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Potential Toxicity of Silver Nanoparticles to Microbial Communities and Macroinvertebrates

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Potential Toxicity of Silver Nanoparticles to Microbial Communities and Macroinvertebrates

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by

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ABSTRACT

Silver Nanoparticle Impacts and Implications on Microbial Communities and Macroinvertebrates

by

Joseph Kusi

Silver nanoparticles (AgNPs) are the most common nanomaterials incorporated in commercial products due to their antimicrobial activity. Recently, AgNPs were detected in surface waters suggesting the potential for bioavailability in the aquatic receptor organisms. This dissertation research attempts to understand the potential toxicity of AgNPs on water quality indicators, focusing on the microbial community and amphipods. This study evaluated whether: (1) the antimicrobial properties of AgNPs pose potential risks to microbial communities in pathogen impaired streams; (2) AgNPs can cause a shift in functional diversity and metabolic fingerprinting of microbial communities; (3) survival and growth of *Hyalella azteca* (amphipods) could be affected by AgNPs; and (4) surface coating agents influence AgNP toxicity in *H. azteca*. Microbial community responses to AgNPs were assessed using standard plate count, microbial enzyme assays, and carbon substrate utilization with Biolog EcoPlates™. Ten-day and 28-d toxicity tests were conducted in a static system to assess AgNP effects on *H. azteca*. AgNPs caused a 69% decrease in microbial concentration and a 77% decrease in β-glucosidase activity at 0.32 mg Ag kg^{-1} dry sediment. The substrate utilization pattern of the microbial community was altered by AgNPs at 0.33 mg Ag kg^{-1} dry sediment. Ten-day LC50s for the survival of *H. azteca* were 3.3, 9.2, and 230.0 µg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively, whereas the 28-d LC50s

were 3.0, 3.5, and 66.0 μg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively. The EC20s for growth (calculated as biomass) for the 10-d test were 1.6, 4.7, and 188.1 μg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP; while the 28-d EC20s for $AgNO_3$, citrate-AgNP and PVP-AgNP were 3.2, 0.5, and < 50 μg Ag L^{-1}. The NOECs for dry weight were 4, 1, and 100 μg Ag L^{-1}, while those for biomass were 2, 0.5, and < 50 μg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively. The overall toxicity followed the trend: $AgNO_3$ > citrate-AgNP > PVP-AgNP. The studies suggest that AgNPs pose potential risks to microbial communities and epibenthic macroinvertebrates used as bioindicators of water quality to protect public health and ecosystem health.

DEDICATION

I dedicate this dissertation to my parents, John Kwaku Addai and Regina Kyeraa, my senior brother, Augustine Amoako, and my wife, Beatrice Amponsah for their support and encouragement.

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CHAPTER 1

INTRODUCTION

Background and Problem Statement

Nanotechnology and Nanomaterials

The manipulation of matter at the nanoscale (between 1 and 100 nanometers (Hansen et al., 2013; USEPA, 2012) known as nanotechnology has led to increased production and incorporation of nanomaterials (NMs) in products. Currently, there is little knowledge of the potential impacts of nanomaterials on sediment organisms due to limited toxicological data (Coll et al., 2015). Nanotechnology can be defined as engineered structures, devices, and systems (CDC, 2018). What makes NMs so interesting and useful is their unique properties such as shape, size, charge, reactivity, and large surface area to volume ratio (Nowack et al., 2011). Examples of NMs include fullerenes (C60, carbon nanotubes, graphene), ceramics (iron oxides, ceria, titanium), metals (silver nanoparticles, gold nanoparticles), quantum dots (cadmium chalcogenides, zinc oxide, cadmium selenide), and polymers (copolymer assemblies, dendrimers) based on the materials and techniques used for their synthesis (NAS, 2012; Zhang & Webster, 2009). Among NMs, nanoparticles, nano-objects with all three external dimensions in the nanoscale (EU-OSHA, 2014), are currently of greater environmental concern due to the increasing quantity produced annually and a greater number of available products on the market containing nanoparticles (Fabrega et al., 2011; Hansen et al., 2013; McGee et al., 2017; USEPA, 2014).

Most nano-enabled products contain silver nanoparticles (AgNPs) due to their antimicrobial activity and conductivity (Buzea et al., 2007; Fabrega et al., 2011; Piccinno et al., 2012). In medicine, AgNPs are incorporated in wound dressings and catheters to

prevent bacterial infections. AgNPs are also used in consumer products such as textiles and personal care products to reduce the growth of odor-producing bacteria (EC, 2014). AgNPs are also used for groundwater remediation to remove hexachlorobenzene (Corsi et al., 2018). About 220 to 312 tons of AgNPs are produced globally per year (McGee et al., 2017), suggesting a potential release of the nanoparticles into the environment during manufacturing, transportation, use, and disposal (Fabregas et al., 2011). Silver nanoparticles discharged from industries, washing machines, and bathtub drains are deposited into sewage systems and treatment facilities (USEPA, 2012). Thus, it is not surprising that AgNPs have been detected in the wastewater and surface water (Kim et al., 2010; Peter et al., 2018). This phenomenon suggests a potential release of AgNPs into the aquatic systems.

Fate and Transport of Nanomaterials in Aquatic Systems

Nanomaterials undergo several transformations including aggregation, agglomeration, dissolution, and speciation (e.g., formation of silver chloride and silver sulfide by AgNPs) when released into the environment (EC, 2014). Fate and transport of nanoparticles in environmental media depend on several factors. Material properties and water chemistry influence the distribution of nanoparticles in aquatic systems (USEPA, 2014). The large surface area to volume ratio exhibited by nanoparticles makes them highly reactive to ligands in water column. Nanoparticles readily agglomerate and settle at the bottom of a solution (USEPA, 2014), thus, keeping the particles suspended in solutions is very important in obtaining desired results. To prevent agglomeration, surface coating agents are added to the solution during

nanoparticle synthesis. The addition of surface coatings to nanoparticle solutions also increases particle solubility and suspension (USEPA, 2012). Surface coating agents can influence particle mobility. El Badawy et al. (2013) demonstrated that AgNPs coated with polyvinylpyrrolidone were transported rapidly through quartz sand compared to citrate-coated AgNPs. Considering the effects of surface coating agents on nanoparticle behavior, it is important to understand how different surface coating agents can affect the toxicity of nanoparticles. Although some studies have shown potential influence of surface coating agents on AgNP toxicity (Button et al., 2016; Moore et al., 2016; Pokhrel et al., 2012), little is known about the influence of citrate and polyvinylpyrrolidone capping agents on AgNP toxicity to microbial communities in pathogen impaired streams and macroinvertebrates that serve as bioindicators of water quality.

Nanomaterials may undergo transformation through oxidation, sulfidation, dissolution, or aggregation processes depending on water chemistry such as pH, hardness, and organic matter (Abbas et al., 2020). These processes may cause nanoparticles to be transformed and transported from water column to sediment. Thus, sediment is considered as the sink for nanoparticles entering aquatic systems (Baun et al., 2008; Colman et al., 2012; Ramskov et al., 2015). Sediment can adsorb to particles and hold contaminants (Oetken et al., 2004), which may increase the accumulation of metal-based nanoparticles in sediments. For example, zinc oxide and copper oxide nanoparticles added to estuarine water exhibited an accumulation of their metals in sediment (Hanna et al., 2013). In another study, metal oxide nanoparticles entering the marine system settled rapidly at the bottom due to low total organic compounds and high ionic strength in water column (Keller et al., 2010). Similarly, AgNPs are very

reactive and have a short time in the water column (Levard et al. 2012); they interact with ligands and settle quickly in sediment (Colman et al., 2012; Ramskov et al., 2015).

Recent studies show that benthic organisms are more responsive to nanoparticle exposure (Das et al., 2012; Hanna et al., 2013; Pokhrel et al., 2012; Ramskov et al., 2015). Amphipods are among the benthic invertebrates, which are highly affected by metal pollution. They are among the first benthic species to disappear in contaminated sediments because their ability to hide in sediments to avoid predation may be impacted (Burton, 1992). Silver is highly toxic to *H. azteca* (Berry et al., 1999), but to our knowledge, only one study has evaluated potential toxicity of AgNPs on this species in wastewater (Kühr et al., 2018). Thus, it is important to evaluate the effects of AgNPs on *H. azteca* and other aquatic organisms, which are likely to be affected by AgNPs in sediment. While microbial responses to AgNPs exposure have been studied in the soil, constructed wetland, and estuarine system (Button et al., 2016; Echavarri-Bravo et al., 2015; Samarajeewa et al., 2017), evaluation of potential effects of AgNPs on microbial functional diversity in natural freshwater sediment is limited.

Benthic Invertebrates and Microorganisms

Invertebrates form a large and diverse group of aquatic organisms (Baun et al., 2008). This group of organisms plays an important role in aquatic ecosystems by feeding on primary producers and bacteria to control their populations, serving as a food source for other secondary consumers, and nutrient cycling to maintain the flow of energy in aquatic ecosystems. In addition, invertebrates are used as model organisms to generate toxicity data for assessing, regulating, and monitoring the quality of water

bodies (Baun et al., 2008). The use of invertebrates as water pollution indicators promotes the relationship between ecosystem and human health because water bodies that do not support the growth and reproduction of invertebrates are sometimes not good for their designated benefits (TDEC, 2014).

Microorganisms like amphipods play an important role in ecosystem health and function. The microbes provide ecosystem services such as organic matter decomposition and nutrient cycling (Colman et al., 2012; Jomini et al., 2015). Some groups of microorganisms such as fecal coliforms are used as pathogen indicators in surface waters. Some streams with high levels of pathogen indicators are listed on the 303(d) List by the Tennessee Department of Environment and Conservation (TDEC) as impaired, and their recreational uses are prohibited (TDEC, 2016). Despite the health and ecological benefits of microorganisms and amphipods, their survival and growth could be affected by the potential release of AgNPs into aquatic systems (Colman et al., 2012; Echavarri-Bravo et al., 2015; Pokhrel et al., 2012).

Considering the strong antimicrobial properties of silver, which is known for many years (Fabrega et al., 2011), releasing AgNPs into aquatic systems may cause adverse effects to microbes and invertebrates. Evidence from previous studies demonstrates the potential impacts of AgNPs on beneficial microorganisms in the environment, but studies investigating their effects on microbial communities in the natural environment are limited (Fabrega et al., 2011). AgNPs in aquatic systems can accumulate in sediment, but few studies have evaluated AgNP toxicity in freshwater sediment. Further studies are needed to explain the impacts and implications of AgNPs in aquatic

systems. Based on the current information, the following knowledge gaps in AgNPs toxicity studies were identified:

(1) limited studies on microbial community responses to AgNPs in pathogen impaired streams,

(2) *Hyalella azteca* responses to AgNPs in aquatic systems,

(3) comparison of citrate-coated AgNPs and polyvinylpyrrolidone-coated AgNPs toxicity to *H. azteca*, and

(4) implications of AgNPs toxicity on public health.

Research Objectives

This study was designed to achieve the following objectives:

Objective 1: To determine the potential effects of AgNPs on microbial communities in pathogen impaired streams and their implications on human health.

Objective 2: To determine the potential effects of AgNPs on microbial functional diversity in pathogen impaired streams and their implications on aquatic ecosystems.

Objective 3: To evaluate benthic macroinvertebrate responses to AgNP exposure.

Dissertation Outline

This dissertation consists of five chapters. Chapter 1 contains background information, problem statement, and research objectives. Chapter 2 provides an investigation of microbial concentration and enzyme activity responses to AgNPs in a pathogen impaired stream. Chapter 3 contains the evaluation of AgNP effects on microbial community diversity in a pathogen impaired stream. Chapter 4 consists of the

evaluation of acute and chronic endpoints of AgNPs in *H. azteca*. Chapter 5 presents a summary of the important findings of this dissertation and provides the future direction of the research. Chapter 2 is structured based on reviewer comments and suggestions received from the journals- Applied and Environmental Microbiology and Environmental Pollution.

CHAPTER 2

ANTIMICROBIAL PROPERTIES OF SILVER NANOPARTICLES MAY INTERFERE WITH FECAL INDICATOR BACTERIA DETECTION IN PATHOGEN IMPAIRED STREAMS

Joseph Kusi, Phillip R. Scheuerman, Kurt J. Maier

Keywords: alkaline phosphatase, enzyme activity, β-glucosidase, microbial responses, toxicity

Abstract

Silver nanoparticles (AgNPs) are expected to enter aquatic systems, but there are limited data on how they might affect microbial communities in pathogen impaired streams. We examined microbial community responses to citrate-AgNP (10.9 ± 0.7 nm) and polyvinylpyrrolidone (PVP)-AgNP (11.0 ± 0.7 nm) based on microbial concentration and enzyme activity in sediment from a pathogen impaired stream. Addition of each nanoparticle to sediment yielding nominal 0.323 mg Ag/kg caused, at least, a 69% decrease in microbial concentration (1,264 ± 93.6 to 127 ± 29.5 CFU/g) and a 77% decrease in β-glucosidase activity (11.7 ± 2.1 to 2.7 ± 1.4 μg/g/h) compared to corresponding controls. Alkaline phosphatase activity was reduced by both AgNPs but the effect was not statistically significant. The addition of silver nitrate yielding 0.108 mg Ag/kg to sediment at a concentration equivalent to the lowest concentration of AgNPs led to a 92% decrease in microbial concentration compared to the controls, but enzyme activity was not affected. Measured total silver in sediments treated with AgNPs which

exhibited significant inhibition effects ranged from 0.19 ± 0.02 to 0.39 ± 0.13 mg Ag/kg which are much lower than the expected concentrations (2 to 14 mg Ag/kg) in freshwater sediments. The results of this study demonstrate that AgNPs can alter microbial community activity and population size, which may lead to false negative fecal indicator bacteria detection and enumeration using methods that rely on β-glucosidase activity. We conclude that the presence of AgNPs in impaired streams and recreational waters can influence pathogen detection methods, potentially influencing public health risk estimates.

1. Introduction

Silver nanoparticles (AgNPs) are known to express antimicrobial activity in stream water and sediments (Bao et al., 2016; Colman et al., 2012), yet no study has evaluated their effects on pathogen impaired streams. In the United States (U.S.), more than 270,000 km of rivers and streams are pathogen impaired dominating the 303 (d) list of Clean Water Act (Gilfillan et al., 2018; Pandey et al., 2014). Pathogens enter surface waters through discharges of untreated sewage, stormwater runoff, failing septic systems, livestock grazing, manure application, and wildlife (Arnone & Walling, 2007; Qiu et al., 2018; Soupir et al., 2010). The U.S. Environmental Protection Agency (USEPA) and public health agencies have been using fecal indicator bacteria (FIB) as indicators of fecal pollution and the possible presence of pathogens to protect public health (USEPA, 2015).

Escherichia coli and enterococci are indicators of fecal contamination and a high correlation between these indicators and gastrointestinal illness in humans have been

reported (Arnone & Walling, 2007; Boehm & Sassoubre, 2014; Tallon et al., 2005). The US EPA recommends the use of *E. coli* and enterococci as FIB for fresh recreational water and enterococci as FIB for marine recreational water. Several states and territories have adopted this standard as the basis for bacteria water quality standards (USEPA, 2015; USEPA, 1998). Enterolert, a commonly used standard method for enterococci detection and enumeration in ambient water, relies on β-glucosidase activity (Boehm & Sassoubre, 2014). Evidence from previous studies demonstrates that AgNPs are capable of inhibiting microbial concentration, enzyme activity, and function (Button et al., 2016; Choi et al., 2009; Echavarri-Bravo et al., 2015; Samarajeewa et al., 2017) suggesting their potential effects on FIB detection methods that rely on β-glucosidase. We hypothesized that AgNPs could inhibit β-glucosidase activity which may interfere with the enterococci detection method.

AgNPs are the most commonly used nanomaterials in consumer products due to their antimicrobial properties (Buzea et al., 2007; Xu & Zhang, 2018). Continuous application of AgNPs in products and a recent detection of the nanoparticles in municipal wastewater (Kim et al., 2010) and surface water (Peter et al., 2018) raise concerns about potential risks to microorganisms in aquatic systems. AgNPs inhibited bacterial function in estuarine sediment by reducing the rate of carbon substrate utilization and bacterial abundance (Choi et al., 2009; Echavarri-Bravo et al., 2015). The uptake of AgNPs by sediment bacteria inhibited cell growth and reduced *Pseudomonas putida* biofilm formation (Khan et al., 2015). Silver ions released from AgNPs interact with bacterial enzymes and proteins to cause inhibition of cellular respiration and ion transport across membranes which may result in cell death. Exposure to AgNPs also

induces the formation of free radicals and the accumulation of reactive oxygen species, potentially leading to cell death (Fabrega et al., 2011; Pulit-Prociak & Banach, 2016).

In this study, we evaluated microbial responses to citrate-coated silver nanoparticle (citrate-AgNP) and polyvinylpyrrolidone-coated silver nanoparticle (PVP-AgNP) exposure in a pathogen impaired stream. These two AgNPs are commonly incorporated in products and have unique properties that may influence their stability and toxicity in sediments (Pokhrel et al., 2012; Salih et al., 2019; Silva et al., 2014). Although some previous studies have examined the effects of these AgNPs on microorganisms (Table 1), no study has compared their effects on bacterial concentration and enzyme activity in sediments from a pathogen impaired stream. Changes in the presence or concentration of pathogens and pathogen indicators (e.g., fecal indicator bacteria) could influence the classification of stream impairment. In addition, we compared the effects of AgNPs on microbial communities with those reported in previous studies (Table 2.1). The nanoparticles were coated with different capping agents to increase particle stability. We expected the effects of citrate-AgNP and PVP-AgNP on microbial activity to differ due to the unique characteristics of their surface coating materials. We performed microbial assays to examine the effects of AgNPs on microbial concentration and enzyme activity in sediments collected from a local pathogen impaired stream.

Table 2.1. Effects of silver nanoparticles on microbial communities

Stabilizing agent	Environmental compartment	Effect	References
Carboxy-functionalized	Freshwater	Reduced bacterial production and enzyme activity	Das et al. (2012)

Citrate	Freshwater	Altered biofilm structure	Grun et al. (2018a)
Sulfide	Freshwater	Altered microbial community structure	Liu et al. (2018)
Polyoxyethylene glycerol trioleate and Tween 20	Marine water	Reduced bacterial abundance	Echavarri-Bravo et al. (2017)
Polyoxyethylene glycerol trioleate and Tween 20	Marine estuarine sediment	Inhibited carbon substrate utilization	Echavarri-Bravo et al. (2015)
Polyvinylpyrrolidone	Freshwater sediment	No effect on microbial activity	Colman et al. (2012)
Uncoated and polyvinylpyrrolidone	Freshwater sediment	Altered microbial community biomass and enzyme activity	Bao et al. (2016)
Gum Arabic	Freshwater sediment	Altered microbial community structure and composition	Moore et al. (2016)
Uncoated	Freshwater sediment	Altered microbial community profiling	Welz et al. (2018)
Uncoated	Soil	Altered microbial community function	Kumar et al. (2011)
Slurry	Soil	Altered bacterial biomass and composition	Colman et al. (2013)
Uncoated	Soil	Decreased enzyme activity and bacterial community composition	McGee et al. (2017)
Polyvinylpyrrolidone	Soil	Inhibited microbial growth, enzyme activity, and community diversity	Samarajeewa et al. (2017)
Polyacrylate	Soil	Reduced microbial biomass, enzyme activity, and abundance of nitrogen-fixing bacterial	Grun et al. (2018b)
Citrate and polyvinylpyrrolidone	Constructed wetland	Reduced catabolic activity	Button et al. (2016)
Citrate and polyvinylpyrrolidone	Pathogen impaired freshwater sediment	Reduced microbial concentration and enzyme activity	Current study

2. Materials and methods

2.1. Sediment collection

Freshwater and sediment samples were collected from Sinking Creek in Johnson City, Tennessee. Sinking Creek is listed as pathogen impaired on the 303(d) List by the Tennessee Department of Environment and Conservation (TDEC). Discharges from MS4 area storm sewers and pasture grazing are suggested as the primary causes of the creek's contamination (TDEC, 2016). The sampling site for this study contains the highest number of microorganisms according to the results of monthly sampling at 14 sites conducted by our laboratory to monitor the presence of pathogens in the creek (Hall, 2012).

Two liters of creek water and about 1 kg of sediment, 5 cm below the sediment surface, were collected by sterile spatula into three sterile polyethylene carboys and three polyethylene bags, respectively. The samples were carried on ice to the laboratory and refrigerated at 4 ^{o}C to preserve the samples for microbial analysis. The streamwater was filtered through a 0.45 μm filter to remove microorganisms, and the sediments were sieved through a 2 mm sieve to remove any large particles. All three sediment samples were pooled together and homogenized. The sediment pH was determined using the method described by Colman et al. (2012).

2.2. Nanoparticles synthesis, purification, and characterization

Citrate-AgNP and PVP-AgNP were selected for this study based on their stabilizing properties and their use in consumer products. Citrate-AgNP is electrostatically stabilized while PVP-AgNP is sterically stabilized (El Badawy et al.,

2013). These nanoparticles were synthesized in our laboratory using the method of El Badawy et al. (2010). Briefly, citrate-AgNP was synthesized by adding 2 parts 1 mM silver nitrate ($AgNO_3$) (Fisher Science) to 1 part 10 mM sodium citrate dihydrate (Fisher Science). The solution was heated in a water bath at 70 ^oC for 4 hours until it turned yellow indicating the formation of citrate-AgNP. For PVP-AgNP, 1 part 5 mM $AgNO_3$ solution was added to 3 parts 2 mM sodium borohydride (Acros Organics) in a 1 % PVP K60 solution (Sigma Aldrich) on ice with vigorous stirring.

