform metallic ions into NPs during their growth and development; although the mechanism of such *in vivo* transformation is not known (Gardea-Torresdey et al., 2003).

Given the notable inconsistency among the studies assessing phytotoxicity of NPs (Stampoulis et al., 2009; Yin et al., 2011; Lee et al., 2012) and the lack of understanding of whether alterations might occur in the cellular morphology of the vital structures (e.g., in the root tip, the zone of elongation, and the metaxylem vessels) during plant development, a comprehensive study of the root anatomy would then allow uncovering any cellular anomalies following exposure to metal-based NPs. Alterations in the shape and size of the cells at earlier stages of growth and development are known to cause severe functional impairments associated with tissue differentiation and solute transport in young plants (Puertas-Mejia et al., 2010; Delmail et al., 2011). Hence, it becomes critically important to address these knowledge gaps and explore the proximate mechanisms of the observed toxicity of NPs.

In this study, we investigate the potential developmental phytotoxicity of citrate-coated silver (Citrate–nAg) and zinc oxide (nZnO) nanoparticles against two agriculturally significant crop plants, maize (*Z. mays* L.) and cabbage (*Brassica oleracea* var. *capitata* L.), by systematically providing complimentary information through histological examinations of the primary root morphology and anatomy, and analyses of metal biouptake, moisture content, germination rate,
and root elongation. Toxicity profiles of the ionic salts (i.e., AgNO₃ and ZnSO₄), including of the untreated controls (moderately hard water), are developed for comparison purposes. While we determine variability in moisture content and metal biouptake, other proximate mechanisms (involving growth hormones and transcription factors) explaining the observed toxicity are discussed.

2. Materials and methods

2.1. Materials characterization

As previously stated, nanoparticles of citrate-coated silver (Citrate–nAg) and zinc oxide (nZnO) were tested for potential developmental phytotoxicity against maize (Z. mays L. cv. NK-199) and cabbage (B. o. var. capitata L. cv. Golden Acre). The synthesis procedure for Citrate–nAg is presented in the Supplementary information. Citrate–nAg was purified using a tangential flow filtration (TFF) process (detailed in the following section). nZnO was procured as an aqueous suspension from Meliorum Technologies, Inc., NY, USA (purity – 99.9%) and was used as-obtained. For comparison, AgNO₃ and ZnSO₄ were tested for their potential toxicities. The ionic salts were purchased from Fisher Scientific, Inc., USA (AgNO₃, USP grade; ZnSO₄, ACS grade, purity: 99–102%).

NPs were characterized using Uv-vis spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM). The surface Plasmon resonance (SPR) spectra were recorded using a HACH DR5000 spectrophotometer (HACH Company, CO, USA). A PSS Nicomp 380ZLS particle sizer/zeta potential unit (Particle Sizing Systems, CA, USA) was calibrated at 23°C with a Duke 500 (491 nm) NIST 3490A standard (PSS Nicomp, FL, USA) before measuring hydrodynamic diameters (HDD) and zeta (ζ) potentials. The Smoluchowski equation was used to estimate ζ potential from electrophoretic mobility of the particles. A Philips
EM 420 transmission electron microscope (TEM) was used to image NPs by dropping an aliquot of sample onto a carbon-coated copper formvar grid (Ted Pella, Cat # 01813-F) and, allowing it to air dry before recording images, operating TEM at 120 kV in the bright-field mode. By importing representative TEM images into ImageJ 1.44 program (www.rsb.info.nih.gov/ij/), particle size distributions (PSD) were determined. An inductively coupled plasma-mass spectrometer (Bruker 820-ICP-MS) was used to quantify total metal concentrations in the test suspensions, prior to which samples were digested using nitric acid (trace metal grade) following the standard USEPA method 3050B.

2.2. Purification of Citrate–nAg

As-synthesized Citrate–nAg was cleaned using polysulfone (PS) 10 kD hollow fiber diafiltration membranes (P/N: X31S-300-02P, surface area = 145 cm²) connected with a KrosFlo Research IIi TFF System and controlled via KF COMM software (ver. S16; Spectrum Laboratories, CA). A suitable shear pressure generated by pump head enabled maintaining a tangential peristaltic flow of aqueous suspension through the hollow fiber membranes. These membranes are designed to permit lesser than 10 kD (~1–2 nm) sized particles including dissolved ions and residual impurities to pass across the membranes and forming the permeate, while allowing greater than 10 kD sized particles to flow through the membrane’s lumen and forming the retentate. This process involved buffer exchange of the residual impurities and ions present in the as-synthesized Citrate–nAg suspension with nanopure water (electrical conductivity = 2 µS/cm). A measure of electrical conductivity was used as an established surrogate (El Badawy et al., 2010; Kanel and Al-Abed, 2011; Pokhrel et al., 2012), indicating likely absence of ions and/or impurities accounting for suspension conductance (Appendix Table D1). Characterization of permeate and cleaned Citrate–nAg was explained earlier (Fig. 5.1,
2.3. Seed preparation and exposure

Untreated seeds of maize and cabbage were purchased from Eden Brothers, Dahlonega, GA, USA. Seeds were visually inspected for any morphological damage or discoloration, cleaned three times with sterile water, followed by immersion in 70% ethanol for two minutes to ensure seed surface sterility. Soon the seeds were washed several times with sterile water to remove any left-over ethanol from the surface. Onto each Petri dish (100 mm x 15 mm) was placed a filter paper (Yang and Watts, 2005; Atha et al., 2012) moistened with 3 mL of sterile nanopure water to prevent it from soaking the test chemical, while, at the same time, allowing the seeds to be thoroughly exposed to the test chemical.

Ten seeds were randomly assigned onto each Petri dish, and were manually spaced apart from each other by at least a cm. Each dish received 5 ml of the test chemical, or a 5 ml of sterile moderately hard water (MHW) as an untreated control (Yang and Watts, 2005). NPs or their ionic forms were diluted in sterile MHW to obtain the desired concentrations. The suspended NP stability or the state of aggregation in MHW (as a diluent) was evaluated using the DLS method (Appendix Table 5.5). All test runs were conducted in triplicates, including that of the controls, unless noted otherwise. Seeds were allowed to germinate in dark for about a week at 23 ± 1 °C to offer optimal growth condition, prior to which seeds were soaked in sterile MHW for 24 h to allow water imbibition. Seed preparation and assignment into the dish were performed under the laminar flow hood to maintain sterile working environment. The pH of all the test chemicals was maintained within a narrow range (6.8–7.2).

2.4. Developmental phytotoxicity assessment

The seeds were exposed for six (cabbage) or seven (maize) days to each test chemical,
following which experiments were ended when the controls displayed over 80% germination; the root length was measured (in mm) and germination rate was recorded for each replicate. The USEPA OPPTS 850.4200 guideline that seeds are considered germinated when the root measures at least 20 mm in length for the controls was followed (USEPA, 1996). Relative seed germination inhibition and relative root growth inhibition were calculated using the following standard equations (USEPA, 1996).

\[
\text{Relative Seed Germination Inhibition (\%) = } \left( \frac{\text{Germination}_{\text{Control}} - \text{Germination}_{\text{Treat}}}{\text{Germination}_{\text{Control}}} \right) \times 100 \quad \text{(i)}
\]

\[
\text{Relative Root Growth Inhibition (\%) = } \left( \frac{\text{RE}_{\text{Control}} - \text{RE}_{\text{Treat}}}{\text{RE}_{\text{Control}}} \right) \times 100 \quad \text{(ii)}
\]

Where RE denotes root elongation, and Treat indicates treatment group.

As shown in Fig. 5.3, the concentrations at which significant effect was observed in root elongation measure were chosen for each test chemical for comprehensive microscopic studies in maize. Maize was chosen because of its sturdy nature of the root, which enabled free-hand sectioning. Detailed investigations of the root morphology and anatomy were conducted focusing on potential alterations that might occur in the cellular structure and alignment in the root tip and zone of elongation, including the metaxylem vessel counts. The sectioned root piece was stained using Toluidine Blue O (0.05%) for 30 s, washed with running water for one min, and temporarily mounted on the glass slide using glycerin. Both longitudinal and transverse sections of fresh young roots were observed under microscope within three hours of slide preparation. Samples were analyzed using an Olympus BX41 system microscope and images were acquired using an Olympus MicroFire™ color digital camera and PictureFrame imaging software (Olympus America, Inc. NY).
2.5. Moisture analysis

To quantify moisture content in the seedlings and that retained in the filter papers, separate experiments were conducted. For this, each Petri dish was assigned one filter paper and nine maize seeds. The experiment comprised of treatments with Citrate–nAg (73.4 µg total Ag/mL) or AgNO₃ (127 µg total Ag/mL), and comparisons were made with the control. To analyze the moisture content that retained in the filter papers, after 7 days each (wet) filter paper was weighed and oven dried at 105 °C overnight (Gubbins et al., 2011). The difference between the wet weight and the dry weight of filter paper was calculated as the moisture content; the data were averaged for two replicates and expressed as % moisture per dry weight of filter paper. After 7 days of experimental period, the seedlings (whole seedlings analyzed) were weighed (initial fresh weight), then oven dried at 105 °C overnight to estimate dry weight (Gubbins et al., 2011). The difference between the wet- and dry weight of nine seedlings was calculated as moisture content, and the values were averaged for two replicates (n = 18 seedlings) and expressed as % moisture per dry weight of the seedlings.

2.6. Biouptake of silver

Nine oven-dried seedlings of maize from each replicate were ashed in the Muffle furnace at 550 °C for 30 min and digested following the USEPA method 3050B (using ultrapure HNO₃). The samples were analyzed using an ICP-MS (Bruker 820-ICP-MS) to determine total Ag concentration in each replicate; data were averaged for two replicates (n = 18 seedlings).

2.7. Data analysis

Our data were normally distributed (Kolmogorov-Smirnov (K-S) test: \( p > 0.1 \) in all cases); except for a measure of root elongation in cabbage with nZnO treatment (K-S test: \( Z = 1.644, p < 0.01 \)), which were then log-transformed for use in further analyses as they satisfied normality (K-
S test: $Z = 1.194, p > 0.1$; Pokhrel et al., 2013a). Data were analyzed for significant differences in the variances and means between the treatment and the control. When sample variances were significantly different from the control, the t- and p-values were adjusted for independent samples t-test, which tested the null hypothesis that the treatment means are not significantly different from the control at the $p \leq 0.05$ level. The Chi-square goodness-of-fit test was used to test if all treatments, including the control, contained the same proportion of the metaxylem counts. Data for Citrate–nAg are reported as total Ag, while other test chemicals are reported as the respective compounds, unless otherwise noted. Data were plotted as the concentration-response curves for the measures of root elongation and seed germination (%). EC$_{50}$ (i.e., effective concentrations for 50% inhibition) values were estimated using the linear regression analysis.

3. Results and discussion

3.1. Characteristics of nanoparticles

Both types of NPs had the same HDD (11 ± 0.7 nm) and their characteristic SPR spectra are presented in Appendix Fig. 5.9. On average, TEM diameter was larger for Citrate–nAg than nZnO (Table 5.1), with particles appearing polymorphic for Citrate–nAg, and roughly oval for nZnO (Fig. 5.1). Detailed particle characteristics are presented in Fig. 5.1, Table 5.1, and Appendix Table 5.5. Evaluation of HDD and $\zeta$ potential values revealed that both types of NPs were fairly stable in MHW (Appendix Table 5.5).

3.2. Impact of metal nanoparticles on the primary root tip in maize

Exposure to nZnO (1000 $\mu$g/mL) caused ‘tunneling-like effect’, characterized by a deep invagination in the primary root tip in maize (Fig. 5.2b; Appendix Fig. 5.5). It is likely that the tender cells of apical meristem upon contact with nZnO suspension led to cell dissolution from
Fig. 5.1. Representative transmission electron microscopy (TEM) images of Citrate–nAg (scale 50 nm; a), and nZnO (scale 20 nm; c). Particle size distributions of Citrate–nAg (b) and nZnO (d) were estimated using ImageJ 1.44 program and are provided in Table 5.1. Number of particles analyzed for both types of nanoparticles = 215. Cellular structural disintegration; this, however, was not observed with dissolved Zn\(^{+2}\) treatment (Figs. 5.2b and 5.2c). Because the frequency of ‘tunneling-like effect’ was low (one out of total four samples analyzed), it is possible that the effect could be a rare occurrence as no significant impact on the physical root growth of the seedlings was observed (Fig. 5.3a). Alternately, the
variability in individual sensitivity to nZnO might explain this discrepancy. In maize, an erosion of apical meristem cells were observed upon exposure to free Ag\(^+\) (as AgNO\(_3\); 200 µg/mL; Fig. 5.2e) which led to reduced root growth (Fig. 5.3c; Appendix Table 5.7).

**Table 5.1**

Characteristics of nanoparticles evaluated for developmental phytotoxicity in maize and cabbage.

<table>
<thead>
<tr>
<th>Material</th>
<th>pH</th>
<th>Particle size distribution (PSD)</th>
<th>Average zeta potential** (mV)</th>
<th>Plasmon resonance spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>manufacturer reported mean particle size (nm)</td>
<td>hydrodynamic diameter* (Mean ± S.D.) nm</td>
<td>TEM diameter (Mean ± S.D.) nm</td>
</tr>
<tr>
<td>Citrate–nAg</td>
<td>7.24</td>
<td>na</td>
<td>11.0 ± 0.7</td>
<td>56.1 ± 13.8 (n = 215)</td>
</tr>
<tr>
<td>nZnO(^a)</td>
<td>7.03</td>
<td>10</td>
<td>11.0 ± 0.7</td>
<td>17.4 ± 4.9 (n = 215)</td>
</tr>
</tbody>
</table>

\(^a\) Purchased from Meliorum Technologies, Inc., NY, USA; * Volume weighted particle size distribution; ** Average zeta potential approximated using Smoluchowski equation by NICOMP 380 ZLS Zeta Potential unit; \(\lambda_{max}\) represents maximum wavelength at which surface Plasmon peak was observed; na, data not available (lab. synthesized); n = number of particles measured for sizing from TEM images using ImageJ 1.44 program.