The newly prepared AgNP solutions were sonicated for 10 minutes to ensure even dispersion of suspended particles. The nanoparticles were purified using a polysulfone 10 kD hollow fiber filter in Kros Flo Research II*i* tangential flow filtration system (Spectrum Laboratories, CA, USA) to remove residual impurities and larger particles. Hydrodynamic diameter (HDD) and zeta potential of the nanoparticles were determined by dynamic light scattering (DSL) using a NICOMP 380 ZLS Particle sizer/zeta potential analyzer (PSS NICOMP Particle Sizing Systems, Santa Barbara, CA, USA). A standard solution with 92 nm DLS purchased from the manufacturer was analyzed as a check standard before the instrument was used to determine HDD and zeta potential. The shape of the particles was determined by transmission electron microscopy (TEM) at the Joint Institute for Advanced Materials, the University of Tennessee followed by image analysis to estimate particle size using ImageJ software (National Institute of Health, USA). Silver ion solution was prepared by dissolving $AgNO_3$ crystals in milli-Q water. To test for the toxic effects of surface coating agents, nominal concentrations of sodium citrate and polyvinylpyrrolidone (PVP) equivalent to

the amount in the highest AgNP exposure concentrations were prepared separately to distinguish their effects from the AgNPs.

2.3. Sediment exposure

Five nominal concentrations of each nanoparticle type (Table 2.2), were prepared using the filtered streamwater. A 25 mg Ag L^{-1} (0.108 mg Ag kg^{-1} dry sediment) nominal as $AgNO_3$ solution was prepared to serve as a positive control. Thirty grams of sediment (equivalent to 23.2 ± 0.3 g dry weight) were weighed into 250 mL clean glass jars (Appendix Figure 2.7A) and treated with 100 mL of the test solutions or stream water (negative control) in triplicate. Treated sediments were manually mixed with test solutions using a glass rod and incubated at room temperature for 48 hours. Sediment samples were taken from each treatment jar after 48 hours of exposure for microbial analyses.

Table 2.2. Nominal concentrations of AgNPs expressed in mg Ag/L and mg Ag/kg dry sediment

Nominal concentration (mg Ag/L)	Nominal concentration (mg Ag/kg dry sediment)
25	0.108
50	0.215
75	0.323
100	0.431
125	0.538

2.4. Chemical analysis

Total silver in overlying water and sediment were determined by acid digestion using standard methods (USEPA, 1996), and their measured concentrations were determined using the flame atomic absorption spectrophotometry (FAAS). All glassware

ware cleaned with 5% nitric acid to remove metal residues before used for AgNPs synthesis or toxicity testing. Five standard solutions prepared from a pure silver solution were analyzed to obtain a calibration curve and one quality control standard solution (QCI-34) purchased from NSI Lab Solutions was analyzed with the samples as a check standard.

2.5. Microbial concentration inhibition

Heterotrophic plate counts (Appendix Figure 2.7B) were used to determine the effects of AgNPs on the microbial concentration. Two grams of the treated sediment was added to 18 mL milli-Q water in 50 mL centrifuge tubes and vortexed for 30 seconds. The tubes remained undisturbed for 30 minutes to allow sediment particles to settle. The supernatant was diluted 10-fold and 100 µL was pipetted into a petri-dish. About 5 mL molten Difco^{TM} tryptic soy agar was poured onto each petri-dish and incubated at 20^{o}C for 48 hours. Colony-forming units (CFU) were counted after 48 hours.

2.6. Enzyme activity

The influence of AgNP exposure on alkaline phosphatase and glucosidase activities was evaluated using standard microbial enzyme assays (Sayler et al., 1979). Four test tubes containing 0.5 g wet sediment (Appendix Figure 2.7C) were used for each treatment. One tube was used for a blank and the other 3 tubes were used for the replicates. For alkaline phosphatase, 5 mL of TRIS buffer (pH 8.6) was added to the blank and 4 mL was added to the replicate test tubes. One milliliter of phosphatase substrate (pH 7.6) was added to each test tube except the blank. For glucosidase, 5 mL

of phosphate buffer (pH 9.0) was added to the blank and 4 mL was added to the replicate test tubes. One milliliter of glucosidase substrate (pH 7.6) was added to each test tube except the blank. All the test tubes were vortexed for 30 seconds and incubated at 30 ^{o}C for 24 hours. After the incubation period, 150 µL aliquot of the samples was pipetted into wells on a clear 96-well plate, and the absorbance was measured at 418 nm using a Multiskan ascent plate reader (Appendix Figure 2.7D). Enzyme activity was quantified by expressing the absorbance in units of µg g^{-1} h^{-1}, which is the amount of substrate hydrolyzed by enzymes in 1 g sediment per hour (Huang et al., 2015).

2.7. QC/QA for sampling and microbial treatment

Sterile equipment was used for sampling to avoid cross-contamination. A blank was used to identify field or laboratory contaminants and both positive and negative controls were tested. Pseudoreplicate samples were used to obtain equal distribution of microorganisms and uniform sediment samples. Standard errors were calculated to determine the consistency of observed values using variance among replicates (Kumari et al., 2014). Samples were incubated at appropriate temperature and time (25 ± 0.5 ^{o}C for 48 hours). Standard Methods for Examination of Water and Wastewater (Greenberg, 1995) were followed to determine the number of bacteria using standard plate count. Instrument efficiency and accuracy were checked by calibrating all measuring instruments using externally supplied standards.

2.8. Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 24). All data were examined for normality using the Kolmogorov-Smirnov test and those which were not normally distributed ($p < 0.05$) were log-transformed. Differences in total silver concentrations between the overlying water and sediment were determined using an independent-sample t-test. One-way analysis of variance (ANOVA) was used to determine the antimicrobial effects of AgNPs on the microbial community and the statistically significant difference between the effects of the two nanoparticles. The effect of each treatment was compared with the control using the Dunnett multi-comparison test.

3. Results

3.1. Nanoparticles characterization

The volume-weighted hydrodynamic diameters of AgNP stock suspensions determined by the dynamic light scattering (DLS) method were the same for citrate-AgNP and PVP-AgNP. The average particle diameters for citrate-AgNP and PVP-AgNP stock suspensions were 10.9 nm (standard deviation, SD = 0.7 nm) and 11.0 nm (SD = 0.7 nm), respectively (Table 2.3). The distribution of these particle sizes dominated the stock suspensions (Appendix Figures 2.5 & 2.6). The small particle diameters of the AgNP indicate that any impurities and large particles were removed during the purification process. The morphology of the nanoparticles characterized by TEM images was spherical with an average particle size distribution of 23.4 nm (SD = 4.4 nm) and 18.7 nm (SD = 2.9 nm) for citrate-AgNP and PVP-AgNP, respectively (Figure 2.1). PVP-

AgNP had a lower magnitude of zeta potential (-9.3 mV) compared to citrate-AgNP (-19.2 mV) which had a higher magnitude of zeta potential (Table 2.3). Both citrate-AgNP and PVP-AgNP had a neutral pH (Table 2.3) suggesting that the pH of the suspensions did not affect AgNP toxicity. The size of the AgNPs (< 20 nm), the spherical shape, and the negative charges of the nanoparticles are similar to the commercially prepared (Echavari-Bravo et al., 2015).

Table 2.3. Characteristics of synthesized silver nanoparticles (mean ± standard deviation)

Nanoparticle	pH (Mean)	Hydrodynamic diameter (Mean ± SD) nm	Average zeta potential (mV)
Citrate-AgNP	7.03	10.9 ± 0.7	-19.2
PVP-AgNP	7.17	11.0 ± 0.7	-9.3

Properties of silver nanoparticles (AgNPs) stock solutions were measured immediately after synthesis (n = 3). PVP = polyvinylpyrrolidone, SD = standard deviation.

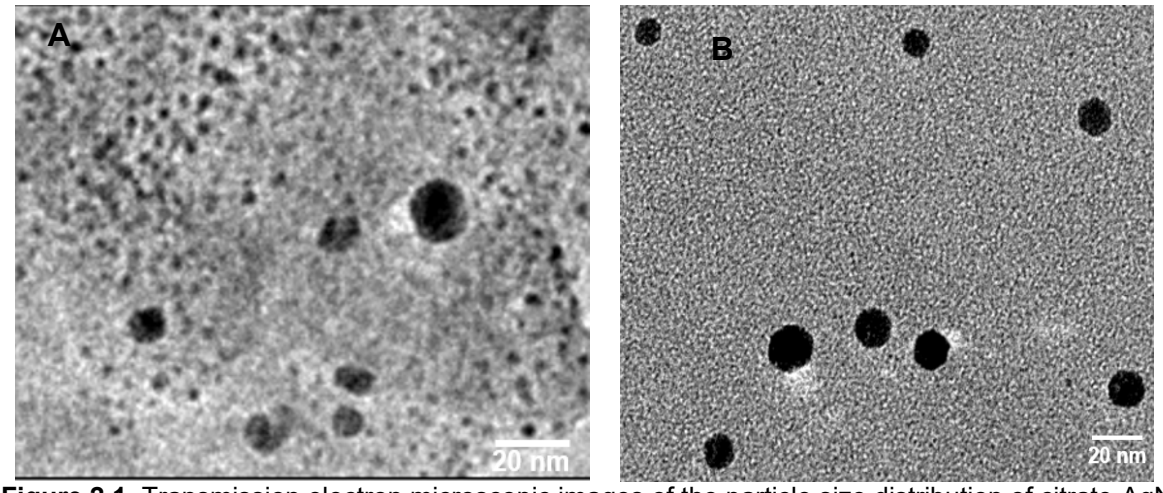


Figure 2.1. Transmission electron microscopic images of the particle size distribution of citrate-AgNPs (A) and PVP-AgNPs (B) in stock solutions. Scale bars represent 20 nm.

3.2. Chemical analysis

Concentrations of total silver for the nanoparticles ranged from 2.3 ± 0.12 to 104.2 ± 19.96 mg Ag/L (Table 2.4). Total silver in the overlying water and sediment measured at the end of the exposure period showed a similar trend for both citrate-AgNP and PVP-AgNP treated samples (Table 2.4). As expected, total silver was always higher in the sediment compared to the overlying water. Measured total silver in sediments treated with citrate-AgNP ranged from 25.7 ± 0.10 to 104.2 ± 19.96 mg Ag/L compared to the overlying water which ranged from 2.3 ± 0.12 to 20.4 ± 1.75 mg Ag/L (Table 2.4). For PVP-AgNP treated samples, measured total silver ranged from 13.4 ± 0.82 to 76.4 ± 6.35 mg Ag/L in the sediment and 3.5 ± 0.15 to 50.1 ± 0.02 mg Ag/L in the overlying water (Table 2.4). Differences between the overlying water and sediment total silver concentrations were statistically significant in only citrate-AgNP treated samples ($p < 0.01$). Total silver in the creek water sediment and silver ion treated sediment were 0.8 ± 0.11 and 21.1 ± 4.75 mg Ag/L (0.0035 mg Ag/kg), but silver in their overlying water was below the detection limit (Table 2.4).

Table 2.4. Measured total silver in test solutions, overlying water, and sediments (n = 3).

Nanoparticle	Ag added (mg Ag/L)	Measured total Ag (mean ± SD) (mg Ag/L)		
		Test solution	Overlying water	Sediment
Citrate-AgNP	0	NA	ND	0.8 ± 0.11
	25	24.5 ± 0.6	2.3 ± 0.1 a	25.7 ± 0.1 b
	50	46.2 ± 4.7	6.1 ± 0.6 a	38.1 ± 0.1 b
	75	69.7 ± 15.9	7.8 ± 0.9 a	67.3 ± 7.8 b
	100	90.9 ± 13.6	13.7 ± 2.3 a	71.6 ± 13.4 b
	125	107.9 ± 20.6	20.4 ± 1.8 a	104.2 ± 20.0 b
PVP-AgNP	25	23.4 ± 2.3	3.5 ± 0.2	13.4 ± 0.8
	50	43.7 ± 1.5	15.1 ± 2.0	27.9 ± 10.2

	75	71.1 ± 3.8	36.2 ± 1.1	41.3 ± 1.6
	100	86.1 ± 2.5	44.1 ± 2.5	51.9 ± 10.2
	125	113.5 ± 2.8	50.1 ± 0.1	76.4 ± 6.4
$AgNO_3$	25	21.1 ± 4.8	ND	20.8 ± 0.9

Statistically significant differences between total silver in sediment and overlying water are denoted by different letters and those without letters are not significantly different ($p < 0.05$). SD = standard deviation. The unit for total silver in sediment is mg Ag/kg. It is shown here as mg Ag/L for the purposes of clearer comparison to total silver in water.

3.3. Microbial responses to AgNPs

Microbial concentration was inhibited by both citrate-AgNP and PVP-AgNP after 48 hours of exposure (Figure 2.2A). Microbial concentrations declined significantly as AgNP concentrations increased. A post-hoc analysis showed that PVP-AgNP and citrate-AgNP significantly ($p < 0.05$) inhibited microbial concentration at 25 and 75 mg Ag/L nominal (nominal concentration), respectively compared to the negative control. The effects of the two nanoparticles on microbial concentration were significantly different ($p < 0.01$). PVP-AgNP demonstrated greater inhibition of microbial concentration than citrate-AgNP. At 25 mg Ag/L nominal, PVP-AgNP decreased microbial concentration by 81.5% while citrate-AgNP decreased the microbial concentration by 69.4% at 75 mg Ag/L as compared to the control. Citrate used as coating material enhanced an increase in microbial concentration while PVP decreased microbial concentration, but the effect was not statistically different from the control (Figure 2.2B). In contrast, $AgNO_3$ at nominal 25 mg Ag/L had a significant inhibition effect on microbial concentration (Figure 2.2B) and decreased microbial concentration by 92% compared to what was observed in the negative control. Citrate-AgNP decreased microbial concentration from 25 to 125 mg Ag/L but the effect was

statistically significant at 75, 100 and 125 mg Ag/L nominal only. On the other hand, PVP-AgNP inhibition effect on microbial concentration was statistically significant at all exposure levels (Figure 2.2A).

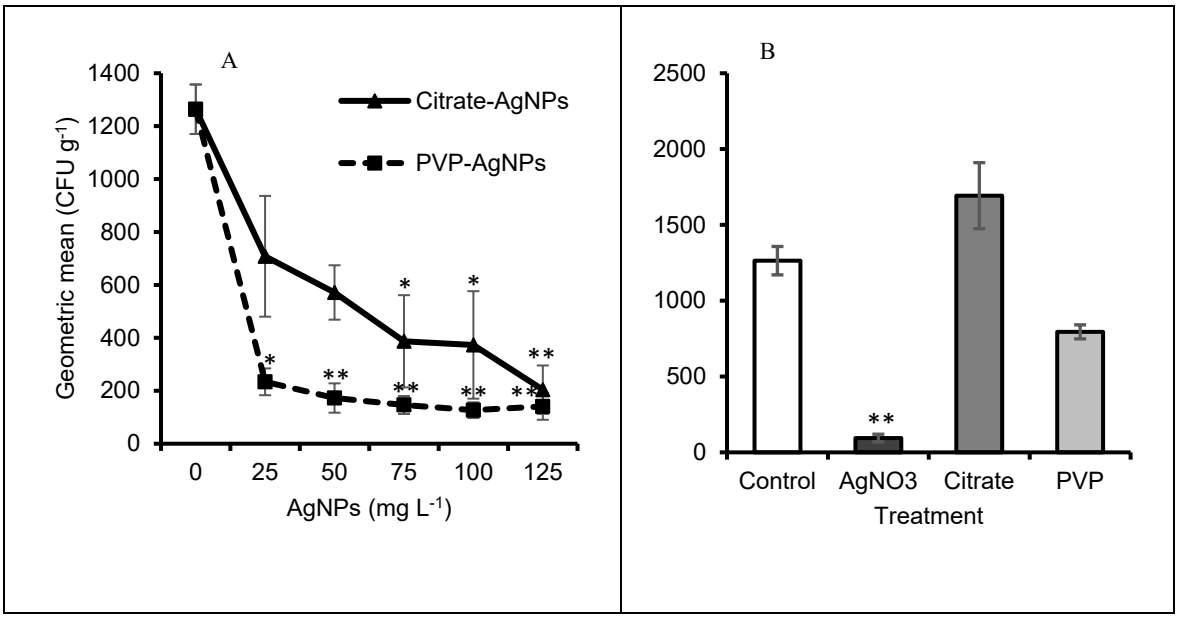


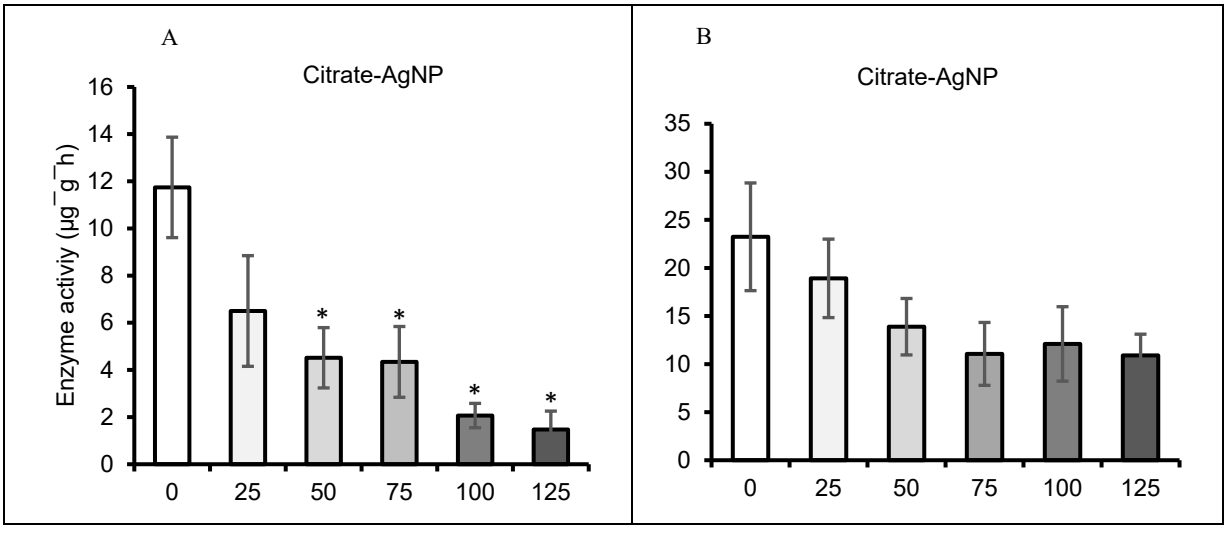
Figure 2.2. Effects of AgNPs and $AgNO_3$ on microbial concentrations in freshwater sediments after 48-h of exposure (n = 4). A = AgNPs and B = silver ion and AgNP surface coating agents. Error bars are standard errors. In bar and line graphs, error bars with asterisks denote statistically significant differences between AgNPs or treatment and the control. One asterisk (*) symbolizes $p < 0.05$ and two asterisks (**) symbolize $p < 0.01$.

3.4. Enzyme activity

Alkaline phosphatase and β-glucosidase responded differently to AgNP exposure (Figure 2.3). The β-glucosidase activity was significantly ($p < 0.05$) inhibited by both citrate-AgNP and PVP-AgNP (Figure 2.3A, C). For alkaline phosphatase, there was an observable decrease in the enzyme activity at the concentrations tested for citrate-AgNP exposure although the differences were not statistically significant (Figure. 2.3B). PVP-AgNP did not have an observable effect on alkaline phosphatase at the

concentrations tested (Figure 2.3D). Both AgNPs demonstrated a similar dose-response pattern for the inhibition of β-glucosidase activity. As AgNP exposure concentrations increased, β-glucosidase activity decreased. The β-glucosidase activity decreased at all tested concentrations but only 50, 75, 100, and 125 mg Ag/L nominal for citrate-AgNP and 75, 100, and 125 mg Ag/L nominal for PVP-AgNP were statistically significant (Figure 2.3A, C). Citrate-AgNP and PVP-AgNP at 75 mg Ag/L nominal decreased β-glucosidase activity by 63% and 77%, respectively compared to the control.

Although PVP-AgNP affected β-glucosidase activity at a lower concentration, the overall effect was not significantly different from that of citrate-AgNP ($p > 0.05$). Citrate, PVP, and $AgNO_3$ did not affect alkaline phosphatase. On the other hand, Citrate, PVP, and $AgNO_3$ caused an observed reduction in β-glucosidase activity but not statistically significant (Figure 2.4).



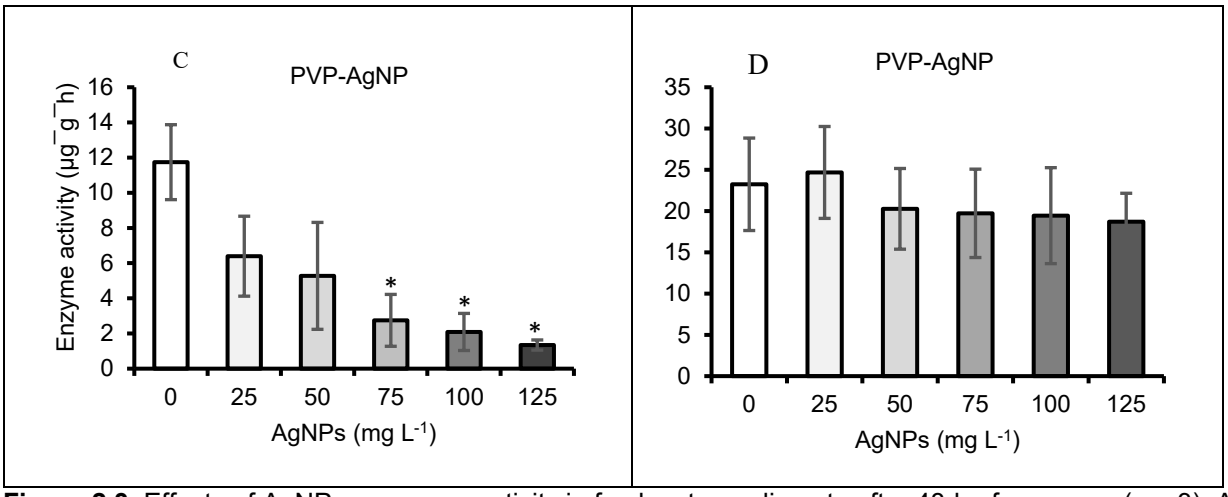


Figure 2.3. Effects of AgNP on enzyme activity in freshwater sediments after 48-h of exposure (n = 3). A = citrate-AgNP effects on β-glucosidase; B = citrate-AgNP effects on alkaline phosphatase; C = PVP-AgNP effects on β-glucosidase; and D = PVP-AgNP effects on alkaline phosphatase. Error bars are standard errors of the means. Error bars with asterisks denote statistically significant differences between AgNPs and the control ($p < 0.05$).