Root hair density, however, appeared unaffected with NPs or with corresponding dissolved ions at the evaluated concentrations (Appendix Fig. 5.6). On exposure to 100 µg AgNO\(_3\)/mL, maize roots appeared generally thinner with tips dried out and red pigmented that severely progressed as concentration increased to 200 µg AgNO\(_3\)/mL, followed by significant germination and root growth inhibitions that occurred at 500 µg AgNO\(_3\)/mL (Fig. 5.3c; Appendix Fig. 5.12, Tables 5.6 and 5.7).
Fig. 5.2. Impacts of metal nanoparticles or their corresponding ionic salt on the primary root tip in maize (*Zea mays*). Exposure to nano-ZnO (1000 µg/mL) caused ‘tunneling-like effect’ (b), while apical meristem was eroded with AgNO₃ (200 µg/mL) treatment causing reduced root elongation in maize seedlings (e). Control (a), ZnSO₄ (1000 µg/mL; c), and Citrate–nAg (73.4 µg/mL; d). Magnifications are shown at the bottom right corner of each panel. Arrows indicate areas of potential impacts.
Fig. 5.3. Concentration response curves showing effects of nanoparticles or their corresponding ionic salts on the root growth and *Zea mays* (top panels a–c) and *Brassica oleracea* var. *capitata* (bottom panels d–e). Error bars represent ± 1 standard error (SE) of the means. Significant difference between the treatment means and the control was tested using the Independent samples t-test at the 0.05 level (*, p < 0.05). nAg denotes Citrate–nAg; C denotes control.

3.3. Biouptake of Ag and analysis of moisture content

Quantification of silver in maize seedlings (whole seedlings analyzed) showed more than five order of magnitude (55x) higher Ag (as total Ag) uptake with AgNO$_3$ (200 µg/mL) treatment than the baseline control. In contrast, this biouptake was 4.5 times higher with Citrate–nAg treatment than the control. The biouptake concentrations were 22 and 1.8 ng Ag/mg dry weight of seedlings for AgNO$_3$ and Citrate–nAg treatments, respectively (Fig. 5.4; Appendix Table 5.11). This was 12.2-fold lower uptake of Ag with Citrate–nAg treatment compared to AgNO$_3$ treatment. When exposure concentrations were normalized, this biouptake was 7.06-fold lower (as total Ag) with Citrate–nAg compared to AgNO$_3$ treatment, suggesting that minimal dissolution of Ag might have emanated of Citrate–nAg surface during the test period.
Analysis of average moisture content in the seedlings revealed greater moisture content with AgNO₃ (168.7% - dry wt.) or Citrate–nAg (163.9% - dry wt.) treatment compared to the control (147.3% - dry wt.). A similar trend was observed for moisture content that was retained in the growth substrate (Fig. 5.5; Appendix Table 5.11). This is consistent with a previous study that showed greater seed moisture content in tomato treated with multi-walled carbon nanotubes in a two day experiment, while the mechanism behind enhanced water uptake by the seeds remained unclear (Khodakovskaya et al., 2009). It is possible that increased water uptake by the seedlings might have facilitated greater Ag biouptake with AgNO₃ treatment compared to Citrate–nAg treatment in the current study.

3.4. Impact of metal nanoparticles on root elongation

In maize, the primary root cells were structurally modified in shape and/or size at the zone of elongation upon exposure to metal NPs or the dissolved ions evaluated (Fig. 5.6). Cells were elongated with both NP treatments (Figs. 5.6b and 5.6d); they appeared thinner and irregular with AgNO₃ treatment (Fig. 5.6e); while with ZnSO₄ treatment cells exhibited shorter and wider morphology, compared to the control (Figs. 5.6a and 5.6c). These contrasting observations for metal NPs versus corresponding ionic salts suggested that the same mechanism(s) could not be responsible for the observed toxicity pattern, and/or this effect might be due to differential metal uptake as observed in maize seedlings. Phenotypic root elongation measurement (Figs. 5.3a–5.3c) was in good agreement with the microscopic observations of the cells at the zone of elongation (Fig. 5.6). In maize, unlike with the AgNO₃ or ZnSO₄ treatment that resulted in significant dose-dependent effects, Citrate–nAg or nZnO treatment had little to no effect on root growth (p > 0.05 in all cases; Figs. 5.3a, 5.3b and 5.3c; Appendix Table 5.7). In cabbage, root elongation was not affected by nZnO or ZnSO₄ in an excess of 100 µg/mL exposure level (Fig.
Fig. 5.4. Increased silver biouptake occurred with AgNO₃ (exposed concentration = 127 µg total Ag/mL) treatment compared to Citrate–nAg (exposed conc. = 73.4 µg total Ag/mL) treatment in Zea mays seedlings. When exposure concentrations were normalized, Ag biouptake (as total Ag) was 7.06-fold greater for AgNO₃ compared to Citrate–nAg treatment. Two replicates were analyzed (n = 18), each containing 9 seedlings, to determine total Ag concentrations using an ICP-MS; data represent means of two replicates. Ag recovery was 103%.

Fig. 5.5. Variation in average moisture content in Zea mays seedlings and that retained in the filter paper with Citrate–nAg or AgNO₃ treatment. Each data point represents an average of % moisture content (dry weight basis) pooled from two replicates, with each replicate consisting of nine seedlings or a filter paper used as the growth substrate.
Table 5.2
Comparison of effective concentration for 50% inhibition (EC$_{50}$) in the rate of germination (%) and root elongation in maize and cabbage.

<table>
<thead>
<tr>
<th>Plant type</th>
<th>EC$_{50}$ for germination rate</th>
<th>EC$_{50}$ for root elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/mL)</td>
<td>(µg/mL)</td>
</tr>
<tr>
<td>nZnO</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.05</td>
<td>192</td>
</tr>
<tr>
<td>Citrate–nAg</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Ag$^+$</td>
<td>1.42</td>
<td>78.2</td>
</tr>
<tr>
<td>Citrate–nAg</td>
<td>na</td>
<td>8.89</td>
</tr>
<tr>
<td>Ag$^+$</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Zea mays</td>
<td>136</td>
<td>53.2</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>9.72</td>
<td></td>
</tr>
<tr>
<td>var. capitata</td>
<td>1.42</td>
<td></td>
</tr>
</tbody>
</table>

‘na’ indicates data not available as EC$_{50}$ was not estimated due to less than 50% inhibition at the highest concentration tested; ZnSO$_4$ was used as a source of Zn$^{2+}$ and AgNO$_3$ was used as a source of Ag$^+$; the EC$_{50}$ values for ionic salts are presented for Zn$^{2+}$ and Ag$^+$.

5.3d). However, root development was completely halted as seeds were unable to germinate at higher ZnSO$_4$ concentrations ($t = 10.288$, $p < 0.001$; Appendix Tables 5.8 and 5.9), unlike nZnO treatment that showed only 40.6% growth inhibition at the highest concentration tested (i.e., 1000 µg nZnO/mL; $p < 0.005$; Fig. 5.3d; Appendix Table 5.9).

At concentrations greater than 10 µg/mL, root growth was significantly inhibited by AgNO$_3$ in a dose-dependent manner ($p < 0.05$ in all cases), while Citrate–nAg inhibited root growth by only 24.1% at the highest tested concentration (73.4 µg/mL as total Ag) compared to the control in cabbage ($t = 2.648$, $p < 0.05$; Figs. 5.3e and 5.3f; Appendix Table 5.9). These data coupled with their EC$_{50}$ values (Table 5.2), defined as an effective concentration at which 50% inhibition is observed, revealed lower toxicity of metal NPs than their corresponding free ions on the primary root growth and development in maize and cabbage. Moreover, Ag$^+$ was found more toxic than Zn$^{2+}$ as shown by the root elongation measurements (Fig. 5.3), or their corresponding
**Fig. 5.6.** Effects of metal nanoparticles and their corresponding ionic salt in the zone of elongation in *Zea mays.* Control (a), nZnO (1000 µg/mL; b), ZnSO$_4$ (1000 µg/mL; c), Citrate–nAg (73.4 µg/mL; d), and AgNO$_3$ (200 µg/mL; e). Note relatively elongated cells with nanoparticles treatments (b, d), but cells appeared thinner and irregular upon Ag$^+$ exposure (e), and showed shorter and wider morphology with Zn$^{2+}$ treatment (c), compared to the control (a).
3.5. Impact of metal nanoparticles on metaxylem vessel count

Unlike the protoxylems, the metaxylem vessels are not transient and are retained throughout plant life representing permanent transportation conduits in mature plants (Esau, 1977). It is known that the xylem vessel number will increase when plants are subjected to water-stress and would decrease upon supply of adequate water (Zimmermann et al., 1993; Holbrook et al., 2001; Schneider et al., 2007). Hence, the measurement of metaxylem number in plants has been used to understand osmotically-induced stress by water loss (Zimmermann et al., 1993; Holbrook et al., 2001; Schneider et al., 2007). Growth-hormones such as auxin and cytokinin are also known to influence xylem vessel formation (Fukuda, 2004; Kubo et al., 2005). Although genetic mechanism of xylem formation has been poorly understood, plant specific transcription factors such as VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 have been recently attributed to induction of trans-differentiation of various cell types into metaxylem- and protoxylem-like vessel elements (Kubo et al., 2005). To date, whether exposure to metal-based NPs could modulate the frequency of metaxylem vessel has not been documented in plants, but contextual evidence from the studies involving heavy metal stress on plants stimulated this part of research (Mufarrege et al., 2010; Delmail et al., 2011).

Differential metaxylem counts were observed upon exposure to metal NPs or their respective ionic salts (Table 5.3). The metaxylem counts ranged from 5 – 6 in the control, which increased to 7 upon exposure to ZnSO$_4$ (1000 µg/mL) or AgNO$_3$ (200 µg/mL; Fig. 5.7; Table 5.3). While with either type of NP treatment (Citrate–nAg: 73.4 µg Ag/mL; nZnO: 1000 µg/mL), metaxylem counts fluctuated in the range of 5 – 6 (Fig. 5.7). The Chi-square test showed Citrate–nAg, ZnSO$_4$, or AgNO$_3$ treatment led to significantly different proportion of metaxylem frequency in
Fig. 5.7. Metaxylem vessel count varied with treatments of metal nanoparticles and corresponding ionic salts in Zea mays. Control (a, b), nZnO (1000 µg/mL; c), ZnSO₄ (1000 µg/mL; d), Citrate–nAg (73.4 µg/mL; e), and AgNO₃ (200 µg/mL; f). Note, induction of 7 metaxylem vessels upon free Ag⁺ (f) or Zn²⁺ treatment (d). mx, metaxylem. Metaxylem count data are presented in Table 5.3 and Appendix Figure 5.14.
maize primary root; however, with nZnO treatment the proportion of metaxylem count was not statistically different ($p > 0.1$; Table 5.3). Because with either ionic salt treatment, formation of seven metaxylem vessels was observed in maize (Table 5.3), this is indicative of higher stress exerted by biolabile metal ions than the evaluated NPs.

**Table 5.3**

Effect on metaxylem count in the primary root of *Zea mays* upon exposure to metal nanoparticles or their corresponding ionic salts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metaxylem count</th>
<th>samples observed (n)</th>
<th>remarks</th>
<th>$\chi^2$, p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µg/mL)</td>
<td>#5 (%)</td>
<td>#6 (%)</td>
<td>#7 (%)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(62.5)</td>
<td>(37.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nZnO (1000)</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(50)</td>
<td>(50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate–nAg (73.4)</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(46.1)</td>
<td>(53.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO$_4$ (1000)</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(85.7)</td>
<td>(14.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgNO$_3$ (200)</td>
<td>2</td>
<td>15</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>(7.4)</td>
<td>(55.5)</td>
<td>(37.0)</td>
<td></td>
</tr>
</tbody>
</table>

The Chi-Square ($\chi^2$) goodness-of-fit test was used to test if all treatments, including the control, contained the same proportion of metaxylem vessel number at the $p = 0.05$ level.

These results suggested that the evaluated NPs had lower effects on the primary root anatomy, possibly due to lower metal biouptake (as shown by total Ag analysis for maize seedlings; Fig. 5.4), or perhaps due to different mode of action between NPs and the respective ionic salts. Given the significance of growth hormones and plant specific transcription factors in metaxylem vessel formation (Fukuda, 2004; Kubo et al., 2005), it remained to be understood if enhanced metal (e.g., Ag, and perhaps Zn) uptake in this study (Appendix Table 5.13) promoted
formation of additional metaxylem vessels. Because water stress did not likely occur under our experimental scenario as demonstrated by the moisture analysis data (Fig. 5.5), involvement of some other mechanisms such as the stimulation of the growth hormones (e.g., auxin or cytokinin) or the transcription factors (such as VND6, VND7) in inducing additional metaxylem vessels in maize cannot be ruled out. Thus, studies designed to address putative signaling mechanisms, which might be involved in differential increase in metaxylem frequency under metal/NM stress, at physiological and biomolecular levels may provide further understanding of the observed toxicity.

3.6. Impact of metal nanoparticles on seed germination

In maize, exposure to a wider range of nZnO concentrations (0.01 - 1000 µg/mL) did not inhibit seed germination (p > 0.1 in all cases; Fig. 5.8a; Appendix Table 5.6), unlike in cabbage a dose-dependent germination inhibition occurred (Fig. 5.8d; Appendix Table 5.8). These data showing little to no inhibition of germination with nZnO treatment in maize are consistent with the previous findings that used the same plant species and similar experimental conditions (El-Temsah and Joner, 2012). In contrast, exposure to ZnSO₄ caused significant germination inhibition in both maize (p < 0.05) and cabbage (p < 0.05; Figs. 5.8a and 5.8d; Appendix Tables 5.6 and 5.8). While complete germination inhibition occurred at 500 µg ZnSO₄/mL in cabbage, it only led to ca. 50% germination inhibition in maize at the same concentration (Appendix Tables 5.6 and 5.8).