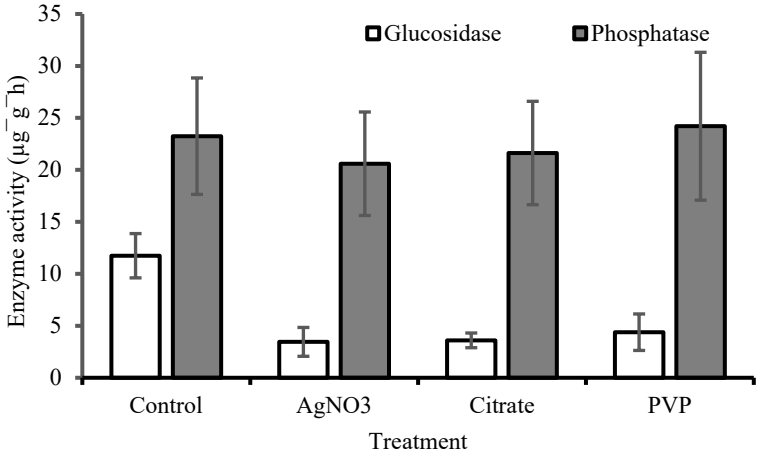


Figure 2.4. Glucosidase and alkaline phosphatase activities responses to silver ion ($AgNO_3$) and AgNP surface coating agents. PVP = polyvinylpyrrolidone

4. Discussion.

4.1. Silver nanoparticle characteristics and toxicity

Particle size, shape, charge, and surface coatings influence the toxicity of nanomaterials in environmental media (Fabrega et al., 2011; El Badawy et al., 2013; Pal et al., 2007; USEPA, 2014). Both nanoparticles had similar HDD (citrate-AgNP = 10.9 ± 0.7 and PVP-AgNP = 11.0 ± 0.7 nm), so it is unlikely that particle size contributed to the differences in their antimicrobial effects. In contrast, the absolute values of the surface charge (zeta potential) of the nanoparticles were different (Table 2.3). The zeta potential of PVP-AgNP (-9.3 mV) was lower indicating greater agglomeration and less stability (Button et al., 2016; Echavarri-Bravo et al., 2015). Lower zeta potential suggests that the behavior of PVP-AgNP in our study may have been altered by agglomeration. On the other hand, citrate-AgNP had a higher zeta potential (-19.2 mV) indicating low aggregation of particles in the suspension (Button et al., 2016).

Total silver in sediments was higher than that of the overlying water at all exposure levels in citrate-AgNP treated samples (Table 2.4), supporting the commonly held understanding that metals in aquatic systems settle in sediments and exposes benthic organisms to a higher risk of metal toxicity (Ramskov et al., 2015; Colman et al., 2012; Baun et al., 2008). The removal of citrate-AgNP but not PVP-AgNP from water column by alum in a previous study (Salih et al., 2019) demonstrates that citrate-AgNP is highly reactive and settles easily at the bottom. Citrate-AgNP had a higher surface charge, which may have contributed to the high amounts of silver in the sediment. A high surface charge of citrate-AgNP decreased agglomeration allowing the particles to interact with biotic and abiotic ligands and remained in the sediment (Xu & Zhang,

2018). PVP is a non-ionic and hydrophobic polymer making PVP-AgNP less reactive (Asadishad et al., 2017). Unlike with Citrate-AgNP, we found no significant differences between the amount of total silver in the overlying water and sediments treated with PVP-AgNP. Since PVP-AgNP had a low surface charge, the particles agglomerated decreasing their surface area and ability to interact more with ligands in the sediment than other ligands in the overlying water. Surface charge and agglomeration of PVP-AgNP may explain the behavior (fate and transport) of the nanoparticles in aquatic systems. A similar pattern has been observed in commercially prepared AgNP with a decreased surface charge which enhanced agglomeration and increased the particle hydrodynamic diameter causing the nanoparticle to settle out and weaken its toxicity (Echarri-Bravo et al., 2015).

4.2. Microbial responses to AgNPs

Changes in the environment can alter microbial concentration, metabolic activity, and community structure due to high surface to volume ratio and low homeostasis in microorganisms (Rodrigues et al., 2013). Reduction in microbial concentration and metabolic activity observed in our study is an indication of microbial responses to AgNP exposure. AgNPs decreased the number of bacteria in sediment collected from a pathogen impaired stream after 48 hours of exposure. For example, at 25 mg Ag/L nominal, the concentration of bacteria was lower in PVP-AgNP (234 ± 50.6 CFU/g) and citrate-AgNP (708 ± 228.2 CFU/g) exposed sediments versus the controls (1264 ± 93.6 CFU g^{-1}) (Figure 2.2A). Thus, PVP-AgNP and citrate-AgNP caused at least 43% and 82% decrease in microbial concentration, respectively. Colman et al. (2012) dosed

freshwater sediments with AgNP concentrations twice as the highest concentration in our study but found little effect on microbial activity. The higher concentration of dissolved organic carbon measured in the sediments used by Colman et al. (2012) may have reduced the AgNP toxicity (Pokhrel et al., 2013; Trenfield et al., 2012).

A decrease in bacterial concentration by PVP-AgNP observed in our study was similar to the results of previous studies that investigated the nanoparticle effects on microbial communities in different environmental compartments (Table 2.1). In contrast, the effects of citrate-AgNP on bacterial concentration are limited in previous studies. To the best of our knowledge, this is the first study to evaluate bacterial responses to citrate-AgNP toxicity in sediment collected from a pathogen impaired stream.

A single exposure concentration of $AgNO_3$ (25 mg Ag L^{-1}) equivalent to the lowest exposure concentration of the AgNPs was used to determine the effect of the silver ion on microbial concentration as measured using standard plate counts. Silver ion inhibited microbial concentration the most (92%) compared to the control; confirming the well-known antimicrobial properties of silver. Although sodium citrate and PVP affected microbial concentration, their effects were not statistically significant compared to the control. This effect was expected because citrate and PVP are not toxic to bacteria (Pokhrel et al., 2012). Although citrate and PVP as coating agents are non-toxic, they influenced the behavior of their respective AgNPs. Citrate stimulated microbial concentration at very low concentrations of citrate-AgNP during our preliminary studies. Therefore, it is not surprising that citrate-AgNP influenced microbial concentrations less than PVP-AgNP (Figure 2.2). Asadishad et al. (2017) also reported stimulated microbial activity associated with exposure to citrate-coated gold

nanoparticles. Citrate is a precursor metabolite for bacterial metabolic pathways to release energy from carbohydrates (White et al., 2012) and may have been used by the microorganisms as an extra carbon source resulting in growth stimulation.

Our results demonstrate that AgNP may inhibit microbial activity at low AgNP concentrations. Although AgNP exposure concentrations (0.108 to 0.538 mg Ag/kg) used in our study are higher than those reported in other studies (Bao et al., 2016; Welz et al. 2018), they are much lower than the expected concentrations (2 to 14 mg Ag kg^{-1}) in freshwater sediments predicted by models as Ag (Blaser et al., 2008). AgNPs inhibiting microbial concentration at concentrations lower than what can be found in sediments raises concerns about potential risks that these nanoparticles may pose to microbial communities and their ecological significance such as nutrient mineralization, nitrogen cycling, and organic carbon degradation (Hegde et al., 2016) in aquatic systems.

4.3. Enzyme activity

Alkaline phosphatase and β-glucosidase activities were examined in this study, but only β-glucosidase activity was significantly inhibited by AgNP compared to the control (Figure 2.3). Alkaline phosphatase is an extracellular enzyme used to obtain phosphorus from large substrates (Bao et al., 2016; Das et al., 2012). In freshwater sediment, alkaline phosphatase activity can be used as an indicator of microbial community density and biomass (Sayler et al., 1979). β-glucosidase is an extracellular enzyme that degrades carbohydrates for bacterial metabolism (Liu et al., 2008). Both citrate-AgNP and PVP-AgNP exhibited similar inhibition patterns of β-glucosidase

activity. AgNP reduced β-glucosidase activity in the sediment by at least 75% compared to the control suggesting a rapid enzyme response to AgNP. An inhibition of β-glucosidase activity is an indication of AgNP ability to interfere with the hydrolytic activity that breaks down complex organic compounds to simple molecules for bacterial metabolism. The decline in β-glucosidase activity may be due to the penetration of AgNPs into the bacterial cell resulting in an alteration of the structure and shape of the enzyme causing it to lose its catalytic ability (Fabrega et al., 2011; Tortora et al., 2004). AgNPs are also inhibitors and may bind to the active site or another part of the enzyme or compete with the substrate for the active site (Tortora et al., 2004). For example, citrate-containing compounds are highly negatively charged and have high binding affinities to enzymes which may have altered β-glucosidase activity (Asadishad et al., 2017).

The results of our study demonstrate that AgNP can inhibit β-glucosidase activity in pathogen impaired streams which may lead to false negative enterococci (pathogen indicators) detection and enumeration. The US EPA Method 1600, the International Organization for Standardization (ISO) methods 7899-2, and Enterolert are standardized methods approved for the detection and enumeration of enterococci in water in the U.S. and Europe (Boehm & Sassoubre, 2014; Maheux et al., 2009). These standard methods are enzyme-based and dependent on the action of (β-glucosidase for enterococci detection in fresh and marine recreational waters (Maheux et al., 2009; ISO, 1998; USEPA, 2006). β-glucosidase is present in enterococci and it hydrolyzes glycosides to sugar and non-sugar groups (Mroczyñska & Libudzisz, 2010). Since enterococci detection methods are dependent on the expression of β-glucosidase,

inhibition of the enzyme by AgNP can cause false negative detection of enterococci. False negative results can lead to the classification of pathogen impaired streams as unimpaired and potential public health risks. Although enterococci are used as indicators of pathogen contamination in the U.S., their definitive prediction of pathogen occurrence in recreational waters may be limited by AgNP exposure.

Our results showing that AgNPs affect enzyme activity are similar to the inhibition of microbial activity reported in soil (Grun et al., 2018b; McGee et al., 2017; Samarajeewa et al., 2017), water (Das et al., 2012), and sediment (Bao et al., 2016) by AgNPs summarized in Table 1. According to Table 1, limited data have been reported on AgNP effects on enzyme activity in freshwater sediment. Our results are consistent with other studies in which AgNP had no effects on alkaline phosphatase activity (Bao et al., 2016; Colman et al., 2012) but had significant effects on the activity of other enzymes (Bao et al., 2016). Findings from previous studies demonstrate that AgNP inhibits microbial activity supporting our conclusion that AgNP could affect conventional methods used to detect and enumerate FIB for classification and monitoring of pathogen impaired streams. Nucleic acid-based methods such as standard quantitative polymerase chain reaction (qPCR) and hybridization which rely on DNA sequence rather than enzyme activity can be used to assess microbial concentration in surface waters contaminated with AgNP. The qPCR targets specific genes of pathogen indicators (e.g. lacZ gene and uidA) with the β-glucuronidase enzyme to detect the presence or absence of *E. coli* in water. Hybridization is quantitative and allows for the enumeration of pathogen indicators (Pachepsky et al., 2018; Tallon et al., 2005).

4.4. Mechanism of AgNP toxicity to microbes

Silver nanoparticles are widely used in consumer products, but their mechanisms of action are not well understood (Tambosi et al., 2018). It is unclear whether the toxicity is directly caused by the AgNPs or dissolved Ag ions released from the nanoparticles (Fabrega et al., 2011; Levard et al. 2012; USEPA, 2012). While some studies suggest that AgNP toxicity is mainly caused by the nanoparticles themselves, other studies suggest that it is the dissolved Ag ions released from the AgNPs which enhance toxicity (Fabrega et al., 2011; Levard et al., 2012). It appears that the nanoparticles themselves primarily exert toxic effects on the enzymes since the toxicity of AgNPs is dependent on the properties of the nanoparticles such as particle size, shape, charge, and capping agents (Raza et al., 2016). Evidence from previous studies demonstrates the presence of AgNPs in bacteria and their interactions with bacterial cells to cause membrane damage and cell distortion (Chen et al., 2011; Gopal et al., 2013; Kumari et al., 2014). On the other hand, some studies which used $AgNO_3$ as a positive control found the free Ag^+ more toxic than their nanoparticles (Colman et al., 2012; Silva et al., 2014).

Determining the mechanisms of AgNP influence on microbial activity is beyond the scope of this study since we did not measure dissolved Ag^+ in the samples. However, nanoparticle properties such as size, shape, surface charge, and capping agents have been found to influence the accumulation of AgNPs in animal cells and tissues (Bruneau et al., 2016; Kleiven et al., 2018; Zhao & Wang, 2011). Smaller nanoparticle size and capping agents increase the bioavailability of AgNP compared to larger and uncoated nanoparticles (Kleiven et al., 2018; Scown et al., 2010). AgNPs used for our study had different surface charges and coating agents which may

influence their behaviors in sediment. Significant differences in AgNP accumulation and toxicity in sediments due to nanoparticle properties found in our study suggest that AgNPs and Ag^+ may have different toxicity mechanisms.

5. Conclusion

The results of our study demonstrate that AgNPs entering the aquatic system settle in the sediment and pose potential risks to benthic microbial communities. Citrate-AgNP and PVP-AgNP inhibited microbial concentration and enzyme activity at low concentrations much lower than what is expected in the environment predicted by models as Ag (Blaser et al., 2008). AgNPs induced dose-dependent toxicity in microbial concentration and β-glucosidase activity but not alkaline phosphatase activity. Our results suggest that microbial abundance and carbohydrate degradation in pathogen impaired streams may be altered by AgNP exposure. This is the first study that compared the effects of citrate-AgNP and PVP-AgNP on microbial communities in a pathogen impaired stream. We conclude that AgNPs are inhibitors of β-glucosidase activity and may affect the accuracy of the Enterolert test used for enterococci detection and enumeration in ambient water. The findings of our study may contribute to the understanding of limitations associated with conventional pathogen detection methods (Tallon et al., 2005) to predict the occurrence of pathogens in impaired streams and recreational waters and may impact public health.

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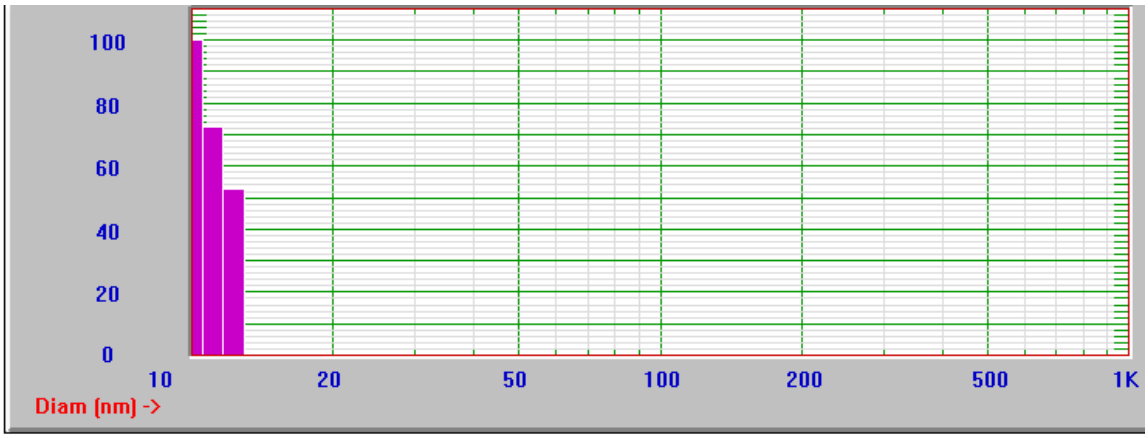

Figure 2.5. The hydrodynamic diameter of citrate-AgNPs dispersed in Milli-Q water measured by NICOMP 380 ZLS Particle Sizer.

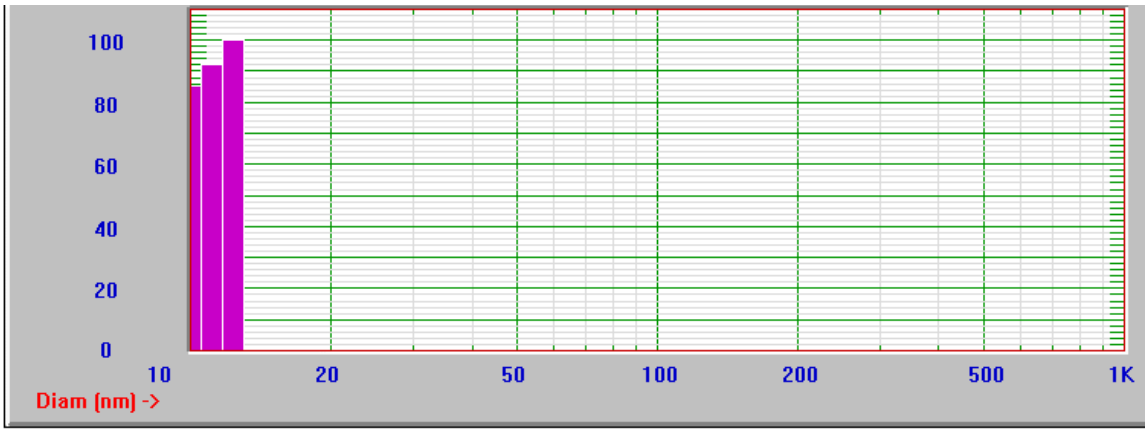

Figure 2.6. The hydrodynamic diameter of PVP-AgNPs dispersed in Milli-Q water measured by NICOMP 380 ZLS Particle Sizer.

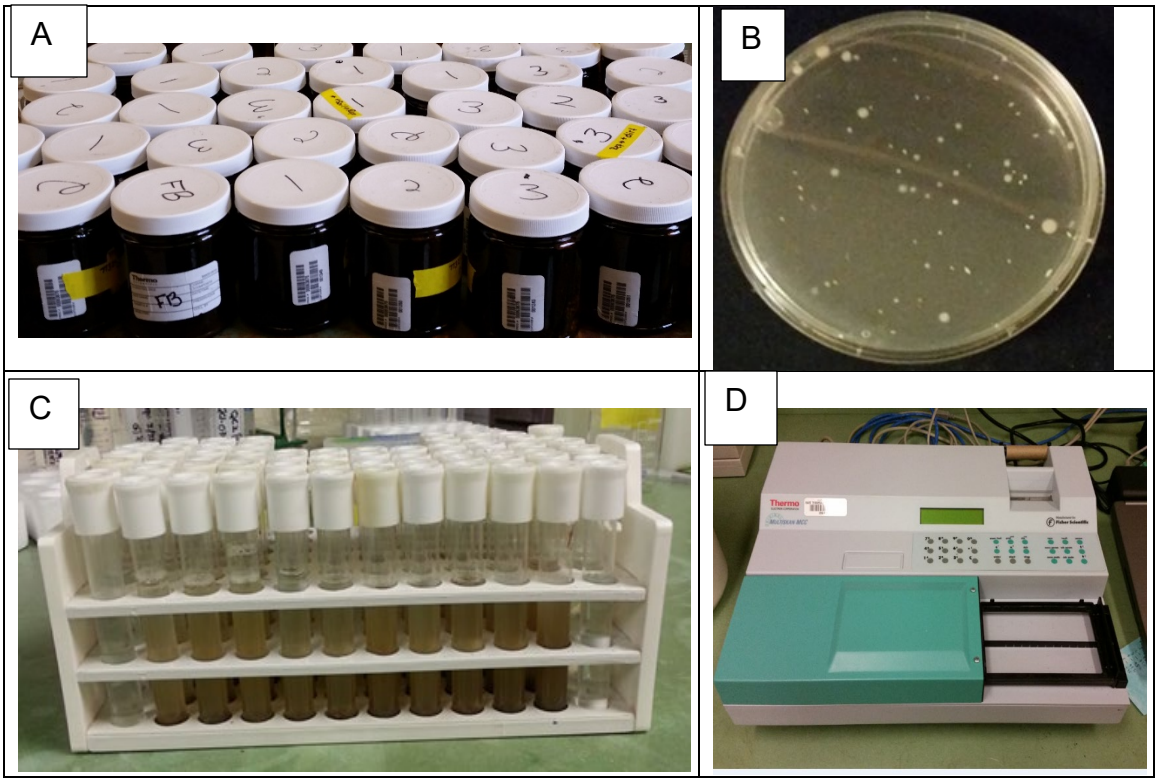

Figure 2.7. Photographs showing glass jars (A), agar plate (B), test tubes (C), and multiskan plate reader (D). The glass jars contained treated samples and the test tubes contained sediment, buffer, and substrates for enzyme activity.