Citrate–nAg showed some potential to inhibit germination in maize; this inhibition was, however, always less than 35% even at the highest concentration tested (73.4 µg/mL; p > 0.1 compared to the control; Fig. 5.8b; Appendix Table 5.6). Germination was not significantly affected in maize upon exposure to an excess of 200 µg AgNO₃/mL (p > 0.1), while at 500 µg
AgNO$_3$/mL germination was significantly inhibited by 84.4% (p < 0.05; Fig. 5.8c; Appendix Fig. 5.12, Table 5.6). In cabbage, a concentration-dependent germination inhibition was observed with AgNO$_3$ treatment, and this was greater than the effect caused by Citrate–nAg at comparable concentrations (Figs. 5.8e and 5.8f; Appendix Table 5.8). While both the evaluated metal-based NPs showed lower inhibition of seed germination in maize, Ag$^+$ ions also exhibited lower

![Graphs showing concentration-response curves for Zea mays and Brassica oleracea var. capitata.](image)

**Fig. 5.8.** Concentration-response curves showing effects of metal nanoparticles and their ionic salts on seed germination in *Zea mays* (top panels a–c) and *Brassica oleracea* var. capitata (bottom panels d–f). Error bars represent ± 1 standard error (SE) of the means. Significant difference between the treatment means and the control was tested using Independent samples t-test at the 0.05 level (*, p < 0.05). nAg, Citrate–nAg; C, control.

toxicity at ≤ 200 µg AgNO$_3$/mL; whereas cabbage displayed higher sensitivity toward both the NPs and their ionic salts (Fig. 5.8). The corresponding EC$_{50}$ values for NPs and their ions against seed germination are presented in Table 5.2.
Although characterization of NP morphology via TEM revealed variability in average particle diameter, the DLS measurements revealed similar average HDDs for both types of NPs and were smaller than the TEM values for particle diameter (Fig. 5.1; Table 5.1; Appendix Table 5.5). Literature suggests that different sizing techniques, such as TEM and DLS, may offer some level of bias, and should be used as complementary to each other (Ito et al., 2004; Pokhrel et al., 2013b). Given that average HDDs of both types of NPs were similar in aqueous suspension (Table 5.1; Appendix Table 5.5), consideration of contrasting seed sizes of the tested plant species indicates that the potential interactions of similar-sized NPs might be greater for the smaller seeds of cabbage compared to the larger seeds of maize, largely due to greater surface-to-volume ratio of the small-sized seeds to that of the large-sized seeds (Lin and Xing, 2008). These results corroborate the findings of an earlier study reporting higher toxicity of the rare earth oxide NPs to the smaller seeds of rape, radish, and cabbage compared to the larger seeds of wheat (Ma et al., 2009). Alternately, this may indicate plant species-specific toxic response as reported for Citrate–nAg (particle diameter = 2–20 nm) showing germination inhibition in ryegrass and barley (El-Temsah and Joner, 2012), but not in flax (El-Temsah and Joner, 2012) or lettuce at as high as 100 µg/mL levels (Barrena et al., 2009).

Nanomaterials are emerging environmental contaminants for which potential impacts on the terrestrial crop plants are far from clear (Stampoulis et al., 2009; Yin et al., 2011; Yin et al., 2013). In nature, plants are exposed to various stressors, including that of chemicals, at different stages of their life cycles. Exposure to engineered NMs has shown to inhibit germination, seedling growth, and development in plants in the laboratory experiments (Gubbins et al., Lin and Xing, 2007, 2008; Stampoulis et al., 2009; Yin et al., 2011; Yin et al., 2013). The inhibition may arise due to several reasons; however, specific ion toxicity, osmotically-induced stress due
to water loss, or hormonal imbalance are among the predominant factors being explored to explain observed toxicity in many plant species (Khodakovskaya et al., 2009; Stampoulis et al., 2009; Nawaz et al., 2010). In the current study, development of toxicity profiles of two predominantly used metal NPs, including their ionic salts (i.e., AgNO₃ and ZnSO₄), against two agriculturally significant plant species (i.e., maize and cabbage) was achieved by evaluation of moisture content in the 7 day old seedlings and the substrate used, and more importantly, the histological details revealing anomalies in maize root anatomy provided information on the potential toxic responses of the maize and cabbage seedlings upon in vitro exposure to a wide exposure range (0.01–1000 µg/mL) of metal-based NPs and ions. As novel properties can be acquired by manipulating surface moieties, surface charge, or size of the nanoscale materials, any changes in NP properties could trigger different biological responses, and thus may lead to unanticipated toxicity (Williams et al., 2010). Our observations of ‘tunneling-life effect’ in maize apical meristem with nZnO treatment and relatively elongated cell morphology at the zone of elongation with nZnO and Citrate–nAg treatments are consistent with the fact that NMs may cause different biological interactions and thus the responses compared to their specified ion toxicity. A study by Stampoulis et al. (2009) has recently shown higher toxicity of Ag (particle diameter = 100 nm) and ZnO (particle diameter = 5 nm) NPs to the seedlings of *Cucurbita pepo* compared to their bulk powder form evaluated or the controls. Although reports on plant species-specific toxicity (Ling and Xing, 2008; Ma et al., 2010) and assay-dependent toxicity (Stampoulis et al., 2009; Lee et al., 2012) tend to complicate the limited understanding of phytotoxicity of NMs, it nonetheless highlights the need to explore various complimentary and measureable endpoints to capture enough information explaining the observed differences in toxicity for metal-based nanomaterials versus their free ions.
Studies have shown that reduced vacuole size could result in decreased cell turgidity, which could subsequently cause cell walls to deform and thus irregular cell alignments as observed in maize leaf or in barley (*Hordeum vulgare*) root with cadmium treatment (Puertas-Mejia et al., 2010). In addition, binding of metals onto the cell walls and to other sub-cellular structures could lead to a loss in cell wall elasticity (Sieghardt, 1984; Barceló et al., 1986). Our results showing cellular alterations in the zone of elongation, especially by ionic Ag\(^+\) and Zn\(^{+2}\), possibly due to higher metal uptake, suggest that similar mechanisms might be responsible for such altered cellular phenotype. Studies show plant hormones, especially gibberellins, can induce seed germination and cell elongation (Celik et al., 2008; Finch-Savage and Leubner-Metzger, 2006). This research provides a basis to test whether our observation of elongated cellular phenotype in maize under metal-based NP treatments (Fig. 5.6) might be associated with metal NP-mediated synthesis of plant hormones, such as gibberellins, causing cells to grow longer than the control.

Inclusion of several anatomical features as examined using light microscopy, which were in good agreement with the plant phenotypic responses (i.e., corresponding germination and root elongation measurements; Figs. 4.3 and 4.8), should offer a comprehensive understanding of the potential toxic responses of the crop plants at early developmental stage on exposure to metal-based NPs or their corresponding ionic counterparts. Although silver biouptake by maize seedlings in our experimental period was lower (ng/mg dry weight), AgNO\(_3\) treatment resulted in 7.06-fold higher uptake than with Citrate–nAg treatment when exposure concentrations were normalized (Fig. 5.4; Appendix Table 5.11). It is important to note that these anatomical anomalies were observed at ng/mg level of biouptake (measured for Ag), which corresponded to higher exposure concentrations (≥ 73.4 µg/mL) that were chosen based on the root elongation measurements as shown in Figure 4.3. Although growing use of NMs and nano-enabled products
particularly associated with metal NPs could inevitably lead to environmental contamination, our observations of cellular alterations in different regions and structures in maize primary root offer valuable information on potential hazard of metal-based NPs to the crop plants. The prospective anatomical impairments that could occur at environmentally realistic concentrations (sub µg/L level) may not be directly emulated from our histological evidence of altered cellular phenotype, but should provide a basis of what could happen if exposure occurs at the tested levels. Statistical analysis of the phenotypic responses as exhibited by both crop species offers an understanding of potential phytotoxicity in a wide range of exposure concentrations (µg/L–mg/L; Figs. 5.3 and 5.8). More research is needed to understand how the observed morphological and anatomical alterations would affect plant development as (or should) they grow to maturity under field environments.