CHAPTER 3

CHANGES IN MICROBIAL COMMUNITY FUNCTIONAL DIVERSITY AND METABOLIC FINGERPRINTING IN A PATHOGEN IMPAIRED STREAM AS A FUNCTION OF SILVER NANOPARTICLE EXPOSURE

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Keywords: activity; diversity; microorganisms; sediment; silver nanoparticle, substrate richness

Abstract

Microbial community diversity and metabolic activity are important for the degradation of organic matter and pollutants in the environment. There is a growing concern that these ecosystem services provided by microorganisms may be altered by the introduction of silver nanoparticles (AgNPs) into the environment. AgNPs may inhibit microbial growth and enzyme activity but their effects on microbial diversity are not fully understood. We used community-level physiological profiling (CLPP) to examine the antimicrobial effects of citrate coated AgNP (citrate-AgNP) and polyvinylpyrrolidone coated AgNP (PVP-AgNP) on the microbial community in freshwater sediments. At 125 mg Ag/L nominal, citrate-AgNP decreased microbial catabolic activity by 80% from 1.16 ± 0.02 to 0.23 ± 08 while PVP-AgNP decreased the catabolic activity by 51% from 1.25 ± 0.07 to 0.61 ± 0.19. Substrate richness decreased by 58% from 29 ±1.2 to 12 ± 5.9 and by 21% from 30 ± 2.3 to 23 ± 4.0 for citrate-AgNP and PVP-AgNP, respectively at 125 mg Ag/L (nominal concentration). The principal component analysis revealed citrate-AgNP caused a shift in carbon source utilization pattern. Our results showed that AgNPs

caused the microbial community to be less diverse and altered the metabolic fingerprinting pattern. This study suggests that AgNP can alter the ability of microorganisms to utilize different carbon sources and degrade a variety of pollutants in the environment.

1. Introduction

Microorganisms perform diverse functions in ecosystems including organic matter decomposition, nutrient cycling, and breakdown of pollutants to maintain ecosystem function (Colman et al., 2012; Escalas et al., 2019). The functional diversity of microorganisms has been used to understand the relationship between biodiversity patterns and ecosystem functioning (Escalas et al., 2019). Microbial functional diversity can be influenced by factors such as habitat alteration, predation, competition, climate change, nutrients, and chemical pollution, but a reduction in species diversity is always found associated with habitat contamination (Johnston & Roberts, 2009; Reed, 1978). Sediment is the most common environmental compartment that has been used to study pollution impacts on biodiversity (Johnston & Roberts, 2009), which may be probably due to the accumulation of contaminants in sediments (Colman et al., 2012). Currently, microbial functional diversity is threatened by emerging environmental contaminants including nanomaterials. Silver nanoparticles (AgNPs) are the type of nanomaterial extensively incorporated in consumer products over the past decade due to their antimicrobial properties that target a wide range of disease-causing microorganisms (Fabrega et al., 2011; Kuraj, 2019; Moeta et al., 2019). About 220 to 312 tons of AgNP are produced globally per year (McGee et al., 2017).

Silver nanoparticles can be released into the environment during manufacturing, transportation, use, and disposal processes (Salieri et al., 2018). Discharges from industries, washing machines, and bathtub drains containing AgNPs are deposited into sewage systems and wastewater treatment facilities (USEPA, 2012). Silver nanoparticles are more likely to affect microorganisms in aquatic systems because the current wastewater treatment processes do not remove nanoparticles effectively before discharge into surface waters (Good et al., 2016). It is not surprising that AgNPs have been detected in treated municipal wastewater and surface waters (Kim et al., 2010; Peters et al., 2018). Sediment-dwelling microorganisms will be affected most by the presence of AgNPs in aquatic systems compared to organisms in other environmental compartments due to the antimicrobial activity of the nanoparticles.

Silver nanoparticles altered microbial functional diversity in a constructed wetland, estuarine sediment, and soil which affected carbon source utilization patterns (Bao et al., 2016; Echavarri-Bravo et al., 2015; Samarajeewa et al., 2017). In addition, bacterial growth, enzyme activity, and function were inhibited by AgNP (Echavarri-Bravo et al., 2015; Khan et al., 2015; Pokhrel et al., 2012). Although previous studies have demonstrated changes in functional diversity patterns of microbial communities exposed to AgNPs in soil and marine estuarine sediment (Bao et al., 2016; Echavarri-Bravo et al., 2015; Samarajeewa et al., 2017), our understanding of how AgNP influences microbial functional diversity in freshwater sediment is limited. A study investigating the effects of AgNPs on microbial functional diversity using community-level physiological profiling (CLPP) is needed to elucidate whether the ability of the microbial community to utilize different carbon sources in freshwater sediments can be altered by AgNPs.

Community-level physiological profiling is a useful technique for measuring the functional diversity of heterotrophic communities (Garland & Mills, 1991; Weber & Legge, 2010). The CLPP provides Biolog^{TM} EcoPlate data on metabolic activity and carbon source utilization patterns needed to assess the functional diversity of microbial communities (Zak et al., 1994). In addition, CLPP has been useful in providing information on microbial community function over space and time (Weber, 2010). This technique has also been used recently to determine the effects of nanomaterials on microbial community function in streamwater, soil, and estuarine sediment (Button et al., 2016; Echavarri-Bravo et al., 2015; Kumar et al., 2011: Rodrigues et al., 2013; Samarajeewa et al., 2017). Thus, CLPP could be used to evaluate the impacts of AgNPs on microbial functional diversity in freshwater sediments. Substrate richness (number of substrates utilized by a microbial community), substrate diversity, and substrate evenness are diversity measures commonly used to determine and describe the impacts of contaminants on functional diversity (Johnston & Roberts, 2009; Weber et al., 2008; Weber & Legge, 2010).

In this study, we evaluated the impacts of citrate coated and polyvinylpyrrolidone (PVP) coated AgNPs on microbial diversity and catabolic activity in freshwater sediments. Since AgNPs caused changes in soil microbial function, we expected AgNPs to alter microbial functional diversity and carbon source utilization patterns. Citrate is a precursor metabolite for bacterial metabolic pathways to release energy from carbohydrates (White et al., 2012). If bacteria used citrate as a carbon source in the exposed samples, we expect a lower inhibition effect by citrate coated AgNP compared to PVP-coated AgNP. We collected sediments from a pathogen impaired

creek to examine potential effects of AgNPs on microbial community functional diversity and metabolic fingerprinting.

2. Materials and methods

2.1. Sample collection

Streamwater and sediment were collected from the downstream of Sinking Creek (Johnson City, Tennessee, U.S.A), an impacted stream of the Watauga River Watershed due to pathogen contamination from agricultural settings (TDEC, 2016). Two liters of streamwater were collected into sterile polyethylene carboys and 1 kg sediment was collected 5 cm below the sediment surface into polyethylene bags in triplicates. Microorganisms in streamwater were removed by filtering the water through a 0.45 μm filter and debris in the sediments was removed through a 2 mm sieve. Samples were kept in a refrigerator at 4 ^{o}C overnight before they were used for microbial analyses. Streamwater pH ranged from 7.9 to 8.3, the conductivity of 367.5 μS cm^{-1} (standard deviation, SD = 19.1 μS cm^{-1}), and dissolved oxygen of 8.5 mg/L (SD = 0.5 mg L^{-1}) were measured during sample collection. The sediment pH ranged from 6.5 to 6.8. Concentrations of Ca^{2+}, Mg^{2+}, Cl^{-}, NO_3^{-}, PO_4^{-3}, and SO_4^{-2} in the stream were 49.6 ± 8.7, 9.4 ± 0.6, 7.8 ± 0.1, 5.8 ± 0.1, 0.3 ± 0.1, and 6.1 ± 0.1 mg L^{-1} (mean ± SD), respectively.

2.2. Silver nanoparticle synthesis and characterization

Stock suspensions of AgNPs were prepared in the laboratory following a method described in previous studies (El Badawy et al., 2010; Pokhrel et al., 2014). The nanoparticles were stabilized with surface coating agents to obtain citrate-coated silver

nanoparticle (citrate-AgNP) and polyvinylpyrrolidone-coated silver nanoparticle (PVP-AgNP). Appropriate amounts of silver nitrate (Fisher Science) and sodium citrate dihydrate (Fisher Science) were dissolved in milli-Q water. Silver nitrate and sodium citrate solutions were mixed in a 1 to 2 ratio and heated in a water bath at 70 ^{o}C for 4 hours to synthesize citrate-AgNP stock suspension. An appropriate amount of sodium borohydride (Acros Organics) was dissolved in a 1% polyvinylpyrrolidone K60 (Sigma Aldrich) solution. Silver nitrate solution was added to the sodium borohydride solution drop by drop in a 1 to 3 ratio while stirring vigorously on ice to obtain PVP-AgNP stock suspension. The AgNP suspensions were sonicated for 10 minutes and purified using a polysulfone 10 kD hollow fiber filter in Kros Flo Research IIi tangential flow filtration system (Spectrum Laboratories, CA, USA). Dynamic light scattering (DSL) method (NICOMP 380 ZLS Particle Analyzer, PSS NICOMP, Santa Barbara, CA, USA) was used to determine hydrodynamic diameter (HDD) and zeta potential of the nanoparticles. The accuracy of the instrument was checked by using a standard solution supplied by the manufacturer. Transmission electron microscopy (TEM) was used to visualize particle morphology and size distribution in stock solutions. ImageJ software (National Institute of Health, USA) was used to estimate the size of the nanoparticles. Sodium citrate and PVP solutions were prepared to test for the influence of surface coating agents on AgNPs toxicity. Silver nitrate stock solution was prepared to serve as a positive control.

2.3. Sediment dosing

Streamwater filtered through 0.45 µm filter was used to prepare exposure nominal concentrations (25, 50, 75, 100, and 125 mg Ag L^{-1}) of each nanoparticle and 25 mg Ag

L^{-1} $AgNO_3$. Thirty grams wet sediment (23.2 g dry weight) was treated with 100 mL of the test solutions in 250 mL clean glass jars in triplicate. A glass rod was used to mix the test solution with the sediment manually and incubated at room temperature for 48 hours.

2.4. Community functional diversity

Community-level physiological profiling (CLPP) based on carbon source utilization patterns from Biolog™ EcoPlate data was used to evaluate the effects of AgNPs on microbial community function in sediments. Each Biolog™ Ecoplate contains 96 wells and 31 unique carbon sources in triplicate with 3 controls (Appendix Table 3.3). CLPP provides useful information on changes in microbial community in the environment and nanomaterial effects on the microbial activity in different environmental compartments (Button et al., 2016; Echavarri-Bravo et al., 2015; Samarajeewa et al., 2017; Weber and Legge, 2010).

Two grams of treated sediments were added to 18 mL of milli-Q water in 50 mL centrifuge tubes and vortexed for 30 seconds. The supernatant was 10-fold diluted, and 150 µL was inoculated in each well on Biolog™ EcoPlate. The Biolog™ EcoPlates were incubated at 20 ^oC for 120 h and color development in each well was measured using a multiskan ascent plate reader at 590 nm after every 24 h up to 120 h. Color development measured at 120 h of incubation was used for all analyses. Average well color development (AWCD) for each Biolog™ EcoPlate was calculated to determine microbial metabolic activity according to the method described by Samarajeewa et al. (2017):

AWCD = ∑ODi/31

Where ODi is the corrected optical density (OD minus blank) for each well and 31 represents the total number of carbon substrates.

Substrate richness, substrate diversity, and substrate evenness were examined to determine AgNP effects on microbial functional diversity based on carbon source utilization patterns (Weber et al., 2008; Weber et al., 2011). Substrate richness (*S*) was calculated by counting the number of wells with a corrected OD greater than 0.25. Shannon diversity index (*H*) represented by substrate diversity was calculated as:

$$H = \sum p_i \ln(p_i),$$

where *H* is the substrate diversity and p_i is the ratio of a substrate activity to the overall substrate activity. Substrate evenness (*E*) was calculated as:

$E = H/\log S$.

2.5. Measurement of total silver

Aliquots of sediment were taken after 48 hours and acid digested using the standard method 3050B (USEPA, 1996). Total silver in the samples was measured using the flame atomic absorption spectrophotometry (FAAS). Nitric acid (5%) was used to wash all glassware before use. Pure silver purchased from NSI Lab Solutions was used to calibrate the instrument before AgNP analysis.

2.6. Statistical analysis

Data were not normally distributed after transformation and they were analyzed using Kruskal-Wallis one-way ANOVA in Statistical Package for the Social Sciences software

(SPSS 24). Two ecological metric analyses, principal component analysis (PCA) and diversity indices (substrate diversity, substrate richness, substrate evenness), were performed to determine shifts in the microbial community due to AgNP exposure. Dunnett's multiple comparison test was performed to compare the effects of the AgNPs on microbial diversity with the control. The PCA was used to separate and differentiate treatments based on carbon sources utilization patterns of the microbial community.

3. Results

3.1. Characteristics of nanoparticles

The average particle diameters for citrate-AgNP, 10.9 nm (SD = 0.7 nm) and PVP-AgNP, 11.0 nm (SD = 0.7 nm) in stock solutions measured by DSL were similar. The nanoparticles had different zeta potential (surface charge). Citrate-AgNP had a greater surface charge (-19.2 mV) compared to PVP-AgNP (-9.3 mV). The shape of both nanoparticles determined by the TEM was spherical (Figure 3.1), and the average diameters were greater than those measured by the DSL. The mean particle diameters for citrate-AgNP and PVP-AgNP stock suspensions analyzed from TEM images were 21.8 nm (SD = 6.5 nm, n = 26) and 16.7 nm (SD = 5.2 nm, n = 27), respectively.

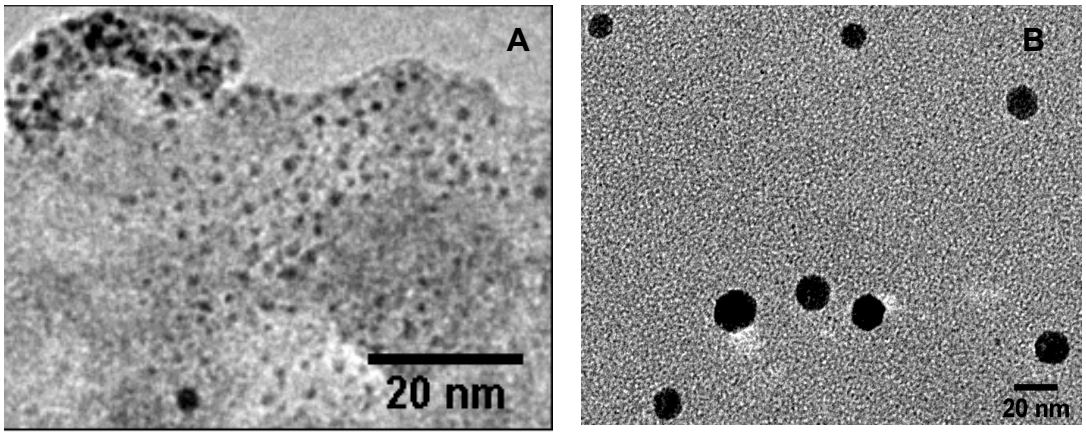

Figure 3.1. Transmission electron micrographs of images showing the particle size distribution of citrate-AgNP (A) and PVP-AgNP (B) in stock solutions. Scale bars represent 20 nm.

3.2. Total silver

The addition of nanoparticle suspensions to the sediment increased the total amount of silver in the sediment to cause toxic effects. Total silver in sediments varied between citrate-AgNP and PVP-AgNP. Measured total silver in sediment after 48 hours of exposure ranged from 0.11 mg Ag kg^{-1} (SD = 0.01 mg Ag kg^{-1}) to 0.43 mg (SD = 0.07 mg Ag kg^{-1}) for citrate-AgNP and 0.06 mg Ag kg^{-1} (SD = 0.01 mg Ag kg^{-1}) to 0.33 mg Ag kg^{-1} (SD = 0.03 mg Ag kg^{-1}) for PVP-AgNP (Table 3.1). Though total silver in sediments treated with citrate-AgNP was higher than that of PVP-AgNP at all exposure levels, their means were not significantly different.

Table 3.1. Measured total silver in sediment after 48-h exposure

Type of silver	Nominal (mg Ag L^{-1})	Total Ag in sediment (mg Ag kg^{-1} dry weight) ± SD
Citrate-AgNP	0	0.01 ± 0.00
	25	0.11 ± 0.01
	50	0.17 ± 0.04
	75	0.29 ± 0.03
	100	0.33 ± 0.03
	125	0.43 ± 0.07

PVP-AgNP	25	0.06 ± 0.01
	50	0.12 ± 0.04
	75	0.19 ± 0.02
	100	0.24 ± 0.04
	125	0.33 ± 0.03

Total silver in sediment was measured at the end of the test (n = 3). SD = standard deviation. Concentration in mg Ag kg^{-1} was calculated by dividing the concentration of the volume of test solution added to sediment by mean dry weight of sediment (Colman et al., 2012).

3.3. Microbial community diversity

Silver nanoparticles altered the community level physiological profiling of the microbial community in the freshwater sediment by causing variations in carbon source utilization patterns (CSUPs) after five days of exposure (see Appendix Figure 3.5). Carbon source utilization based on the catabolic activity (color response) of the microbial community was higher in the control samples compared to the exposed sediments (Figure 3.2). The overall catabolic activity measured by average well color development (AWCD) generally, decreased with increasing citrate-AgNP and PVP-AgNP exposure concentrations (Figure 3.2A). The inhibition effects of the two AgNPs on the catabolic activity differed significantly ($p < 0.05$). Citrate-AgNP decreased AWCD, the overall catabolic activity, at all exposure levels but the effect was statistically significant at 100 and 125 mg Ag L^{-1} nominal. PVP-AgNP also caused an observed decrease in AWCD from 50 to 125 mg Ag L^{-1} but was statistically significant at 125 mg Ag L^{-1} relative to the control (Figure 3.2A). Citrate-AgNP reduced the catabolic activity of the microbial community by 80% from 1.16 (SD = 0.02) to 0.23 (SD = 0.08) while PVP-AgNP reduced the catabolic activity by 51% from 1.25 (SD = 0.07) to 0.61 (SD = 0.19)

at 125 mg Ag L^{-1} nominal. Significant inhibition of the catabolic activity by the AgNPs is an indication of a potential reduction in microbial functional diversity.

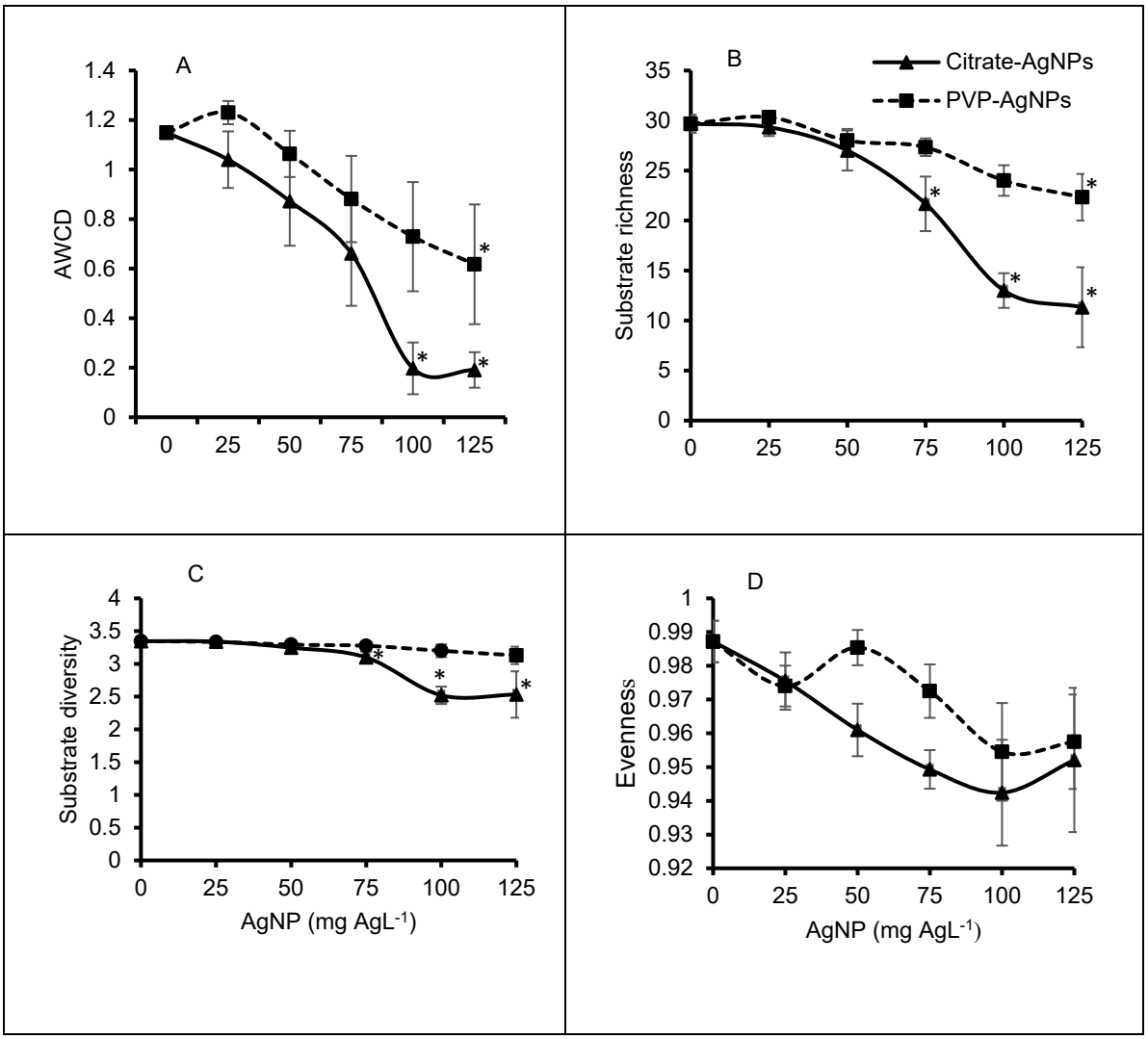

Figure 3.2. Community level physiological profiling showing the inhibition of the microbial community functional diversity by AgNPs in freshwater sediment after 48-h exposure (n = 3). Diversity indices based on carbon source utilization patterns extracted from Biolog™ EcoPlate data at 120 h of incubation were provided as follows: A = average well color development (AWCD); B = substrate richness; C = substrate diversity; and D = substrate evenness. Error bars with asterisks denote a statistically significant difference between AgNPs and the controls ($p < 0.05$).