In summary, these results show that NPs of Ag and ZnO are potentially toxic to the early development and growth in maize and cabbage, *albeit* their toxic responses were generally lower for NPs than their specified ionic salts. Notably, the strategic investigations of root anatomy revealing cellular alterations in apical meristem, zone of elongation, and metaxylem count, coupled with metal biouptake, moisture content, germination, and root elongation under NPs versus ionic salt treatments showed differential potential of metal-based NPs and their ions for developmental toxicity in agriculturally important crop plants. Generally, the standard USEPA OPPTS 850.4200 bioassay for measuring germination rate and root growth appeared to be not sensitive enough to capture the effects (Stampoulis et al., 2009) that we observed at the anatomical level, underscoring the need to consider detailed histological studies involving various anatomical structures as investigated here. Taken together, our observations of ‘tunneling-like effect’ upon nZnO treatment, relatively elongated cells with both NPs treatments
versus differentially altered cells with free ions treatments, and varied metaxylem frequency with NPs or free ions treatments suggest potential risks of metal NPs, including of their free ions, on the growth and development of agriculturally significant plant species.

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Mufarrege MM, Hadad HR, Maine MA. Response of *Pistia stratiotes* to heavy metals (Cr, Ni, and Zn) and phosphorous. Arch Environ Contam Toxicol 2010;58:53–61.


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APPENDIX

Synthesis of Citrate–nAg. 1 mM AgNO₃ (Fisher Scientific; Cat # S486-100) solution was mixed with a solution of 10 mM Sodium citrate dihydrate (Fisher Scientific; Cat # S279-500) in a ratio of 2:1 (v/v), and was heated for 4 hours in a water bath at 70 °C. As a result, citrate capped Ag nanoparticles (citrate-nAg) were formed; the characteristics of which are presented in Table 1 (see main manuscript). The ionic citrate carboxyl groups are known to cap the Ag⁰/Ag⁺, thereby stabilizing the particles electrostatically (Kimling et al., 2006; El Badawy et al., 2010).

Fig. 5.9. Ultraviolet-visible spectra of Citrate–nAg, nZnO, and nanopure water (as a blank).
Fig. 5.10. Light microscopy image showing ‘tunneling-like effect’ (shown by red arrows) observed in the primary root tip of Zea mays seedling upon exposure to 1000 µg nZnO/mL.
Fig. 5.11. Potential effects of nZnO (1000 µg/mL; B), ZnSO₄ (1000 µg/mL; C), citrate-nAg (73.4 µg/mL; D), and AgNO₃ (200 µg/mL; E) on Zea mays root hair density as compared to the control (A). Root hair density appeared unaffected with nanoparticles or corresponding ionic salt treatments.
Fig. 5.12. Exposure of *Zea mays* seeds to 100 µg AgNO₃/mL caused thinner roots with tips drying out and red pigmented (a), which severely progressed as concentration increased to 200 µg AgNO₃/mL (b), followed by severe root growth inhibition at 500 µg AgNO₃/mL (c, d). Arrow showing the affected part of the primary root tip.

Fig. 5.13. Effects of nZnO (left panels) and ZnSO₄ (right panels) on germination and root growth in cabbage (*Brassica oleracea* var. *capitata*).
Fig. 5.14. Alteration in metaxylem frequency in *Zea mays* root upon exposure to ZnSO$_4$ (1000 µg/mL) showing formation of 7 metaxylem (mx) vessels.
Table 5.4
Cleaning protocol developed for purification of as-synthesized Citrate–nAg using Tangential flow filtration (TFF) system (Pokhrel et al., 2012).

<table>
<thead>
<tr>
<th>Purification of As-synthesized Citrate–nAg</th>
<th>Electrical Conductivity (µS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Started Volume = 500 ml</td>
<td>1095</td>
</tr>
<tr>
<td>Ended Volume = 70 ml</td>
<td>1162</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>185</td>
</tr>
<tr>
<td>Ended Volume = 100 ml</td>
<td>283</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>36</td>
</tr>
<tr>
<td>Ended Volume = 75 ml</td>
<td>68</td>
</tr>
<tr>
<td>Volume increased to 500 ml by adding nanopure water</td>
<td>11</td>
</tr>
<tr>
<td>Ended Volume = 150 ml</td>
<td>20</td>
</tr>
<tr>
<td>Volume increased to 500 ml</td>
<td>5*</td>
</tr>
</tbody>
</table>

* obtained as purified citrate-nAg suspension with electrical conductivity of 5 µS/cm and was used for phytotoxicity studies.
Table 5.5

Impact of dilution of nanoparticles suspensions in moderately hard water (MHW) as determined by measuring average hydrodynamic diameters (HDD) and zeta potential values using the DLS method. Data showed that the diluted nanoparticles were fairly stable in the test matrix (MHW).

<table>
<thead>
<tr>
<th>Dilution factor (v:v)</th>
<th>Citrate-nAg</th>
<th></th>
<th>nZnO</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Zeta Potential (mV)</td>
<td>Average HDD ± SD (nm) (% volume)</td>
<td></td>
<td>Average Zeta Potential (mV)</td>
</tr>
<tr>
<td>1x</td>
<td>-34.36</td>
<td>11.3 ± 1.3 (100)</td>
<td>-13.21</td>
<td>11.0 ± 0.7 (100)</td>
</tr>
<tr>
<td>2x</td>
<td>-19.84</td>
<td>10.9 ± 0.8 (100)</td>
<td>-15.6</td>
<td>40.3 ± 5.3 (100)</td>
</tr>
<tr>
<td>10x</td>
<td>-18.03</td>
<td>10.9 ± 0.8 (100)</td>
<td>-15.03</td>
<td>43.2 ± 5.0 (100)</td>
</tr>
<tr>
<td>20x</td>
<td>-16.27</td>
<td>11.0 ± 0.7 (97.7)</td>
<td>na</td>
<td>20.6 ± 2.1 (99.5)</td>
</tr>
<tr>
<td>40x</td>
<td>na</td>
<td>11.0 ± 0.9 (97.7)</td>
<td>na</td>
<td>11.3 ± 1.2 (96.2)</td>
</tr>
</tbody>
</table>

x, dilution factor; HDD, volume-weighted hydrodynamic diameter using dynamic light scattering (DLS) method; SD, Standard deviation; na, data not available due to low signal from the highly diluted sample as shown by DLS.
## Table 5.6

Developmental response of *Zea mays* to a range of exposure concentrations of nZnO, Citrate–nAg, and their respective ionic salts as measured by germination rate (%). All comparisons were made with the untreated control (MHW) using Independent Samples t-test at the 0.05 significance level, prior to which homogeneity of variances was tested using Levene’s test at the 0.05 significance level.

<table>
<thead>
<tr>
<th>Material</th>
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<th>Relative Germination Inhibition %</th>
<th>Levene’s test (F)</th>
<th>t-test p-value</th>
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*+ indicates % growth enhancement; SD, standard deviation of the triplicate runs.*

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Table 5.7
Developmental response of *Zea mays* to a range of exposure concentrations of nZnO, Citrate–nAg, and their respective ionic salts as measured by root elongation. All comparisons were made with the control (MHW) using Independent Samples t-test at the 0.05 significance level, prior to which significant difference in variances was compared with the control using Levene’s test at the 0.05 significance level. + indicates % growth enhancement. SD, standard deviation of the triplicate runs.