Substrate richness, the number of carbon sources utilized by the microbial community, which depends on catabolic activity demonstrated a similar trend of AgNP effects observed for AWCD (Figure 3.2B). Both citrate-AgNP and PVP-AgNP decreased substrate richness, but the observed effect was statistically significant ($p < 0.01$) at concentrations tested above 50 mg Ag L^{-1} nominal for citrate-AgNP and at 125 mg Ag L^{-1} for PVP-AgNP only. At 125 mg Ag/L nominal, PVP-AgNP caused a decrease in substrate richness by 26% from 31 to 23 substrate utilization while citrate-AgNP caused a 61% decrease from 31 to 12 substrate utilization. Substrate diversity was reduced by citrate-AgNP at all exposure levels, but the observed effect was statistically significant at concentrations tested above 50 mg Ag L^{-1}. PVP-AgNP did not exert an observed effect on substrate diversity at all exposure levels (Figure 3.2C). In contrast, substrate evenness ranging from 0.94 to 0.99 was reduced by citrate-AgNP and PVP-AgNP but the observed effect was not statistically significant in either AgNPs (Figure 3.2D). Lack of significant AgNP effects on substrate evenness may be due to a small sample size and high variability in the two highest concentrations. Citrate and PVP used as coating agents did not affect catabolic activity, substrate richness, substrate evenness, and substrate diversity of the microbial community (Figure 3.3). Similarly, $AgNO_3$ had no significant effect on catabolic activity, substrate richness, and substrate diversity at the concentration used but reduce substrate evenness although the effect was not statistically significant (Figure 3.3).

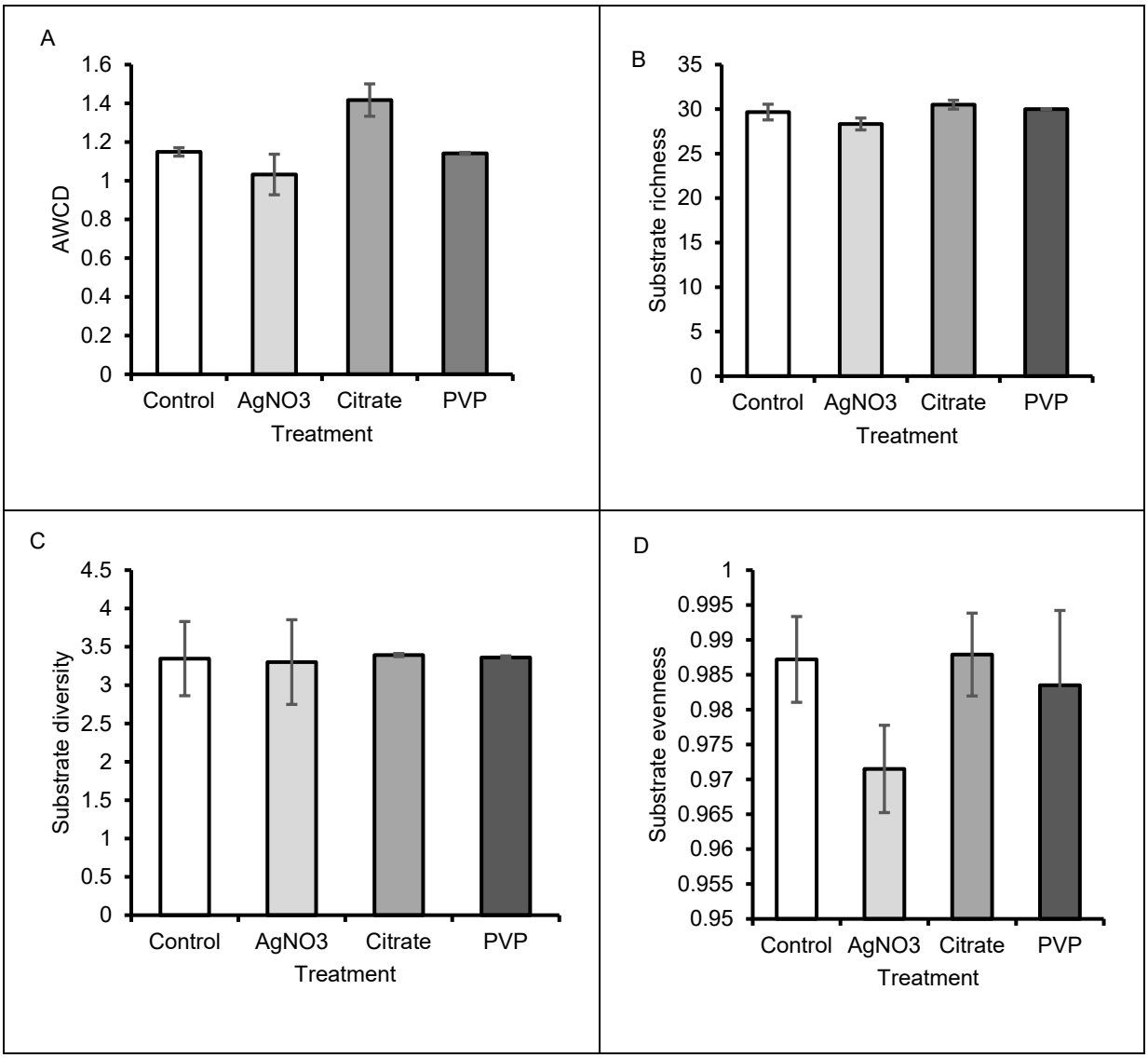

Figure 3.3. Community-level physiological profiling of the microbial community treated with silver ion and AgNP surface coating agents (n = 3). A = average well color development (AWCD); B = substrate richness; C = substrate diversity; and D = substrate evenness.

3.4. Carbon source utilization pattern

Principal component analysis (PCA) revealed groups of treatments having similar or different CSUPs. The PCA showed two different microbial community CSUPs among the treatments. The microbial community in sediments exposed to citrate-AgNP at 100 and 125 mg Ag L^{-1} nominal grouped while the microbial community in all other

treatments including positive and negative controls also grouped (Figure 3.4). The utilization of all the 31 substrates by the microbial community was affected by at least one AgNP exposure concentration except D-mannitol and 4-hydroxy benzoic acid (Table 3.2). Out of the 31 carbon substrates, citrate-AgNP inhibited 20 at 100 mg Ag L^{-1} nominal and 23 at 125 mg Ag L^{-1} nominal. Based on carbon groups, citrate-AgNP inhibited all the amino acids and carboxylic acids, and 7 out of the 10 carbohydrates at 125 mg Ag L^{-1} nominal. On the other hand, PVP-AgNP inhibited only 3 carbon substrates even at the highest exposure concentration. D, L-α-glycerol phosphate surprisingly was not utilized by the microbial community in any of the treatments including the control and was excluded from the PCA.

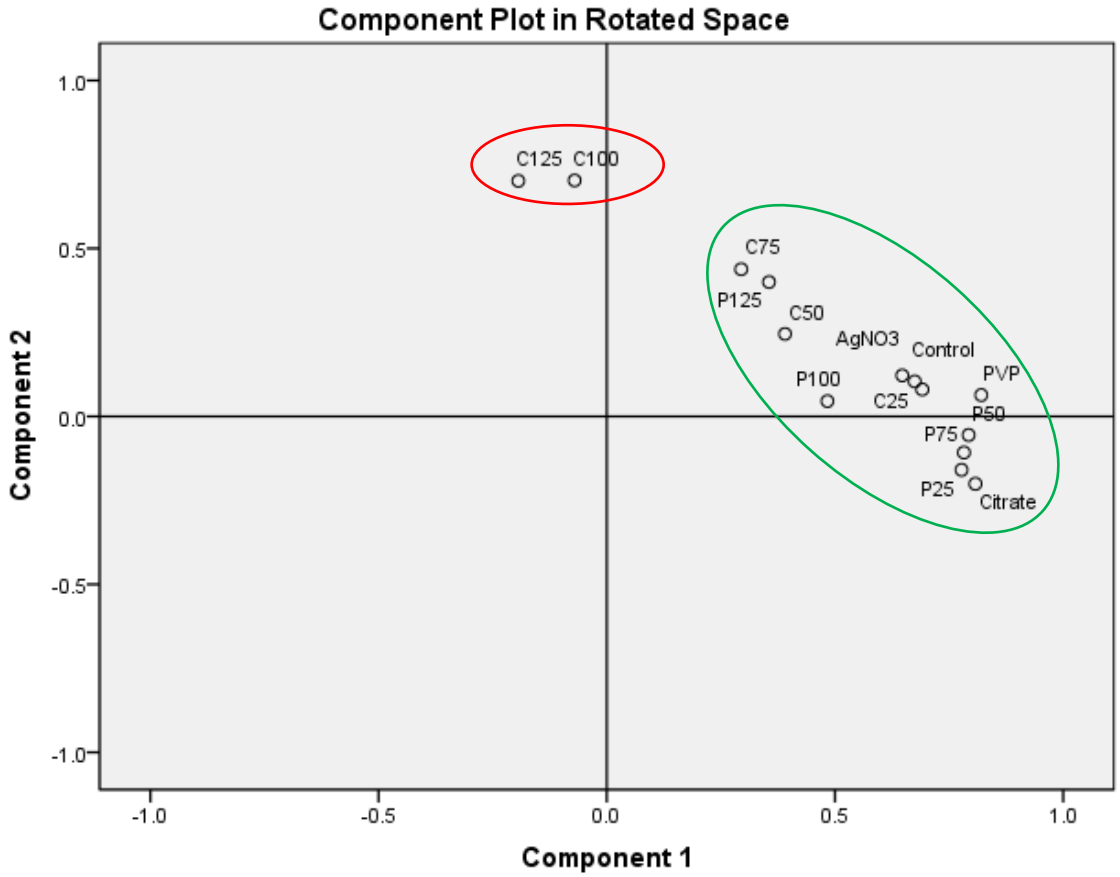

Figure. 3.4. Principal component analysis of community-level physiological profile based on carbon source utilization patterns of the microbial community in freshwater sediments exposed to AgNPs and silver ion (n = 3). Color change in Biolog^{TM} EcoPlates measured at 120 h of incubation was used for the analysis. C = citrate-AgNP; P = PVP-AgNP; and PVP = polyvinylpyrrolidone.

Table 3.2. Types of carbon groups and substrates utilized by the microbial community or inhibited by AgNPs

Carbon group	Substrate	Treatment													
			Citrate-AgNP					PVP-AgNP					Positive control		
		0	25	50	75	100	125	25	50	75	100	125	$AgNO_3$	Citrate	PVP
Amines	Phenylethyl-amine	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	Putrescine	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Amino acids	L-Arginine	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	L-Asparagine	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	L-Phenylalanine	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	L-Serine	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	L-Threonine	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	Glycyl-L-Glutamic Acid	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Carbohydrates	β-Methyl-D-Glucoside	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	D-Galactonic Acid γ-Lactone	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	D-Xylose	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	i-Erythritol	+	+	+-	-	-	-	+	+	-	-	-	+	+	+
	D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	N-Acetyl-D-Glucosamine	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	D-Cellobiose	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	Glucose-1-Phosphate	+	+	+	+	-	-	+	+	+	-	-	+	+	+
	D, L-α-Glycerol Phosphate	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	α-D-Lactose	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Carboxylic acids	Pyruvic Acid Methyl Ester	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	Itaconic Acid	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	α-Ketobutyric Acid	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	D-Malic Acid	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	D-Galacturonic Acid	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	D-Glucosaminic Acid	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	γ-Hydroxybutyric Acid	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Phenolic Compounds	2-Hydroxy Benzoic Acid	+	+	+	-	-	-	+	+	-	-	-	+	+	+
	4-Hydroxy Benzoic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Polymers	Tween 40	+	+	+	+	+	-	+	+	+	+	+	+	+	+

	Tween 80	+	+	+	+	-	-	+	+	+	+	+	+	+	+
	Glycogen	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	α-Cyclodextrin	+	+	+	+	-	+	+	+	+	+	+	+	+	+

Average corrected color responses of each carbon substrate for each treatment were used to determine AgNP inhibition effects on substrate utilization (n = 3). Average color responses < 0.25 are denoted by a negative sign (-) indicating no substrate utilization and those > 0.25 are denoted by a positive sign (+) indicating substrate utilization. AgNPs inhibited the utilization of all the substrates with a negative color response sign. AgNPs = silver nanoparticles and PVP = polyvinylpyrrolidone

4. Discussion

4.1. Microbial community functional diversity

The CLPP provided useful information on functional diversity and carbon source utilization patterns of the microbial community in the freshwater sediment. Most substrates in sediments treated with citrate-AgNP at nominal 100 and 125 mg Ag L^{-1} were not utilized by the microbial community while most substrates were utilized at all other exposure concentrations (Table 3.2). Substrate utilization inhibition was visible on EcoPlates at low exposure concentrations (Appendix Figure 3.5), as a few wells developed a color change confirmed by the low optical density detected using the Multiskan plate reader. A color change occurs when cell densities between 10^5 and 10^8 cells/mL are reached (Weber and Legge, 2010), which suggests that AgNPs reduced the number of bacterial cells in the sediments resulting in a decreased catabolic activity.

The results demonstrated changes in the pattern of catabolic activity of the microbial community caused by AgNP exposure. Although CLPP is also the carbon utilization patterns exhibited by different groups of microorganisms, fungi do not metabolize tetrazolium in the Biolog wells to cause a color change (Rodrigues et al., 2013). Thus, fungi did not contribute to the microbial responses observed in this study.

The overall catabolic activity measured by AWCD was reduced by AgNPs at the concentrations tested (Figure 3.2A). A decrease in overall catabolic activity suggests a decrease in microbial activity or carbon substrate utilization by the microbial community. Since AWCD also represents the functional diversity of the microbial community, its low value is an indication of AgNPs decreasing the ability of microorganisms to use diverse carbon sources on the EcoPlates (Weber et al., 2013; Samarajeewaa et al., 2017).

Low AWCD implies that AgNP exposure can alter the capability of microbial communities to utilize different carbon sources available in the natural environment. While both AgNPs inhibited the catabolic activity, citrate-AgNP caused a greater inhibition effect, possibly because of its higher surface charge and the larger quantity of total silver measured in the exposed sediments (Table 3.1). A similar trend of citrate-AgNP and PVP-AgNP inhibition effects on catabolic activity has been reported in a microcosm study that examined a microbial community response to AgNPs (Button et al., 2016). The morphology of the nanoparticles may have not contributed to the differences in their toxicity effects because both nanoparticles had the same shape (Figure. 3.1). Spherical AgNPs have been shown to enhance toxicity against bacterial strains compared to those with a triangular shape. The spherical shape allows the nanoparticles to penetrate bacterial cells readily to cause antimicrobial activity (Raza et al., 2016).

Other ecological metrics used to determine and describe AgNP effects on the microbial community were substrate richness, substrate diversity, and substrate evenness. These ecological metrics demonstrated a shift in CLPP due to AgNP exposure (Button et al., 2016; Kumar et al., 2011; Samarajeewaa et al., 2017). Based

on the number of carbon sources utilized by the microbial community (OD > 0.25), AgNP decreased substrate richness significantly compared to the control (Figure 3.2B). A decrease in substrate richness followed a decrease in the microbial population and the overall catabolic activity (Weber et al., 2008). While AWCD and substrate richness were affected by both AgNPs, only citrate-AgNP significantly decreased substrate diversity at the tested concentrations above 50 mg Ag L-1 in this study (Figure 3.2C). Substrate diversity was 25% lower than the control at 100 mg Ag L^{-1}. This suggests that citrate-AgNP caused the microbial community to be less diverse in utilizing different carbon sources and can decrease the ability of microbial communities to decompose organic matter and variety of environmental pollutants (Weber et al., 2013).

Substrate evenness is the equal use of all utilized substrates (Weber & Legge, 2010), which can be used to determine the effects of rare species on carbon source utilization patterns (CSUPs) (Weber et al., 2008). Rare species resistant to environmental contaminants can increase microbial activity in some wells, which may have a large impact on the CSUP by increasing substrate richness and decreasing substrate evenness (Weber et al., 2008; Zak et al., 1994). On average, AgNPs reduced substrate evenness from 0.99 to 0.94 but the difference was not statistically significant (Figure 3.4D). This suggests that AgNP affected the bacterial species evenly including the rare species (if any) resulting in no or little change in substrate evenness. If there were the existence of rare species, their activity was inhibited by AgNP making substrate richness the most sensitive indicator of AgNP impacts on microbial functional diversity (Johnston & Roberts, 2009). This implies that if there was the existence of rare species, their activity did not influence the CSUP. Other studies have shown that the

microbial community can recover from AWCD, substrate richness, substrate diversity, and substrate evenness inhibition after several days of exposure to environmental contaminants (Weber et al., 2008), thus, long-term exposure studies are necessary to determine whether AgNP effects could be reversed over time.

4.2. Carbon source utilization pattern

The ability to distinguish between treatments and understand the factors that affect microbial community function provides ecological implications to CLPP through Biolog^{TM} EcoPlate data (Garland & Mills, 1991). Principal component analysis (PCA) was used to determine the differences in CSUPs among treatments. PCA is commonly used to assess the effects of environmental variables and contaminants on CLPP (Button et al., 2016; Echavarri-Bravo et al., 2015; Garland & Mills, 1991; Rodrigues et al., 2013; Weber et al., 2013;), because it is robust and its validity does not depend on the assumptions of normality and homoscedasticity (Weber & Legge, 2010).

The first and second PCA explained 38.9% and 9.1% of the variance in the data and separated CSUPs of microbial communities in sediments treated with citrate-AgNP at 100 and 125 mg Ag L^{-1} nominal from all other treatments (Figure 3.4). The distinctive patterns among treatments were partly due to the inhibition of a large number of carbon sources utilization by citrate-AgNP at the two highest concentrations tested (Table 3.2). Microbial communities involved in the metabolism of amino acids, carbohydrates, and carboxylic acids were highly inhibited by citrate-AgNP exposure (Table 3.2). Similar carbon source utilization inhibition by nanomaterials resulting in a shift in CLPP has been reported in other studies (Echavarri-Bravo et al., 2015; Rodrigues et al., 2013).

This suggests that the use of PCA to separate metabolic patterns is directly related to the differences in carbon source utilization (Garland & Mills, 1991). Although PVP-AgNP inhibited 3 carbon substrates (Table 3.2), their patterns of carbon source utilization were not different from the control (Figure 3.4). This implies that the microbial community is more susceptible to citrate-AgNP resulting in the alteration of its metabolic fingerprint pattern.

While AgNPs showed greater inhibition effects on carbon source utilization, their antimicrobial effects did not affect the utilization of D-mannitol and 4-hydroxy benzoic acid by the microbial community at the tested concentrations in this study. In contrast, D-mannitol, a carbon storage, was inhibited by uncoated AgNPs in estuarine sediments at 6 mg Ag kg^{-1} dry weight (Echavarri-Bravo et al., 2015). The differences in substrate utilization may be due to surface coating agents and organic matter content. The present study used citrate and PVP as AgNP surface coating materials which have been shown to cause less toxic effects compared to the uncoated AgNP (Pokhrel et al., 2012). Organic matter (15%) in freshwater sediment reacted with AgNPs causing aggregation that decreased AgNP toxicity (Colman et al., 2012). Organic matter in marine estuarine sediment in the previous study (Echavarri-Bravo et al., 2015) was low (<1%) and may have caused little aggregation to reduce AgNP toxicity. As reported in another study (Button et al., 2016), some carbon substrates including D-mannitol and 4-hydroxy benzoic acid may not be sensitive to AgNP at low doses for short-term exposure. However, long-term exposure to low doses of AgNPs has shown inhibition of microbial biomass, enzyme activity, and community function while short-term exposure exhibited limited inhibition effects (Grun et al., 2018; Xu & Zhang, 2018).

4.3. Expected environmental concentrations

Environmental concentrations of nanomaterials are expected to increase due to the continuous release of nanomaterials into aquatic systems via the discharge of wastewater effluent, and discharge from landfills (Ganzleben et al., 2011; Good et al., 2016). Determining nanomaterial concentrations in the environment will enable ecologists to use relevant environmental concentrations to evaluate the toxicity of nanomaterials in laboratory studies. Up to date, only a few studies have measured the concentrations of nanomaterials in aquatic systems ranging from 0.001 to 100 µg/L (Kim et al., 2010; Loosli et al., 2019; Peter et al., 2018) while several modelling studies have predicted environmental concentrations of nanomaterials (Blaser et al., 2008; Coll et al., 2015; Good et al., 2016; Mueller & Nowack, 2008; Sun et al., 2014; Sun et al., 2016). Nanomaterial concentrations predicted by models are higher than the measured concentrations. In 2010, it was estimated that 0.4-7% of the global release of nanomaterials ended up in aquatic systems (Keller et al., 2013). Also, low concentrations of nanomaterials are expected in aquatic systems due to the physicochemical transformation of nanomaterials through aggregation, sulfidation, oxidation, and dissolution (Good et al., 2016). Despite the low amounts of nanomaterials potentially released annually into aquatic systems and the transformation of nanomaterials in the environment, Blaser et al. (2008) estimated environmentally relevant concentrations of nanomaterials in freshwater sediment ranging from 2 to 14 mg Ag/kg. The overestimation of environmentally relevant concentrations may be due to the failure of the models to properly account for the physicochemical transformation processes that influence nanomaterial behavior in the environment. Concentrations

tested in our study are higher than the measured concentrations in the environment, but lower than the estimated concentrations, suggesting that our tested concentrations may be environmentally relevant for freshwater sediments.

Nanomaterials released into aquatic systems may originate from the anthropogenic activity and natural processes complicating the detection of engineered nanomaterials in natural samples. Although there are available techniques to quantify nanomaterials in natural samples, the current analytical methods to distinguish between natural and engineered nanomaterials are problematic due to similarity in particle properties and water chemistry influence (Loosli et al., 2019; Nowack, 2017; Sun et al., 2016). Thus, using analytical techniques to distinguish nanomaterial concentrations from the background concentrations of natural origin is complicated at best. Besides, each nanomaterial can have unique characteristics that may influence their behavior in the environment making detection a difficult proposition. Water chemistry including organic matter, temperature, pH, conductivity, and dissolved ions could influence nanomaterial behavior complicating their detection in aquatic systems (Colman et al., 2012; Levard et al., 2012; Lowry et al., 2012).