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<th>p-value</th>
<th>t-test</th>
<th>p-value</th>
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+ indicates % growth enhancement; SD, standard deviation of the triplicate runs.
Table 5.8
Developmental response of *Brassica oleracea* to a range of exposure concentrations of nZnO, Citrate–nAg, and their respective ionic salts as measured by germination rate (%). All comparisons were made with the control (MHW) using Independent Samples t-test at the 0.05 significance level, prior to which significant difference in variances was compared with the control using Levene’s test at the 0.05 significance level.

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<th>Material</th>
<th>Concentration (µg/mL)</th>
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<th>Relative Germination Inhibition %</th>
<th>Levene’s test (F)</th>
<th>p-value</th>
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<td>&lt; 0.05</td>
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<tr>
<td></td>
<td>1</td>
<td>33.3±15.2</td>
<td>60</td>
<td>0.235</td>
<td>&gt; 0.5</td>
<td>4.523</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>60±10</td>
<td>28</td>
<td>0.308</td>
<td>&gt; 0.5</td>
<td>2.646</td>
<td>= 0.05</td>
</tr>
<tr>
<td></td>
<td>36.7</td>
<td>63.3±15.2</td>
<td>24</td>
<td>0.235</td>
<td>&gt; 0.5</td>
<td>1.809</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>73.4</td>
<td>60±10</td>
<td>28</td>
<td>0.308</td>
<td>&gt; 0.5</td>
<td>2.646</td>
<td>= 0.05</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>0.1</td>
<td>50±20</td>
<td>40</td>
<td>0.4</td>
<td>&gt; 0.5</td>
<td>2.646</td>
<td>= 0.05</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>56.6±5.7</td>
<td>32</td>
<td>3.2</td>
<td>&gt; 0.1</td>
<td>3.578</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13.3±11.5</td>
<td>84</td>
<td>0</td>
<td>&gt; 1</td>
<td>7.425</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10±10</td>
<td>88</td>
<td>0.308</td>
<td>&gt; 0.5</td>
<td>8.315</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>12.5</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>12.5</td>
<td>&lt; 0.0005</td>
</tr>
</tbody>
</table>

SD, standard deviation of the triplicate runs.
Table 5.9
Developmental response of *Brassica oleracea* to a range of exposure concentrations of nZnO, Citrate–nAg, and their respective ionic salts as measured by root elongation. All comparisons were made with the control (MHW) using Independent Samples t-test at the 0.05 significance level, prior to which significant difference in variances was compared with the control using Levene’s test at the 0.05 significance level.

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (µg/mL)</th>
<th>Root length (Mean ± SD) (mm)</th>
<th>Relative root growth inhibition %</th>
<th>Levene’s test (F)</th>
<th>p-value</th>
<th>t-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control for Zn-groups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nZnO</td>
<td>0.01</td>
<td>51.13±19.78</td>
<td>17.3+</td>
<td>5.686</td>
<td>&lt; 0.05</td>
<td>-1.638</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>44.08±19.77</td>
<td>1.1+</td>
<td>1.844</td>
<td>&gt; 0.1</td>
<td>-0.362</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>46.84±24.47</td>
<td>7.5+</td>
<td>0.268</td>
<td>&gt; 0.5</td>
<td>-0.529</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>36.87±11.15</td>
<td>15.4</td>
<td>17.828</td>
<td>&lt; 0.001</td>
<td>0.621</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>37.76±13.47</td>
<td>13.3</td>
<td>14.236</td>
<td>&lt; 0.001</td>
<td>0.438</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>37.2±12.44</td>
<td>14.6</td>
<td>7.082</td>
<td>&lt; 0.05</td>
<td>0.484</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>25.85±7.62</td>
<td>40.7</td>
<td>19.467</td>
<td>&lt; 0.0001</td>
<td>3.398</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>0.01</td>
<td>57±20.58</td>
<td>30.8+</td>
<td>0.99</td>
<td>&gt; 0.1</td>
<td>-2.069</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>47.04±20.64</td>
<td>7.9+</td>
<td>1.176</td>
<td>&gt; 0.1</td>
<td>-0.579</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>40.2±19.10</td>
<td>7.8</td>
<td>3.15</td>
<td>&gt; 0.05</td>
<td>0.492</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>55.23±20.33</td>
<td>26.7+</td>
<td>0.609</td>
<td>&gt; 0.1</td>
<td>-1.864</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>36.84±12.06</td>
<td>15.5</td>
<td>14.227</td>
<td>&lt; 0.001</td>
<td>1.333</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>10.288</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>10.288</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td><strong>Control for Ag-groups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate–nAg</td>
<td>0.05</td>
<td>63.66±23.49</td>
<td>2.4+</td>
<td>1.583</td>
<td>&gt; 0.1</td>
<td>-0.227</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>63.1±17.12</td>
<td>1.5+</td>
<td>0</td>
<td>&gt; 0.5</td>
<td>-0.140</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>36.8±19.04</td>
<td>40.8</td>
<td>0</td>
<td>&gt; 0.5</td>
<td>3.670</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>61.05±18.99</td>
<td>1.8</td>
<td>0.031</td>
<td>&gt; 0.5</td>
<td>0.192</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>36.7</td>
<td>51.57±20.63</td>
<td>17</td>
<td>1.126</td>
<td>&gt; 0.1</td>
<td>1.801</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>73.4</td>
<td>47.15±19.09</td>
<td>24.1</td>
<td>0.42</td>
<td>&gt; 0.5</td>
<td>2.648</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>0.1</td>
<td>53.33±22.85</td>
<td>11</td>
<td>0.391</td>
<td>&gt; 0.5</td>
<td>1.042</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>55.76±15.95</td>
<td>10.3</td>
<td>0.474</td>
<td>&gt; 0.1</td>
<td>1.171</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40.5±23.79</td>
<td>34.8</td>
<td>1.201</td>
<td>&gt; 0.1</td>
<td>2.123</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>25.66±3.05</td>
<td>58.7</td>
<td>4.183</td>
<td>&gt; 0.05</td>
<td>3.403</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>17.032</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>17.032</td>
<td>&lt; 0.0005</td>
</tr>
</tbody>
</table>

+ indicates % growth enhancement; SD, standard deviation of the triplicate runs.
Table 5.10

Impact of nanoparticles and corresponding ionic salt on the root cell morphology and anatomy in *Zea mays* as observed using light microscopy.

<table>
<thead>
<tr>
<th>Test Chemicals (µg/mL)</th>
<th>Primary Root Tip</th>
<th>Root Hairs</th>
<th>Zone of Elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>normal</td>
<td>abundant</td>
<td>cells appeared normal</td>
</tr>
<tr>
<td>nZnO (1000)</td>
<td>Tunneling-like effect/invagination occurred, but such observation was infrequent (1 out of 4 samples analyzed)</td>
<td>abundant</td>
<td>cells appeared elongated</td>
</tr>
<tr>
<td>Citrate-nAg (73.4)</td>
<td>normal</td>
<td>abundant</td>
<td>cells appeared elongated</td>
</tr>
<tr>
<td>ZnSO₄ (1000)</td>
<td>normal</td>
<td>abundant</td>
<td>cells appeared shorter and wider</td>
</tr>
<tr>
<td>AgNO₃ (200)</td>
<td>compromised</td>
<td>abundant</td>
<td>cells appeared thinner and irregular</td>
</tr>
</tbody>
</table>

Table 5.11

Variability in biouptake, and moisture content in the filter paper used as the substrate, and in the seedlings, with AgNO₃ or Citrate-nAg treatment in *Zea mays*.