5. Conclusion

This study demonstrated potential toxicity of AgNPs to microorganisms in aquatic systems. Microbial community diversity and carbon source utilization are susceptible to the antimicrobial properties of AgNPs. Microbial diversity, catabolic activity, and metabolic fingerprint patterns were altered by AgNPs. A more diverse microbial community indicates the ability of the community to degrade various pollutants in the

environment (Weber et al., 2013). Thus, a shift in the metabolic fingerprinting pattern of the microbial community as a result of AgNP exposure may affect functional diversity and the ability of microorganisms to decompose organic matter and pollutants in the environment.

Alteration of microbial functional diversity by AgNPs can be explained by silver ion interactions with thiol groups of enzymes and proteins inhibiting cellular respiration and ions transport across membranes. Though the mechanisms of AgNP toxicity in microorganisms are the same, autotrophic microorganisms have a higher affinity for AgNPs leading to stronger growth inhibition effects compared to heterotrophic microorganisms (Fabrega et al., 2011). Exposure to AgNPs may lead to increased membrane permeability and disruption of cell structure increases (USEPA, 2014). Different species vary in their susceptibility to contaminants (Johnston and Roberts, 2009), thus, species that constitute the microbial community may vary in their responses to AgNPs. Identification of individual species within the microbial community is beyond this study, but we assume that not all species within the community responded to the AgNPs. We recommend that future studies should consider identifying species that respond to AgNPs to determine species that are susceptible or resistant to AgNPs. Toxicological data generated in this study can be used as a basis to investigate potential negative impacts of AgNPs on ecosystem services and functions.

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APPENDIX

Table 3.3. EcoPlate showing 31 carbon sources in triplicates and three controls (water) in 96 wells.

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

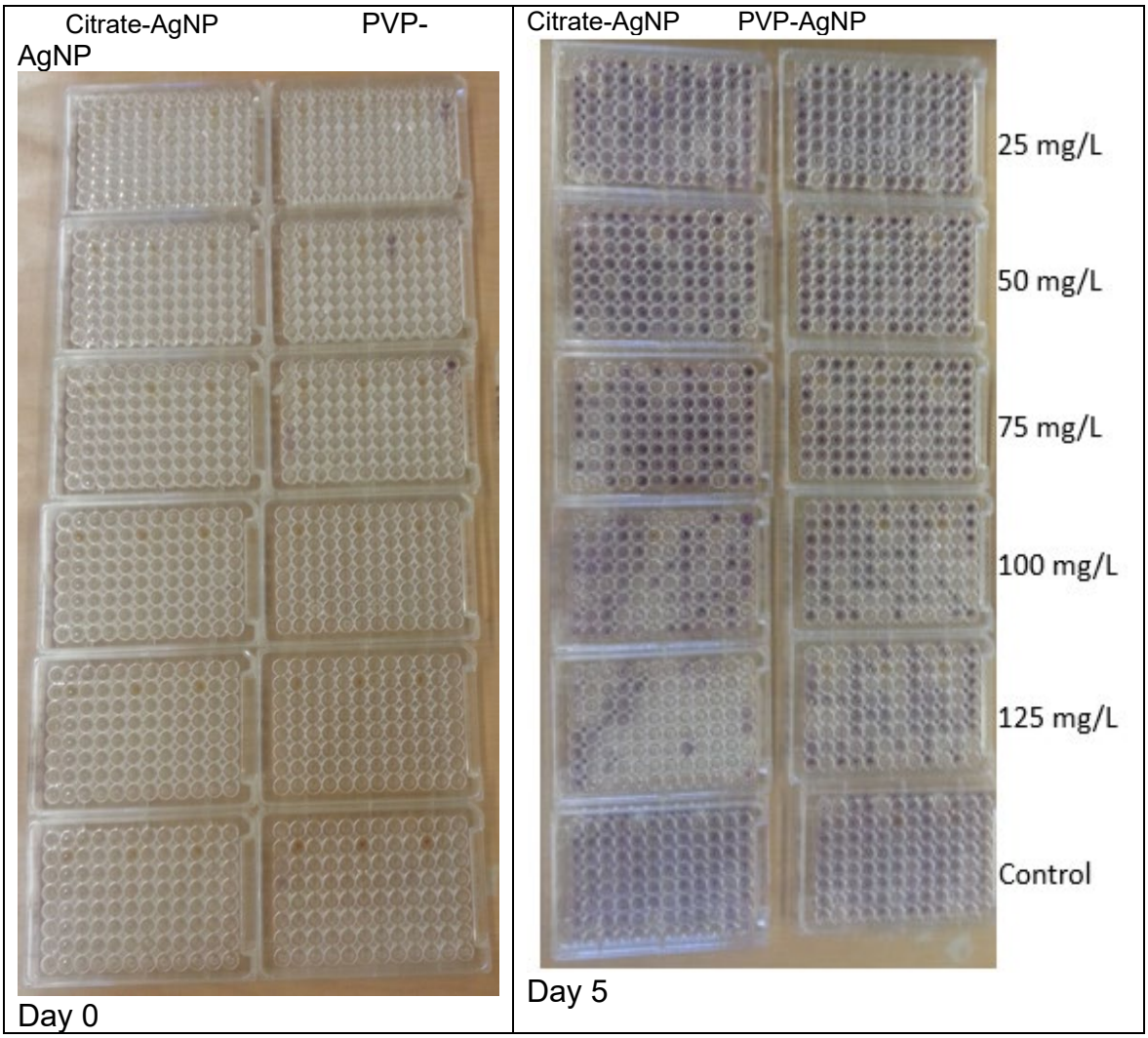

Figure. 3.5. Biolog EcoPlates inoculated with 150 µL of treated samples in each well showing color development at day 0 and day 5. Well with pink colors indicate utilization of carbon source by microorganisms and those without color change indicate no carbon source utilization. Each plate contains 96 wells and 31 carbon sources with three replicates and three controls.

CHAPTER 4

SILVER NANOPARTICLE TOXICITY IN 10-DAY AND 28-DAY SURVIVAL AND GROWTH TESTS OF *HYALELLA AZTECA*

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Keywords: amphipods; aquatic systems; *Hyalella azteca*; nanomaterials, silver nanoparticle; toxicity

Abstract

Silver nanoparticles (AgNPs) are one of many emerging environmental contaminants detected in surface waters, where the effects on biological systems are not fully understood. Amphipods used as bioindicators of sediment and water quality are likely to be affected by AgNP exposure because they are susceptible to ionic silver. We exposed *Hyalella azteca* to silver nitrate ($AgNO_3$), citrate-coated AgNP (citrate-AgNP), and polyvinylpyrrolidone-coated AgNP (PVP-AgNP) to evaluate the effects of AgNPs on amphipods. In this study, AgNPs decreased the survival and growth of *H. azteca*. Median lethal concentration (LC_{50}) and effective concentration (EC_{20}) were calculated using nominal concentrations. The 10-day LC_{50s} for the survival of *H. azteca* were 3.3, 9.2, and 230.0 µg Ag L^{-1} while the 28-d LC_{50s} were 3.0, 3.5, and 66.0 µg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively. The EC_{20s} for growth in 10-d test calculated as the dry weight were 1.7, 4.7, and 249.5 µg Ag L^{-1} while the EC_{20s} for growth also expressed as biomass were 1.6, 4.7, and 188.1 µg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively. Two metrics, EC_{20} and no observed effect

concentration (NOEC) were calculated to determine the growth endpoint in the 28-d test. The 28-d EC_{20} for mean dry weight calculated for citrate-AgNP only due to lack of $AgNO_3$ and PVP-AgNP observed effects on growth was 1.2 µg Ag L^{-1}. The EC_{20s} for biomass were 3.2, 0.5, and < 50 µg Ag L^{-1} for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively. The NOEC for dry weight were 4 and 1 µg Ag L^{-1} while those for biomass were 2 and 0.5 µg Ag L^{-1} for $AgNO_3$ and citrate-AgNP, respectively. The NOEC of survival for $AgNO_3$ and citrate-AgNP were 1 and 2.5 µg Ag L^{-1}, respectively. For PVP-AgNP, the NOEC for dry weight was 100 µg Ag L^{-1} and that of survival and biomass was less than 50 µg Ag L^{-1} because the effects of all tested concentrations were statistically significant. The overall toxicity followed the trend: $AgNO_3$ > citrate-AgP > PVP-AgNP. The toxicity of AgNPs is dependent on the type of surface coating agent. We conclude that AgNPs may affect the survival and growth of freshwater amphipod populations, thus, there may be the need to monitor the release of nanomaterials into aquatic systems.

1. Introduction

Nanomaterials are among environmental contaminants increasingly applied in consumer products for which effects on nontarget biological systems are not completely understood (Pulit-Prociak & Banach, 2016). What makes nanomaterials very concerning is their unique properties such as shape, size, charge, reactivity, and large surface area to volume ratio, which influence their toxicity (Nowack, Krug, & Height, 2011). Among nanomaterials, nanoparticles (nanoparticulate forms of metals with at least two dimensions less than 100 nm) are currently of greater environmental concern due to the

increasing quantity produced annually and the greater number of available products in the market containing nanoparticles (Fabrega, Luoma, Tyler, Galloway, & Lead, 2011; Hansen, Maynard, Baun, &, Tickner, 2013; McGee et al., 2017; USEPA, 2014). Silver nanoparticles (AgNPs) are the most widely used nanoparticles in consumer products including sprays, fabrics, detergent, toothpaste, food storage containers, and cellular phones due to their antimicrobial properties (Buzea, Pacheco, & Robbie, 2007; Fabrega et al., 2011; Pulit-Prociak & Banach, 2016). More than 220 tons of AgNPs are produced annually (McGee et al., 2017). Silver nanoparticles discharged from industries, washing machines, and bathtub drains are deposited into sewage systems and waste treatment facilities (USEPA, 2012). Nanoparticles are also released into the environment during manufacturing, processing, and dilution or degradation from the end-used products (Fabrega et al., 2011). A recent study found 0.1 ng L^{-1} AgNP with a particle size of 14 nm in surface waters (Peters et al., 2018) confirming the presence of AgNP in aquatic systems.

Citrate-coated AgNP (citrate-AgNP) and polyvinylpyrrolidone-coated AgNP (PVP-AgNP) are two of the most common nanoparticles incorporated in consumer products (Silva et al., 2014). Citrate and polyvinylpyrrolidone are coating agents added to the surfaces of nanoparticles to prevent agglomeration, increase particle solubility, mobility, and suspension (El Badawy, Aly Hassan, Scheckel, Suidan, & Tolaymat, 2013; USEPA, 2012). Surface coating agents can cause nanoparticles to behave differently. Studies show that PVP-AgNPs were transported rapidly through quartz sand, ferrihydrite-coated sand, and kaolin-coated sand porous media, and was more toxic to microorganisms compared to citrate-AgNP (El Badawy et al., 2013; Pokhrel et al., 2012). Thus, surface

coating agents may influence the toxicity of AgNPs. In addition, nanoparticle properties such as chemical composition, speciation, and aggregation can change when suspended in water for a long time which may affect their bioavailability (Baun, Hartmann, Grieger, & Kusk, 2008). This implies that long-term exposure to nanoparticles is necessary to determine the chronic endpoints. While 10-d and 28-d endpoints of nanoparticles in smaller aquatic invertebrates have well been studied (Kühr et al., 2018; Pokhrel, 2013; Silva et al., 2014; Wang et al., 2014), little work has been done on the toxicity endpoints of AgNPs with *Hyalella azteca*.

Nanoparticles made from silver are reported to be the most ecotoxic nanomaterials since they affect a wide range of aquatic organisms (Geary, Morris, & Salem, 2016) and its presence in aquatic systems raises concerns about potential risks to benthic invertebrates because sediment is the sink for metals in aquatic systems (Baun et al., 2008). Amphipods are among the first benthic species to disappear in contaminated sediments because they respond quickly to pollution (Burton, 1992). A previous study demonstrated that ionic silver (Ag^+) released from silver nitrate and silver sulfide decreased the survival of *H. azteca* most compared to other invertebrates, algae, fish, and amphibians (Blaser, 2006). The toxicity of Ag^+ to *H. azteca* has been recognized for many years (Berry, Cantwell, Edwards, Serbst, & Hansen, 1999), but little is known about how its nanoparticles could affect the survival and growth of the amphipod. *H. azteca* can be exposed to nanoparticles in streams through different exposure pathways. Nanoparticles adhere to primary producers and have the potential to bioconcentrate in the amphipods which feed on plankton. *H. azteca* may have direct

exposure to nanoparticles through adhesion to the exoskeleton and ingestion of sediments contaminated with nanoparticles (Baun et al., 2008).

The current study evaluated *H. azteca* responses to AgNPs in 10-d and 28-day exposures. *H. azteca* is a euryhaline, widely distributed in North American freshwater bodies (Oviedo-Gómez, Galar-Martínez, García-Medina, Razo-Estrada, & Gómez-Oliván, 2010). The amphipods are shredders involved in the degradation of organic matter and nutrient cycling (Baun et al., 2008). *H. azteca* is highly responsive to pollution and has been used to determine sediment and water quality (Anderson et al., 2015; Hartz et al., 2019; Oviedo-Gómez et al., 2010; Wang et al., 2018). We hypothesized that (1) AgNPs would decrease the survival and growth of *H. azteca* as observed in silver and (2) the toxicity of AgNPs to *H. azteca* would depend on the type of surface coating agent. The main objective of this study was to determine and compare the toxicity to *H. azteca* from exposures of different durations to $AgNO_3$ and AgNPs with two different coating agents.

2. Materials and methods

2.1. Silver nanoparticle synthesis, purification, and characterization

Citrate-coated AgNP (citrate-AgNP) and polyvinylpyrrolidone-coated AgNP (PVP-AgNP) were synthesized in the Environmental Health Science Laboratory at East Tennessee State University, Johnson City, Tennessee, USA using the El Badawy et al. (2010) method. Briefly, 1 mM silver nitrate (Fisher Science) was added to 10 mM sodium citrate dihydrate (Fisher Science) in a 2:1 ratio. The solution was heated in a water bath at 70 ^{o}C for four hours to synthesize the citrate-AgNP stock solution. For the PVP-AgNP stock solution, a 5 mM $AgNO_3$ solution was added to 2 mM sodium

borohydride (Acros Organics) in a 1% PVP K60 solution (Sigma Aldrich) in 1:3 ratio on ice with vigorous stirring. The synthesized nanoparticles were sonicated for 10 minutes for even dispersion of particle suspension followed by purification using a polysulfone 10kD hollow fiber filter in Kros Flo Research IIi tangential flow filtration system (Spectrum Laboratories, CA, USA). Particle size and zeta potential were determined using a particle size analyzer. A dynamic light scattering (DSL) method using a NICOMP 380 ZLS Particle sizer/zeta potential analyzer (PSS NICOMP Particle Sizing Systems, Santa Barbara, CA, USA) was used to determine the hydrodynamic diameter (HDD) and zeta potential of the nanoparticles. A standard solution purchased from the manufacturer was run as a check standard before using the instrument for the particle analysis. Particle shape was visualized using transmission electron microscopy (TEM) at the Joint Institute for Advanced Materials, University of Tennessee. ImageJ software (National Institute of Health, USA) was used to estimate the size of the particles.

2.2. Analytical chemistry

Total and dissolved Ag in the test solutions were measured to understand the mechanism of toxicity. Total Ag was determined by digesting 50 mL of the test solutions in a concentrated nitric acid using the United States Environmental Protection Agency (USEPA) 3050B method (USEPA, 1996). For dissolved Ag, 15 mL of the test solution was ultracentrifuged at 45,000 rpm (205,835 x g) and the supernatant was digested as described above. Digested samples were analyzed for Ag using inductively coupled plasma mass spectrometry (ICP-MS). Pure Ag standard solution purchased from (NSI Lab Solutions) was used for calibrations. Each set of samples was analyzed with 0.25

μg Ag/L solution prepared from quality control standard solution (QCI-34) as a check standard. All glassware were washed in an acid bath (5% HNO_3) and rinsed with Milli-Q water before use to remove any metal residues.

2.3. Test organism culture and maintenance

Several mixed-age *H. azteca* (Appendix Figure 4.2 A) were originally obtained from the United States Geological Survey (USGS), Columbia, Missouri and maintained in culture in a static system (Appendix Figure 4.2C) at the Environmental Health Science Laboratory at East Tennessee State University, Johnson City, USA. The amphipods were cultured according to the USEPA standard culturing procedures (USEPA, 2000) in reformulated moderately hard reconstituted water (R-MHRW) (Poynton et al., 2013) made in the laboratory. The bottom of the culture chambers was covered with plastic mesh to serve a substrate. Amphipods were cultured under 16 h light and 8 h dark each day at temperatures ranging from 21 to 22 ^{o}C (Table 4.1). Dissolved oxygen, pH, hardness, alkalinity, conductivity, and ammonia were measured every week to ensure acceptable water quality (Table 4.1). Amphipods were fed three times per week with fish flakes (Aquatic Biosystems Inc., Fort Collins, Colorado, USA) and *Thalassiosira weissflogii* diatoms (Reed Mariculture, Campbell, CA, USA). Culture water was renewed with R-MHRW once per week. Juveniles were separated from adults using a #25 sieve (710-mm opening) during every water renewal (Wang et al. 2018; USEPA 2000). To obtain juveniles for toxicity testing, less than 24-h old newborns were isolated from adults using the #25 sieve. The newborns were held in a 1 L jar for 7-8 days under the same culturing conditions as the adults before using them for toxicity

testing. The narrow range in age was chosen to reduce variability in growth at the end of the test (USEPA, 2012).

2.4. Toxicity testing

A 10-d and 28-d toxicity tests were conducted to assess the effects of AgNPs on survival and growth endpoints for *H. azteca* following standard methods for conducting sediment toxicity test for freshwater amphipods (USEPA, 2000). Nominal concentrations of citrate-AgNP (0, 2.5, 5, 10, 15, 20, and 25 µg Ag L^{-1}) and PVP-AgNP (0, 100, 200, 300, 400, 600, and 800 µg Ag L^{-1}) were prepared from their respective stock solutions using R-MHRW. These concentrations were selected for the 10-d toxicity test based on range-finding tests conducted before the toxicity testing. For the 28-d toxicity test, citrate-AgNP (0, 0.25, 0.5, 1, 2.5, 5, and 10 µg Ag L^{-1}) and PVP-AgNP (0, 50, 100, 200, 300, and 400 µg Ag L^{-1}) nominal concentrations were prepared as described above based on the LC_{50s} of the 10-d toxicity tests. Appropriated $AgNO_3$ solution was added to R-MHRW to obtain 0, 1, 2, 4, 8, and 16 µg Ag L^{-1} nominal concentrations for positive control tests.

A modular water-renewable system for bulk sediment toxicity testing designed by Leppanen & Maier (1998) was used for the toxicity testing (Appendix Figure 4.4). The system is inexpensive and holds more 300-mL beakers containing water compared to Zumwalt's system (Leppanen & Maier, 1998). A mesh stainless steel fabric (500 µm) rolled into a loose circle was glued below the lips of the beakers to prevent the escape of test organisms (Appendix Figure 4.4E). This system does not only take a short time

(about 10 minutes) for water renewal, it also allows water volume to remain at the same level in test vessels (Leppanen & Maier, 1998).

Five clean 300-mL replicate beakers were assigned to each exposure concentration including the controls (culture water without a toxicant). About 135 g (equivalent to 100 mL water) natural play sand purchased from Home Depot Store was placed in each replicate beaker followed by 200 mL test solution. The beakers were left undisturbed for 24 h for the test solution to equilibrate. Natural play sand was used as a substrate for the toxicity testing, because it supports amphipods growth in culture, reduces variability in results, and makes it easy to find live amphipods after the test. The sand was sterile by washing it with Milli-Q water and drying overnight in an oven at 70 ^{o}C before use.

Ten juveniles (7-8-day old) were randomly placed in each beaker. The test beakers were then placed in plastic chambers with a small hole at the bottom to allow the passage of excess water from the beakers and a plastic container was placed underneath each chamber to collect excess water. The amphipods were fed daily with *Thalassiosira weissflogii* diatoms and fish flake (Poynton et al., 2019). Test solutions were renewed every 24 hours using a syringe (Appendix Figure 4.4B).

Conductivity (ranged from 259 to 274 µS cm^{-1}), pH (range from 7.5 to 7.7), and dissolved oxygen (range from 7.6 to 8.4 mg L^{-1}) were measured at the beginning and end of each test (Appendix Tables 4.7 to 4.12). Live amphipods in each test beaker were counted at the end of the test. Immobilized and missing amphipods were counted dead. Live amphipods were washed with culture water and frozen in a refrigerator until drying. Frozen amphipods removed from the refrigerator were thawed, dried in an oven

at 60 ^{o}C for 24 h, and weighed using a Mettler Toledo AT201 Analytical Balance (0.0001 g) to determine the dry weight (USEPA, 1998).

2.5. Quality control and assurance (QC/QA)

Test organisms were obtained from a reliable supplier with a history of at least one generation. The amphipods were disease-free, and their identification was verified using physical characteristic features. Water quality parameters of the overlying water were kept within acceptable ranges to maintain amphipod health. Test organisms were periodically subject to reference toxicity testing using potassium chloride (KCl) (Appendix Table 4.13) to determine their suitability for the test (USEPA, 1998). All glassware were cleaned with 5% HNO_3 to remove any metal residues before used for toxicity testing. Negative controls were prepared for each test using culture water and sand without AgNPs to show that the concentrations of nanoparticles and their effects on the amphipods were not due to laboratory or external contaminants but were solely due to the nanoparticles (Burton, 1992). Positive controls were prepared using $AgNO_3$ to confirm the sensitivity of test organisms to silver. The experimental design included replication of treatments to obtain consistent results and statistically distinguish treatment effects from the negative control. Amphipods and test beakers were randomly assigned to treatments to avoid experimental biases. All measuring instruments were calibrated before use. Externally supplied standards were used to check for instrument accuracy.

2.6. Data analysis

AgNP effects and endpoint estimates were determined using nominal concentrations. Shapiro-Wilk and Levene's tests were performed to examine normality and homogeneity of variance of the data, respectively. Average amphipod growth was calculated using the final measured dry weight divided by the number of surviving amphipods, and biomass was calculated as total dry weight divided by the initial number of amphipods for each replicate. Survival, dry weight, and biomass dependence on exposure concentrations using a general linear model (GLM). Exposure concentration effects on endpoints were compared using one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way ANOVA followed by Bonferroni and Dunnett's tests in SAS ($\alpha = 0.05$). The NOEC was determined as the highest tested concentration that is not statistically significantly different from the control using Dunnett's test. The effect concentration (EC_{20}) was calculated for growth and biomass using non-linear regression analysis with the logistic equation model in the toxicity relationship analysis program (TRAP). This endpoint was chosen because it is more protective and has a lower proportional effect (Environmental Canada, 2005). Median lethal concentrations (LC_{50s}) were determined by the log-logistic model (Equation 1) using the dose-response curve (DRC) package in R (Rizt, 2009). The fitness of each model to the data was confirmed by the "modelFit" function

$$f(x) = c + \frac{d-c}{1+\left(\frac{x}{e}\right)^b} \qquad \text{Equation 1}$$

Where x = dose, c = lower limit, d = upper limit, b = slope, and e = dose resulting in 50% response.

3. Results

3.1. Nanoparticle characterization

The shape of the nanoparticles was spherical (Figure 4.1) and DSL showed that the particles had the same diameter of 11.0 nm (SD = 0.7 nm). However, analyzed TEM images showed that the mean particle size of citrate-AgNP, 27.8 nm (SD = 8.2 nm), was larger than that of PVP-AgNP, 21.0 nm (SD = 4.4 nm). Citrate-AgNP had a greater zeta potential (-17.6 mV) compared to PVP-AgNP (-9.3 mV). The pH for the stock solutions was neutral (7.2) suggesting that the dissolution of the AgNPs was not affected by the pH.

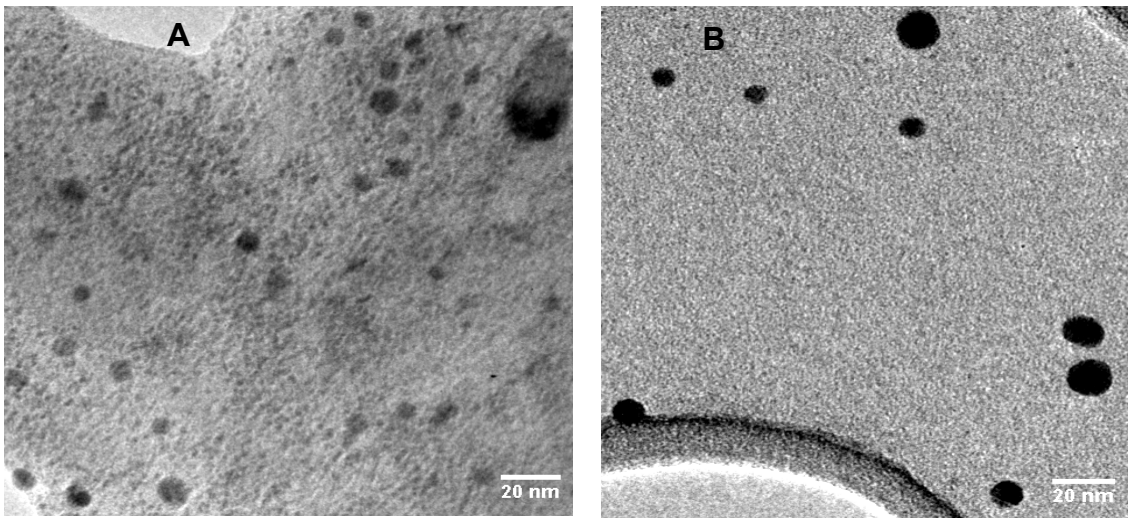

Figure 4.1. Transmission electron micrographs of images showing the particle size distribution of citrate-AgNP (A) and PVP-AgNP (B) in stock solutions. Scale bars represent 20 nm.

3.2. Chemical analysis

Conductivity, pH, dissolved oxygen, alkalinity, hardness, and ammonia measured at the beginning and end of each test (Table 4.1) were within the USEPA recommended values (USEPA, 2000). Hardness was moderate and it was consistent with the culture

water. Ammonia was measured only in 28-d tests and the concentrations were far lower than the maximum limit suggesting that mortality was not influenced by ammonia. Total and dissolved Ag in the water column measured for $AgNO_3$ 10-d and 28-d exposures were similar to nominal concentrations (Table 4.2) indicating the higher dissolution of Ag^+. Generally, the portion of dissolved Ag in citrate-AgNP and PVP-AgNP test solutions was lower than those observed in $AgNO_3$, however, the dissolution of the AgNPs was still high (> 50%) (Tables 4.2 & 4.3) suggesting bioavailability of Ag^+ released from the AgNPs into the test solutions

Table 4.1. Measured water quality parameters

Parameters	Measured value
pH	7.5-7.7
Temperature (°C)	21-22
Conductivity (µS cm^{-1})	259-274
Dissolved oxygen (mg L^{-1})	7.6-8.4
Alkalinity (mg L^{-1} as $CaCO_3$)	58-72
Hardness (mg L^{-1} as $CaCO_3$)	76-88
Ammonia (mg L^{-1})	0.01-0.04

Table 4.2. Measured silver in water column, survival, dry weight, and biomass of *H. azteca* for 10-d exposure. Values in parenthesis are standard deviation.

Toxicant	Nominal (µg L^{-1})	Total Ag (µg L^{-1}; n = 3)	Dissolved Ag (µg L^{-1}; n = 3)	Survival (%; n = 5)	Dry weight (mg; n = 5)	Biomass (mg; n = 5)
$AgNO_3$	0	0	0	100	0.15 (0.02)	0.15 (0.02)
	1	0.9 (0.1)	0.8 (0.0)	100	0.16 (0.03)	0.16 (0.03)
	2	1.7 (0.1)	1.6 (0.0)	92	0.10 (0.03)	0.09 (0.03)
	4	3.5 (0.2)	3.2 (0.1)	30	0.09 (0.04)	0.03 (0.01)
	8	6.9 (0.4)	6.3 (0.1)	0	-	-
	16	13.9 (0.7)	12.7 (0.2)	0	-	-
Citrate-AgNP	0	0	0	98	0.45 (0.03)	0.44 (0.03)
	2.5	1.7(0.3)	1.1 (0.1)	98	0.42 (0.08)	0.41 (0.08)
	5	3.2 (0.4)	2.8 (0.5)	96	0.35 (0.07)	0.33 (0.06)
	10	7.3 (0.6)	6.7 (0.5)	48	0.25 (0.00)	0.12 (0.01)
	15	10.8 (1.2)	8.5 (4)	2	0.2 (0.00)	0.004 (0.00)
	20	14.1 (1.6)	12.5 (3.1)	0	-	-
	25	18.4 (3.2)	18.2 (0.6)	0	-	-

PVP-AgNP	0	0	0	96	0.35 (0.06)	0.34 (0.06)
	100	79.7 (4.7)	73.7 (6.8)	90	0.32 (0.04)	0.29 (0.04)
	200	165.8 (2.9)	146.7 (16.3)	72	0.33 (0.05)	0.23 (0.06)
	300	232.5 (10.9)	209.5 (11.3)	34	0.17 (0.00)	0.058 (0.00)
	400	307.5 (15.2)	251.0 (39.3)	2	0.10 (0.00)	0.002 (0.00)
	600	350.0 (26.5)	278.3 (13.8)	0	-	-
	800	611.7 (23.1)	484.2 (69.5)	0	-	-

Total and dissolved silver in overlying water was measured after each test (n = 3). Live amphipods in each replicate were dried and weighed at the end of each test.

Table 4.3. Measured silver in water column, dry weight, and biomass of *H. azteca* for 28-d exposure. Values in parenthesis are standard deviation.

Toxicant	Nominal ($\mu g\ L^{-1}$)	Total Ag ($\mu g\ L^{-1}$; n = 3)	Dissolved Ag ($\mu g\ L^{-1}$; n = 3)	Survival (%; n = 5)	Dry weight (mg; n = 5)	Biomass (mg; n = 5)
$AgNO_3$	0	0	0	94	0.26 (0.02)	0.25 (0.03)
	1	0.9 (0.0)	0.9 (0.0)	96	0.34 (0.03)	0.33 (0.03)
	2	1.8 (0.0)	1.7 (0.0)	82	0.37 (0.06)	0.30 (0.08)
	4	3.6 (0.1)	3.4 (0.0)	22	0.44 (0.16)	0.08 (0.03)
	8	7.3 (0.1)	6.8 (0.4)	0	-	-
	16	14.5 (0.2)	13.6 (0.1)	0	-	-
Citrate-AgNP	0	0	0	88	0.63 (0.12)	0.56 (0.13)
	0.25	0.2 (0.0)	0.1 (0.0)	84	0.63 (0.21)	0.49 (0.16)
	0.5	0.3 (0.0)	0.3 (0.0)	84	0.54 (0.11)	0.45 (0.11)
	1	0.7 (0.1)	0.6 (0.1)	76	0.51 (0.12)	0.38 (0.13)
	2.5	2.2 (0.4)	1.3 (0.8)	78	0.46 (0.09)	0.35 (0.07)
	5	4 (0.5)	3.1 (1.1)	48	0.43 (0.07)	0.21 (0.09)
	10	7.3 (1.0)	6.4 (1.4)	14	0.40 (0.00)	0.14 (0.01)
PVP-AgNP	0	0	0	98	0.33 (0.04)	0.33 (0.05)
	50	41.5 (13.4)	28.0 (7.2)	76	0.23 (0.05)	0.20 (0.04)
	100	66.0 (0.0)	60.0 (7.2)	16	0.28 (0.13)	0.08 (0.01)
	200	145.0 (12.7)	110.7 (11.0)	0	-	-
	300	174.0 (22.6)	158.0 (2.8)	0	-	-
	400	246.0 (17.0)	212.7 (10.1)	0	-	-

Total and dissolved silver in overlying water was measured after each test (n = 3). Live amphipods in each replicate were dried and weighed at the end of each test.

3.3. 10-day toxicity

All the 10-d survival and growth tests for *H. azteca* met the established test conditions and acceptable mean control survival (U.S. EPA, 2000). Ten-day exposure to citrate-

AgNP and PVP-AgNP resulted in dose-dependent responses for survival and the true mean dry weight of *H. azteca*. Growth calculated as biomass was also affected by $AgNO_3$ and AgNPs. Organisms exposed to citrate-AgNP and PVP-AgNP exhibited a decrease in survival, mean weight, and biomass for 10-d tests. Mean control survival was 100% for $AgNO_3$, 98% for citrate-AgNP, and 96% for PVP-AgNP exposures (Table 4.2). There were no survivors at the two highest concentrations tested for all 10-d tests.

Analysis of the treatment effects showed that $AgNO_3$, citrate-AgNP, and PVP-AgNP had significant effects ($p < 0.01$) on the survival and growth of the amphipods (Table 4.4). Multiple comparison tests (Bonferroni) showed differences in effects on survival among treatments. Dunnett's test showed that the amphipod survival significantly decreased from controls in all treatments except exposures at 1 and 2 µg Ag L^{-1} for $AgNO_3$, 2.5 and 5 µg Ag L^{-1} for citrate-AgNP, and 100 µg Ag L^{-1} for PVP-AgNP. $AgNO_3$ exhibited the highest toxicity while PVP-AgNP exhibited the lowest toxicity (Table 4.5). The PVP-AgNP LC_{50} for the amphipods was 70-fold and 25-fold greater than those of $AgNO_3$ and citrate-AgNP, respectively, while the LC_{50} for citrate-AgNP was 2.8-fold greater than that of $AgNO_3$. The EC_{20} for growth based on the mean dry weight of the amphipods in PVP-AgNP was 146.8-fold and 53-fold greater than those of $AgNO_3$ and citrate-AgNP, respectively, while the EC_{20s} for citrate-AgNP was also 2.8-fold greater than that of $AgNO_3$ as observed in the survival endpoint (Table 4.5). For biomass, PVP-AgNP EC_{20} was 117.6-fold and 40-fold greater than the EC_{20s} of $AgNO_3$ and citrate-AgNP respectively, whereas citrate-AgNP EC_{20} was 2.9-fold greater than that of $AgNO_3$ (Table 4.5).

Table 4.4. Effects of $AgNO_3$ and AgNPs on *H. azteca* survival, growth, and biomass

Endpoint	Treatment	10-d		28-d	
		F-value	P-value	F-value	P-value
Survival	$AgNO_3$	27.28	< 0.001	21.95	< 0.001
	Citrate-AgNP	31.32	< 0.001	20.08	0.003
	PVP-AgNP	31.67	< 0.001	22.10	< 0.001
Growth	$AgNO_3$	5.85	0.008	3.42	0.043
	Citrate-AgNP	4.85	0.014	4.42	< 0.001
	PVP-AgNP	5.11	0.012	1.09	0.374
Biomass	$AgNO_3$	21.00	< 0.001	24.43	< 0.001
	Citrate-AgNP	16.01	< 0.001	6.30	< 0.001
	PVP-AgNP	7.17	0.003	36.55	< 0.001

Table 4.5. The LC_{50} and EC_{20} for *Hyalella azteca* for 10-d and 28-d exposures. Values in parenthesis are confidence intervals.

Test	Treatment	Survival	Dry weight	Biomass
		LC_{50} (µg L^{-1})	EC_{20} (µg L^{-1})	EC_{20} (µgL^{-1})
10-d	$AgNO_3$	3.3 (3.0-3.7)	1.7 (0.7-4.6)	1.6 (1.1-2.3)
	Citrate-AgNP	9.2 (8.4-10.1)	4.7 (2.4-9.4)	4.7 (3.5-6.3)
	PVP-AgNP	230.0 (208.2-251.8)	249.5 (185.2-336.1)	188.1 (152.0-232.8)
28-d	$AgNO_3$	3.0 (2.7-3.4)	NA	3.2
	Citrate-AgNP	3.5 (2.4-4.6)	1.2	0.5 (0.1-2.2)
	PVP-AgNP	66.0 (58.4-73.6)	NA	< 50

The LC_{50} and EC_{20} were calculated using nominal concentration. NA = not analyzed due to the lack of significant differences between controls and treatments.

3.4. 28-day toxicity

All the 28-d toxicity tests met the standard requirements for survival and growth of *H. azteca* (USEPA, 2000). Mean control survival was 94, 88, and 98% for $AgNO_3$, citrate-AgNP, and PVP-AgNP, respectively (Table 4.3). Survival of *H. azteca* in all 28-d tests decreased from the control to the highest concentrations tested. The mean weight of *H.*

azteca reduced from the control to higher exposure concentrations in citrate-AgNP only, while their biomass decreased from the controls to higher exposure concentrations in citrate-AgNP and PVP-AgNP only. In contrast, the mean dry weight of *H. azteca* in $AgNO_3$ exposures increased with increasing concentration through the first three concentrations (Table 4.3), which was related to feeding. The effect of PVP-AgNP on amphipod mean weight did not follow any clear pattern. The average initial mean dry weight of test organisms was 0.10 mg (SD = 0.03 mg).

The survival and biomass of *H. azteca* declined significantly ($p < 0.01$) in $AgNO_3$, citrate-AgNP, and PVP-AgNP treatments after the 28-d test (Table 4.4). Among the three forms of Ag, only citrate-AgNP significantly reduced the amphipod mean weight for the concentrations tested. (Tables 4.3 and 4.4). In contrast, the mean weight of amphipods increased significantly with increasing $AgNO_3$ tested concentrations. (Tables 4.3 and 4.4). As observed in the 10-d test, the survival of amphipods was also reduced with increasing concentrations of $AgNO_3$, citrate-AgNP, and PPVP-AgNP with LC_{50s} of 3.0, 3.5, and 66 μg Ag L^{-1}, respectively (Table 4.5). The EC_{20} of citrate-AgNP was 1.2 μg Ag/L^{-1} but the EC_{20s} for $AgNO_3$ and PVP-AgNP were not calculated. Amphipod biomass showed a different trend in response to treatments where $AgNO_3$, C-AgNP, and PVP-AgNP had EC_{20} of 3.2, 0.5, and 40.6 μg Ag L^{-1}, respectively. This trend may be due to the inconsistent pattern of the amphipod mean weight among concentrations tested for $AgNO_3$ and PVP-AgNP. The NOEC of $AgNO_3$ was 1, 4, and 2 μg Ag L^{-1} for survival, mean weight, and biomass, respectively. For citrate-AgNP, the NOECs for survival, mean weight, and biomass were 2.5, 1, and 0.5 μg Ag L^{-1}, (Table 4.6). PVP-AgNP

NOEC for mean weight was 100 μg Ag L^{-1} but was less than 50 μg Ag/L^{-1} for survival and biomass (Table 4.6).

Table 4.6. The NOEC of $AgNO_3$ and AgNPs for *H. azteca* 28-d exposure

Treatment	Survival (μg Ag L^{-1})	Dry weight (μg Ag L^{-1})	Biomass (μg Ag L^{-1})
$AgNO_3$	1	4	2
Citrate-AgNP	2.5	1	0.5
PVP-AgNP	< 50	100	< 50

The NOEC was determined by the Dunnett's test. NOEC = No observed effect concentration, NA = Not applicable.

4. Discussion

4.1. *Hyalella azteca* responses to AgNPs

We showed that *H. azteca* is highly responsive to the adverse effects of $AgNO_3$ and AgNPs. $AgNO_3$ caused 78% mortality and 68% growth (biomass) reduction at 4 μg Ag L^{-1} (nominal concentration) while citrate-AgNP caused 86% mortality and 63% growth reduction in *H. azteca* at 10 μg Ag L^{-1} for 28-d exposure (Tables 4.3). $AgNO_3$ and AgNPs showed a statistically significant reduction of amphipod survival and growth in this study (Table 4.4). Effects of AgNPs on *H. azteca* demonstrate the toxicity of silver to amphipods in different forms also reported in other studies (Blaser, 2006; Call et al., 2006; Diamond et al., 1990). The results of this study also confirm the acute responses of *H. azteca* to nanomaterials observed in similar studies (Poynton et al., 2013; Poynton et al., 2019; Revel et al., 2015).

Comparing the toxicity of citrate-AgNP and PVP-AgNP, we observed that the amphipods were affected more by citrate-AgNP at lower tested concentrations. The number of survivors decreased significantly when amphipods were exposed to at least 10 and 5 µg Ag L^{-1} (nominal concentrations) of citrate-AgNP for 10 and 28 days, respectively (Tables 4.2 & 4.3). On the other hand, the number of amphipods also reduced rapidly at 200 and 100 µg Ag L^{-1} (nominal concentrations) of PVP-AgNP for 10 and 28 days, respectively. The LC_{50s} and EC_{20s} of citrate-AgNP for *H. azteca* were far less than those of PVP-AgNP (Table 4.5) indicating that the amphipods provided more rapid responses to citrate-AgNP. The differences between the toxicity of the two nanoparticles may be due to the chemical properties of their surface coating agents reported in other studies (Asadishad et al., 2017; Pokhrel et al., 2012). Our results are consistent with a study that observed differences between citrate-AgNP and PVP-AgNP effects on *Escherichia coli* and concluded that the toxicity of AgNPs is partly dependent on their surface coatings (Pokhrel et al., 2012).

Although citrate-AgNP and PVP-AgNP affected the amphipods differently, they showed a common pattern of an effect on the survival endpoint where greater mortality was observed in the 10-d test compared to the 28-d test (Table 4.5). Notwithstanding, $AgNO_3$ caused the highest mortality in both 10-d and 28-d tests at the lowest tested concentrations compared to citrate-AgNP and PVP-AgNP indicating the high toxicity of ionic silver to *H. azteca*. The results demonstrate biomass decrease with increasing tested concentrations for $AgNO_3$ and AgNPs (Tables 4.2 and 4.3). Compared to mean weight, the biomass endpoint showed major statistically significant differences between tested concentrations (Table 4.4) resulting in the calculation of the EC_{20} for all

treatments in both 10-d and 28-day tests (Table 4.5). The biomass endpoint provides a strong indication of $AgNO_3$ and AgNP effects on amphipod growth. The ecological significance of using biomass is to provide information on the overall success of the amphipods in response to the toxicants under different exposure concentrations (Environment Canada, 2005).

Low amphipod growth response to PVP-AgNP in the 28-day test can be misleading due to an artifact of the feeding regimen. We observed that a few live amphipods in the exposure beakers were larger compared to those that survived in the control beakers. Although a few numbers of amphipods survived in the exposure beakers, the amount of food added to the beakers was not adjusted. Therefore, there was excess food in the exposure beakers available to survivors resulting in more growth per survivor that might have overshadowed nanoparticle effect. Anderson et al. (2015) observed similar results in bifenthrin toxicity to *H. azteca* where the pesticide had no clear effect on growth for long-term exposure. The authors attributed the lack of bifenthrin effect on the amphipod growth to the lack of food adjustment. It is also possible that the survivors were more robust and grew faster during the test period making it difficult to explain the actual cause of low sensitivity for growth. We recommend that feeding should be adjusted to account for mortality during toxicity testing to limit the effect of food on growth in future studies.