<table>
<thead>
<tr>
<th>Treatments (µg/mL)</th>
<th>Average moisture (%)/replicate-dry weight</th>
<th>Average biouptake of Ag (as total Ag) (ng/mg dry wt. of seedling)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Filter paper*</td>
<td>Seedlings#</td>
</tr>
<tr>
<td>Control</td>
<td>47.5</td>
<td>147.3</td>
</tr>
<tr>
<td>AgNO₃ (200)</td>
<td>66.2</td>
<td>168.7</td>
</tr>
<tr>
<td>Citrate–nAg (73.4)</td>
<td>57.5</td>
<td>163.9</td>
</tr>
</tbody>
</table>

*Average moisture content for two filter papers, one in each of the two replicates;
#Average moisture content pooled for 18 seedlings in two replicates.

Note that Ag (as total Ag) biouptake by *Z. mays* seedlings was 55 times higher with AgNO₃ treatment, and 4.5 times higher with citrate-nAg treatment, as compared to the control. The biouptake was 12.2 times higher with AgNO₃ treatment compared to citrate-nAg treatment. The Ag (total) concentration in the control seedlings was similar to that of blank (1% HNO₃, trace metal grade), which was, on average, ~15 µg/L in the 50 mL of the digested samples. The samples were acid digested using the standard USEPA 3050B method.
References Cited in Appendix


CHAPTER 6

SUMMARY AND FUTURE OUTLOOK

Because engineered nanomaterials are emerging contaminant of environment, this study was designed to investigate the potential colloidal stability and ecotoxicity of metal-based nanoparticles to provide insights into comparative understanding of nanoparticle- versus ion-specific toxicity using multiple model organisms (*Escherichia coli*, *Daphnia magna*, *Zea mays*, and *Brassica oleracea var. capitata*) and endpoints. Our results revealed lower toxicity of metal-based nanoparticles than of the ions. Employing multiple test organisms enabled to identify *D. magna* as the most sensitive among the four species tested against metal nanoparticle insults. We found that dissociated Ag was inadequate to explain AgNP toxicity and that the combined effect of AgNPs and dissociated Ag\(^+\) under variable test conditions was lower than of AgNO\(_3\) alone.

While higher DOC or pH conferred effective protection against Citrate–AgNPs and free Ag\(^+\), increase in solution hardness appeared to promote toxicity in *E. coli*, however. Toxicity of Citrate–AgNPs or Ag\(^+\) in the representative river water samples revealed no seasonality and was comparable to the baseline controls. Aqueous DOC (i) modified particle size and surface properties, (ii) promoted formation of larger DOC-AgNP aggregates, (iii) which were likely filter-fed by daphnids leading to higher internal dose, and (iv) finally resulted in higher mortality of the animals. Comparison of AgNP toxicity between *E. coli* and *D. magna* under DOC-enrichment clearly showed disparate toxicity, highlighting an important role species biology (e.g., feeding behavior here) can play in explaining AgNP toxicity.

An application of Trolox changed AgNP surface characteristics, inhibited Ag\(^+\) dissociation, perhaps by quenching reactive oxygen radicals present or formed during the experiment, which otherwise could have promoted oxidative dissolution of Ag\(^+\), and consequently protected
daphnids against AgNP toxicity. Oxidative stress-mediated toxicity of Citrate–AgNPs thus emerged using trolox as an antioxidant. Contrarily, an inability of trolox to attenuate free Ag$^+$ toxicity is indicative of an absence of oxidative stress, while effective attenuation of AgNO$_3$ toxicity by cysteine indicated direct effect of Ag$^+$ as the cause of *D. magna* mortality. Furthermore, an addition of organic thiol modified AgNP surface properties, forming cysteine-AgNP complex, inhibited Ag$^+$ release, likely quenched free Ag$^+$ from the nanosuspension, and therefore enabled *D. magna* survival.

The premise that nanoparticles may have different biologic interactions than their ions is supported by our observations of ‘tunneling-life effect’ with ZnONP exposure and relatively elongated cells at the zone of elongation with Citrate–AgNP or ZnONP treatment in maize root; such anomalies were not observed with respective ionic salt treatments. Generalized linear models developed can be usefully applied to probe and quantify the toxicity of AgNPs with modified surface properties, including for other metal nanoparticles released into the aquatic systems. As tangential flow filtration (TFF) system could effectively purify metal nanoparticles with high purity and reproducibility of the colloidal properties (as evidenced for AgNPs), its elimination of residual ions and impurities from Citrate–AgNP suspension enabled differentiating nanoparticle versus ionic toxicity.

This study shows that particle size, surface characteristics, and ion release kinetics of AgNPs modify upon release into aquatic environment, suggesting potential implications to ecosystem health and functions, and that caution be applied when extending one species toxicity to another because obvious differences in organism biology, as the species sensitivity paradigm explains (Pokhrel & Dubey, 2013a), could significantly alter both the nanoparticle- and ion-specific toxicity. Further, our phytotoxicity results underscore the need to consider detailed anatomical
studies applying histological methods in parallel with the standard developmental phytotoxicity assays (germination and root elongation) as the latter appear not sensitive enough to capture the effects that were observed at the anatomical level upon metal nanoparticle insults.

Because of the enigma it presents and new questions that emerge after answering previous questions, nanomaterial research has encouraged scientists from all disciplines to address the critical research needs, a concise list of which is outlined below. As interdisciplinary groups of scientists are involved in elucidating the causal mechanism(s) of bioactivity of both the pristine and the environmentally-modified nanomaterials, it is expected that a better understanding of the nanoscience and nanotoxicology can be achieved in the foreseeable future.

- Need for standardizing protocols to synthesize and purify nanomaterials, and modify existing test protocols (if necessary) to specifically address toxicity issues associated with impurities and/or ions in the nanomaterial suspension;
- To understand the fate and toxicity of various nanomaterials as they leach from the nano-enabled consumer products;
- To identify the target site(s) of action and the associated toxic endpoints for various nanomaterials;
- To understand if nanomaterials would persist (for how long? in what form?), bioaccumulate, or biomagnify in the food chain;
- To identify additional factors and conditions which might influence nanomaterial toxicity in the environments;
- To understand how nanomaterials would behave in the complex natural matrices such as multiple soil types, biosolids, and natural waters;
• To understand how nanomaterials would partition into different environmental matrices including the landfill leachate, residual ash, and wastewater effluent;

• To invent sensors for precise detection and quantification of nanomaterials *in vitro* and *in vivo*, including in different environmental matrices such as soils, sediments, biosolids, and natural waters; and

• To determine if nanomaterials pose risk to human health and the environment.
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Featured on ETSU News, Jul 2012
Featured on ASPH Friday Letters, May 2012
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Featured on ASPH Friday Letters, Jun 2011