The NOEC is another metric for survival and growth endpoints determined for the 28-d test in this study. Citrate-AgNP had the lowest NOEC for amphipod growth expressed as mean weight and biomass. The NOECs of citrate-AgNP for mean weight and biomass were 4-fold lower than those of $AgNO_3$ (Table 4.6) indicating that citrate-

AgNP was less protective to the amphipods. Higher NOEC of $AgNO_3$ may be due to increased weights in concentrations tested compared to the control. Similarly, citrate-AgNP NOEC for weight was 100-fold lower than that of PVP-AgNP which demonstrates that the amphipods were more responsive to citrate-AgNP. The NOECs of PVP-AgNP for survival and biomass were less than 50 μg Ag L^{-1}. A specific NOEC was not determined for the growth endpoint because the effects of all the concentrations tested were statistically significant. The least PVP-AgNP concentration was 50 μg Ag L^{-1}, thus, it is expected that the NOECs for survival and biomass would be less than this concentration tested.

4.2. Influence of surface coating agents on AgNP toxicity

Nanoparticles have a high tendency to form aggregates due to the large surface area to volume ratio, which may cause them to lose their unique properties (Salih et al., 2019). Thus, nanoparticle surfaces are coated to increase stability and prevent aggregation (Asadishad et al., 2017; El Badawy et al., 2013). Surface coating agents influence the chemical properties and behavior of nanoparticles in the environment. For example, AgNPs coated with PVP became less toxic to *H. azteca* upon passing through wastewater (Kühr et al., 2018). No significant effect of PVP-AgNP on *H. azteca* observed at 68.70 μg L^{-1} in the previous was due to a reduction of the nanoparticle bioavailability and toxicity by organic matter in the wastewater (Kühr et al., 2018). In general, surface coated nanoparticles are less bioavailable and less toxic, but uncoated nanoparticles are increasingly bioavailable and more toxic (Fabrega et al., 2011; Pokhrel et al., 2012). Surface coatings influenced the toxicity of citrate-AgNP and PVP-

AgNP in our study since there were observed differences between their effects and properties.

The surface charge of citrate-AgNP was high (-17.6 mV) while that of PVP-AgNP was low (-9.3 mV). It has been shown that gills have negatively charged proteins with a high affinity for positively charged particles (Kleiven et al., 2018). Thus, the high surface charged citrate-AgNP may have reduced aggregation and enhanced the uptake of Ag^{+} (released from the nanoparticles) by the gills of the amphipods (Xu & Zhang, 2018). Oxidized AgNPs produce ionic silver which becomes available to organisms through dissolution (USEPA, 2012). On the other hand, the low surface charge of PVP-AgNP may have increased aggregation and decreased particle uptake by the gills. Moreover, PVP, unlike citrate, is a non-ionic polymer (Asadishad et al., 2017), making PVP-AgNP less attractive to the negatively charged gill proteins to cause toxic effects. Low surface charge and non-ionic nature of PVP-AgNP may explain why it is less toxic to *H. azteca* compared to citrate-AgNP. A previous study showed that coating agents such as citrate and PVP are non-toxic (Pokhrel et al., 2012), suggesting that these surface coating agents did not enhance AgNP toxicity observed in our study. However, the results of the current study demonstrate that PVP can reduce the toxicity of nanoparticles, therefore, AgNPs coated with PVP may be more protective to aquatic organisms than citrate-coated nanoparticles.

4.3. Comparison of $AgNO_3$ and AgNP toxicity

Amphipods responded differently to the nanoparticles compared to ionic silver in the form of $AgNO_3$. As expected, amphipods exhibited greater responses to $AgNO_3$

compared to the nanoparticles confirming the known Ag toxicity to *H. azteca* (Berry et al., 1999; Call et al., 2006). The LC_{50s} and EC_{20} of $AgNO_3$ for the amphipods were lower than those of citrate-AgNP and PVP-AgNP. The overall toxicity followed the trend: $AgNO_3$ > citrate-AgP > PVP-AgNP. We determined that $AgNO_3$ was the most toxic form of Ag compared to the two nanoparticles used in this study. As observed in PVP-AgNP exposures, amphipods growth was less sensitive to $AgNO_3$ in 28-d exposure. Survival decreased from the control to the highest $AgNO_3$ tested concentration, however, growth increased from the control to the first three concentrations tested (Table 4.3). An increase in growth may be due to the continuous normal feeding without adjustment (as explained above), making more food available to survivors in exposure beakers to grow faster than the survivors in the control beakers.

Furthermore, the difference in $AgNO_3$ and AgNP toxicity may be due to the differences in Ag accumulation in test organisms. A comparison of Ag accumulation in salmon exposed to waterborne $AgNO_3$ and citrate-AgNP showed interactions of Ag^+ with gills rather than nanoparticles (Kleiven et al., 2018). The uptake of Ag in the form of Ag^+ was higher than the uptake of Ag in the form of nanoparticles by the fish. The difference in Ag^+ accumulation increases when dissolution is the exposure route for nanoparticles (Kleiven et al., 2018). Other studies also found AgNPs more toxic to *Daphnia magna* than Ag^+ (Asghari et al., 2012; Zhao and Wang, 2011). Both Ag^+ and AgNPs may have contributed to toxic effects observed in the amphipods in our study, but Ag^+ released from $AgNO_3$ caused greater adverse effects.

4.4. Mechanism of action for AgNP toxicity

Silver nanoparticle uptake in biological systems have been studied, but their mechanisms of action are not well understood. It is unclear whether the toxicity is directly caused by AgNPs themselves or Ag ions (Fabrega et al. 2011; Levard et al. 2012; USEPA 2012). After exposure, it is believed that AgNPs release Ag ions which are attracted by the negatively charged gill proteins known to bind to cationic metals (Call et al., 2006). Ag ions are active inhibitor of Na^+ uptake in gills (Kleiven et al., 2018). Ag ions interact with the gill membrane to block sodium ions (Na^+) and potassium ions (K^+) channels. This mechanism reduces the regulation of Na^+ and chloride ions (Cl^-) across the gills (Fabrega et al., 2011), which may eventually kill the amphipods. The generation of oxidative stress (imbalance between reactive oxygen species and antioxidants) can also be used to explain the mechanism of AgNP toxicity (Zhang et al., 2018). Antioxidants are produced by cells to offset the effects of reactive oxygen species (ROS) and have been used as biomarkers for diclofenac (a nonsteroidal anti-inflammatory drug) toxicity in *H. azteca* (Oviedo-Gomez et al., 2010). *H. azteca* exhibited oxidative stress by producing ROS, which increased the toxicity of the drug. The use of antioxidants to characterize *H. azteca* response to AgNPs has not been evaluated.

4.5. Ecological implications of AgNP toxicity

Considering large volumes of AgNPs produced annually worldwide and their recent detection in surface waters (McGee et al., 2017; Peters et al., 2018), there are concerns about their potential accumulation in sediment and subsequent toxic effects on benthic

organisms. Nanoparticle deposition may occur on the surface of sediment in freshwater increasing epibenthic organisms' such as *H. azteca* exposure to higher concentrations of nanoparticles. Evidence from previous studies demonstrates that *H. azteca* can also accumulate nanoparticles through water column (Poynton et al., 2019).

Silver nanoparticles in the form of citrate-AgNP and PVP-AgNP commonly used in commercial products are considered environmentally friendly, although they could persist in the environment (USEPA, 2012). Although our results demonstrate that citrate-AgNP can reduce amphipod survival and growth at 10 μg Ag L^{-1}, this tested concentration is 31-fold greater than the maximum estimated concentration (0.32 μg Ag L^{-1}) in freshwaters (Blaser et al., 2008). Considering the growing applications of AgNPs in consumer products, the observed effect concentration in our study would be environmentally relevant in the near future. AgNPs released from products during manufacturing, use, and disposal could enter aquatic systems through wastewater effluent. Although AgNP toxicity can be reduced when passing through the sewage treatment plant (Kühr et al., 2018), they can reduce the amphipod population, modify bioavailability and toxicity of other toxicants, and serve as carriers of other chemicals to increase organisms' exposure (USEPA, 2012). Amphipods such as *H. azteca* are detritivores and play an important role in aquatic ecosystems, where they degrade organic matter to release nutrients (Baun et al., 2008), feeds on algae and bacteria controlling the populations of these organisms, and serve as food for other organisms. The current results demonstrate that amphipods are susceptible to AgNPs, thus, the release of these nanoparticles into aquatic systems should be monitored to protect this group of organisms.

5. Conclusion

The current study demonstrates that ionic silver is more toxic than AgNPs to *H. azteca*. The results of this study also revealed for the first time that AgNPs can inhibit *H. azteca* survival and growth in aquatic systems. *H. azteca* response to AgNPs depends on the type of surface coating agent. AgNPs coated with citrate ligand was more toxic to the amphipod than those coated with PVP. Higher surface charge and the ionic surface may have increased citrate-AgNP interaction with the target site in amphipods to cause toxic effects. Amphipod survival and growth reduced rapidly by citrate-AgNP and PVP-AgNP exposures. Growth was more sensitive to citrate-AgNP compared to the survival endpoint. In contrast, growth was less sensitive to PVP-AgNP exposure. Low growth response to PVP-AgNP may be due to an unadjusted feeding regimen which resulted in excess food in exposure vessels for few survivors to grow faster. $AgNO_3$ used as a positive control exhibited the most toxic effect with the lowest LC_{50s} and EC_{50s} supporting *H. azteca* high sensitivity to the conventional material. The NOEC values demonstrate that PVP-AgNP is more protective to the amphipod survival and growth. The results of our study show that AgNPs may cause toxic effects in *H. azteca* and other epibenthic invertebrates in surface waters that receive water from wastewater treatment facilities that lack modern technologies to remove nanomaterials.

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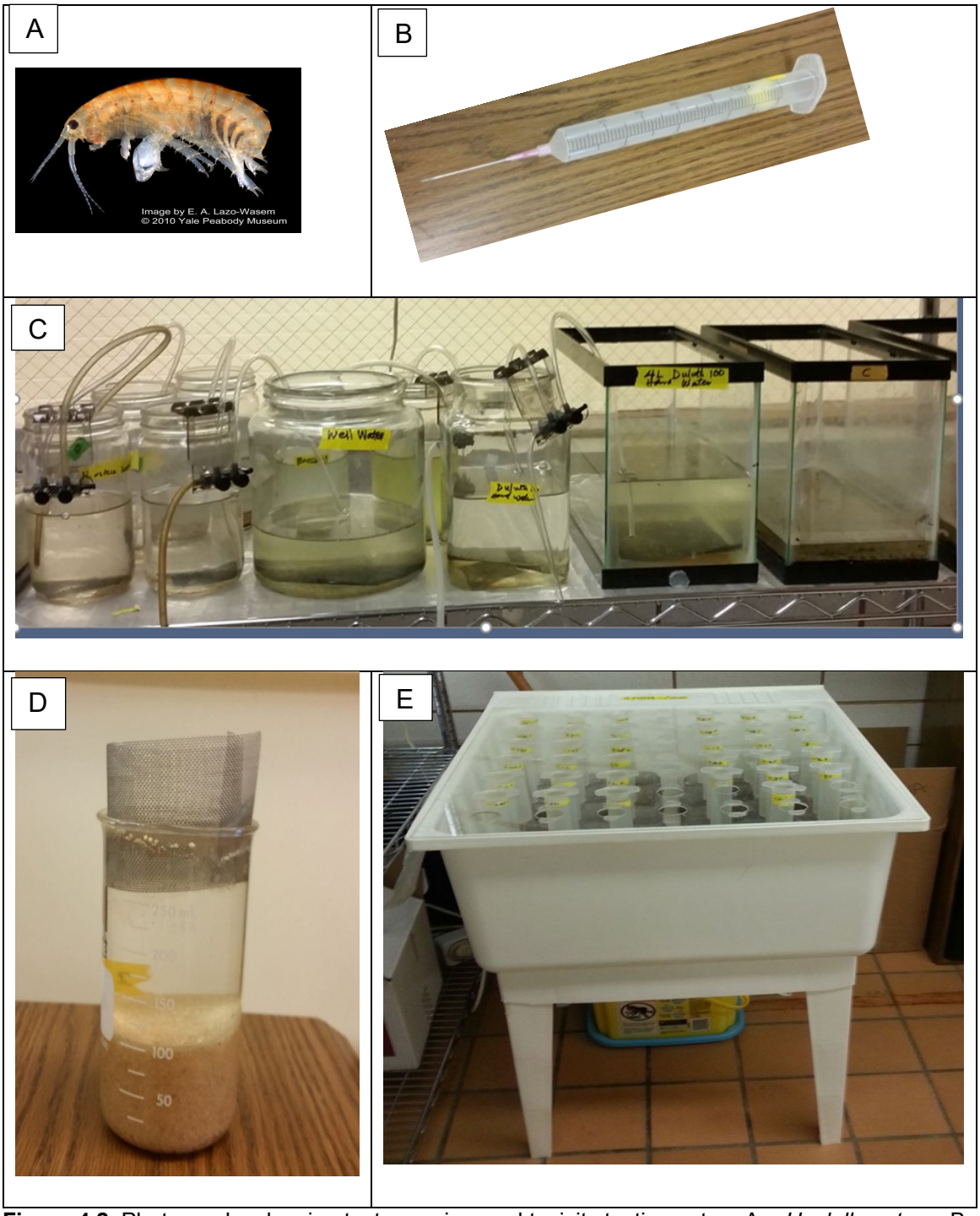

Figure 4.2. Photographs showing test organism and toxicity testing setup. A = *Hyalella azteca*, B = syringe, C = culture vessels, D = test beaker containing substrate (sand) and test solution, and E = plastic chamber containing test beakers and syringe for water renewal.

Table 4.7. Average water quality parameters for $AgNO_3$ 10-d toxicity testing

Treatment (µg/L)	Day 0 pH	Conductivity (µS/cm)	DO (mg/L)	Day 10 pH	Conductivity (µS/cm)	DO (mg/L)
0	7.1	299.3	8.8	6.8	308.3	6.5
1	7.4	302.3	8.3	6.9	307.0	6.3
2	7.3	329.0	8.4	6.8	332.7	6.1
4	7.1	325.0	8.7	6.8	329.3	6.1
8	6.9	254.0	8.6	6.5	260.3	6.0
16	6.8	328.3	8.5	6.4	331.7	6.0

Table 4.8. Average water quality parameters for $AgNO_3$ 10-d toxicity testing

Treatment (µg/L)	Day 0 pH	Conductivity (µS/cm)	DO (mg/L)	Day 10 pH	Conductivity (µS/cm)	DO (mg/L)
0	7.1	301.0	8.7	6.7	306.7	6.4
1	7.4	306.3	8.1	6.7	304.0	6.4
2	7.5	327.7	8.3	6.6	331.3	6.4
4	7.2	323.7	8.5	6.7	327.0	6.0
8	7.0	293.3	8.6	6.5	299.0	6.1
16	6.9	327.7	8.1	6.3	332.7	5.9

Table 4.9. Average water quality parameters for citrate -AgNP 10-d toxicity testing

Treatment (µg/L)	Day 0 pH	Conductivity (µS/cm)	DO (mg/L)	Day 10 pH	Conductivity (µS/cm)	DO (mg/L)
0	7.1	273.6	7.5	6.6	261.2	6.1
2.5	7.0	267.6	8.4	6.4	263.0	5.3
5	7.0	268.6	8.3	6.3	261.8	5.4
10	7.1	267.6	8.4	6.3	262.6	5.4
15	7.1	267.4	8.4	6.2	263.8	5.2
20	7.2	265.8	8.4	6.3	265.6	5.1
25	7.2	266.8	8.3	6.3	264.4	5.2

Table 4.10. Average water quality parameters for citrate-AgNP 28-d toxicity testing

Treatment (µg/L)	Day 0 pH	Conductivity (µS/cm)	DO (mg/L)	Day 28 pH	Conductivity (µS/cm)	DO (mg/L)
0	7.6	250.2	7.8	7.2	254.0	5.6
0.25	7.5	245.8	8.9	6.9	238.8	5.2
0.5	7.5	246.8	8.5	6.7	235.0	5.5
1	7.5	245.8	8.6	6.8	246.2	5.3
2.5	7.5	245.4	8.1	6.9	241.0	5.4
5	7.5	245.4	7.9	6.9	247.0	5.2
10	7.5	244.6	7.9	6.9	246.2	5.1

Table 4.11. Average water quality parameters for PVP-AgNP 10-d toxicity testing

Treatment (µg/L)	Day 0 pH	Conductivity (µS/cm)	DO (mg/L)	Day 10 pH	Conductivity (µS/cm)	DO (mg/L)
0	7.1	259.0	8.9	6.6	255.4	4.0
100	7.0	252.6	8.6	6.6	258.4	6.1
200	7.1	252.4	7.8	6.4	256.4	3.4
300	7.2	251.4	8.4	6.3	260.2	3.8
400	7.2	250.6	8.0	6.3	259.2	3.5
600	7.2	250.0	8.4	6.3	260.0	4.0
800	7.2	250.0	7.9	6.2	260.6	3.2

Table 4.12. Average water quality parameters for PVP-AgNP 28-d toxicity testing

Treatment (µg/L)	Day 0 pH	Conductivity (µS/cm)	DO (mg/L)	Day 28 pH	Conductivity (µS/cm)	DO (mg/L)
0	8.3	292.9	9.9	7.8	303.7	7.4
50	8.2	295.7	9.9	7.7	322.7	5.5
100	7.9	292.3	8.2	7.7	314.3	5.7
200	7.6	298.3	7.5	7.8	355.0	6.4
300	7.5	299.0	7.4	8.0	358.3	6.8
400	7.5	299.0	7.6	8.0	358.7	6.8

Table 4.13. LC50 for *Hyalella azteca* 96-h water-only static acute toxicity tests using potassium chloride (KCl) as a reference toxicant.

Test	KCl LC50 (mg/L)	Confidence intervals Lower	Upper	Percentage control survival
1	233	128	337	90
2	259	200	318	100
3	199	122	163	90
4	204	164	235	100
5	386	-	-	100
6	196	124	249	100
N	6			
Mean	246			
SD	72.7			
CV	29.5%			

Tests that did not meet minimum control survival of ≥ 80% were excluded. N = number of tests;

SD = standard deviation; and CV = coefficient of variation.

CHAPTER 5

SUMMARY AND FUTURE OUTLOOK

Over the past decade, AgNPs have been used extensively as antimicrobial agents to target a wide range of disease-causing microorganisms in products, but there is limited knowledge of their environmental, health and safety implications. In an attempt to understand the implications of emerging environmental contaminants on human health and the environment, this study evaluated the microbial community and *H. azteca* responses to two forms of AgNPs (citrate-AgNP and PVP-AgNP). This study revealed six key findings in which AgNPs (1) reduced the microbial population, (2) inhibited microbial enzyme activity, (3) altered carbon source utilization, (4) decreased the survival and growth of *H. azteca*, (5) surface coating agents influenced toxicity, and (6) that Ag^{+} ions were more toxic than both types of AgNPs tested.

The results of the study revealed that both AgNPs caused a drastic decline in microbial concentration after 48 h of exposure. A rapid reduction of microbial concentration demonstrates the already known antimicrobial properties of AgNPs, which may alter microbial populations in aquatic systems. Thus, the role of microorganisms in aquatic ecosystems including nitrogen cycling, degradation of organic matter, and food source for other organisms may be impacted if the release of AgNPs into the environment is not monitored or controlled.

AgNPs also inhibited microbial enzyme activity that is responsible for the degradation of carbohydrates. AgNPs decreased β-glucosidase activity significantly in exposed sediments while alkaline phosphatase activity was reduced but the effect was not statistically significant. In the United States, federal and public health agencies use the Enterolert test to determine the presence of pathogens in surface waters. The

Enterolert test relies on β-glucosidase activity to detect pathogen indicators, thus, inhibition of this enzyme reaction by AgNPs may interfere with the pathogen detection method and pose potential risks to public health. On the other hand, microorganisms use alkaline phosphatase to obtain inorganic phosphorus, a major component of bacterial DNA, from the environment. AgNPs effects on alkaline phosphate activity imply that the acquisition of inorganic phosphorus by microorganisms in aquatic systems may be affected.

In addition, AgNPs reduced the catabolic activity resulting in a shift in microbial functional diversity and metabolic fingerprint pattern. A shift in metabolic fingerprinting pattern suggests that AgNPs caused less diversity in carbon source utilization, thus, the ability of microorganisms to decompose organic matter and a variety of environmental pollutants may have been altered. Our results demonstrate that AgNPs released into the environment may cause negative impacts on ecosystem function in aquatic systems. Nominal concentrations at which the AgNPs caused inhibition effects were below expected Ag concentrations in freshwater sediments.

AgNPs significantly reduced survival and growth (dry weight and biomass) in 10-d and 28-d exposures. Growth was more sensitive to citrate-AgNP compared to survival in both exposures. We demonstrate for the first time that AgNPs pose a potential risk to the survival and growth of freshwater *H. azteca* populations. We conclude that epibenthic invertebrates are likely to be affected by AgNPs because *H. azteca* exhibited rapid responses to AgNPs at exposure durations.

Surface coating agents, citrate and PVP, were non-toxic but they influenced AgNP toxicity in environmental media. While citrate-AgNP and PVP-AgNP affected

microbial activity, the magnitude of their effects differed significantly. citrate-AgNP exhibited a greater effect on carbon source utilization while PVP-AgNP exhibited a greater effect on microbial concentration and enzyme activity. Similarly, both citrate-AgNP and PVP-AgNP reduced the survival and growth of *H. azteca*. Test organisms were more responsive to citrate-AgNP compared to PVP-AgNP. In general, PVP-AgNP was more protective of the amphipods compared to citrate-AgNP. Differences between the toxicity of the two forms of AgNPs were partly due to the ability of surface coating agents to influence the behavior of nanoparticles.

The outcome of this research has led to a good future direction with prospects of providing additional information on how nanomaterials may affect public health. Based on the research findings, future studies should consider investigating the following research topics to expand our understanding of nanomaterial implications on human health and the environment:

1. AgNP influence on existing pathogen detection and enumeration methods
2. Develop models and simulations to explain whether silver ions or/and AgNPs are responsible for the nanoparticle toxicity
3. A meta-analysis of human exposure to nanomaterials
4. Fate and transport of AgNPs in aquatic systems

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