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An Evaluation of Coating Material Dependent Toxicity of Silver Nanoparticles

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An Evaluation of Coating Material Dependent Toxicity of Silver Nanoparticles

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ABSTRACT

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Silver nanoparticles (AgNPs) synthesized using numerous types of coating materials may exhibit different toxicity effects. The study evaluated coating material dependent toxicity by selecting 3 types of AgNP synthesis methods with different coating materials (citrate, polyvinyl pyrrolidone, and branched polyethyleneimine, coated AgNPs as citrate-AgNPs, PVP-AgNPs, and BPEI-AgNPs respectively). Two acute aquatic toxicity tests were performed; 48hr *D. magna* and MetPLATE *E. coli* toxicity tests. Significantly different toxicity effects were observed in *D. magna* test exhibiting lethal median concentrations (LC_{50}) for citrate-AgNPs, PVP-AgNPs, and BPEI AgNPs respectively as, 2.7, 11.2, and 0.57μg/L. Median inhibitory concentrations (EC_{50}) for MetPLATE tests were 1.27, 1.73, and 0.31mg/L respectively with significant different toxicity effects. Silver ion fractions were detected in the range of 2.4-19.2% in tested NP suspensions. Study suggests the toxicity effects are due to the cumulative action of ionic and nanoparticle fractions in the suspensions.

DEDICATION

Dedicated to My Loving Parents

ACKNOWLEDGEMENTS

thank my loving parents, my sister, and my best friend, for their endless love and

encouragement they have given me throughout my endeavors.

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CHAPTER 1

INTRODUCTION

<u>Background and Problem Statement</u>

Nanotechnology is a newly emerged and rapidly developing technology, with new

technical and utility advances, that is being propagated in both scientific and industrial

sectors. The term "nano" originates from Greek and implies "dwarf", indicating an extremely

small size (Rai et al. 2009). The idea of manipulating nano-sized compounds (compounds in

atomic levels) was first developed in 1959 by Nobel Prize winner physicist Richard Feyman.

He explained the manipulation of nano-sized compounds as, "There's plenty of room at the

bottom", realizing the potential nature of manipulating atoms and/or atomic levels (Feyman

1959). Nanoparticles (NPs) can be broadly divided into 3 categories; natural, incidental, and

engineered. Natural sources of NPs include ocean spray, volcanic ash, volcanic dust, forest

fire smoke, clouds, soil, ice cores, biogenic compounds, and so forth. Incidental NPs may

arise from anthropogenic activities such as combustion, frying, cooking, mining, metal

working, sandblasting, and so forth. Engineered NPs are anthropogenic in origin and include

quantum dots (CdS, CdSe, CdSe/ZnS), metal oxide NPs (AgO, Fe_2O_3, ZnO, CuO),

fullerenes, semiconductor wires, and metal NPs (Ag and Au). Engineered NPs have given

rise to nanoproducts with better performance and better quality than ordinary products that

perform the same function with cheaper methods (Goldman and Coussens 2005). NPs can be

defined as compounds with at least one dimension less than 100 nm. However NPs may have

a range of 0.1 nm- 500 nm, resembling the size of big protein complexes (Ratner and Ratner

2002, Piotrowska et al. 2009). NPs exist in one dimensional (thin films or coatings), two

dimensional (nanowires and nanotubes), or three dimensional (precipitates, colloids, or

quantum dots) means (Nanowerck 2007) and are composed of different shapes; spherical,

tubular, triangular, irregular, aggregated, or fused. Properties of NPs greatly vary with its bulk materials due to high surface area to volume ratio, small size, and quantum effects. These aspects enable changes of reactivity, strength, and electrical properties of the engineered nanomaterials (Nowack and Bucheli 2007).

Due to their tremendous economic and resource saving capabilities, nanoproducts are widely used in medical, remediation, cosmetic, electronic, engineering, and environmental sectors (Nanowerk 2010, Niemeyer 2001). Use of Nanotechnology in the ever-developing medical sector is increasing. This is giving rise to techniques on bio-applications such as biological sensing, imaging, probing, medical diagnostics, and cancer therapy, while integrating particularly metal NPs in bio-systems (Jain et al. 2007). Engineered NPs are used in a wide variety of consumer products. It has been estimated that over 15% of the products in the market will have nano-based materials by the year 2014 (Dawson 2008). The 2011 Inventory of Woodrow Wilson International Center for Scholars for Nanoproducts claims that there are 1317 consumer-based nanoproducts in the market (PEN 2011) (Figure 1). Due to the immense advantageous properties of NPs, research and development funding has been raised and is estimated at US $10 billion worldwide from governmental and industrial sectors (Lux research 2006). According to the 2004 Royal society and Royal Academy of Engineering report, the rate of increase in the quantity of engineered NPs was 58,000 metric tons per year from 2011 to 2020 (Maynard et al. 2006). Therefore, further increase in the number of consumer-based nanoproducts research, and the development of synthesis methods and new technological uses, are expected in the future. Table 1 tabulates a few examples of engineered NPs, their relative properties, and their consumer products found in the U. S. Commerce.

Besides the vast range of benefits, the rapid growth of environmental and human health issues associated with nanomaterials has captured the attention of environmental scientists and researchers.

Table 1 Examples for engineered NPs, properties, and incorporated consumer products

Nanoparticle	Property	Nanoproduct	References
Silver	Antimicrobial	Fridges, vacuum cleaners, textiles, paints, varnish	Schmid and Reidiker 2008, Choi et al. 2008
Carbon nanotube	Excellent electrical conductors, semiconductors	Electronics, rechargeable batteries	Ju-Nam and Lead 2008
Iron oxide	Water soluble	Biomedical applications	Ju-Nam and Lead 2008
Zinc oxide	Large energy band gap energy, high dielectric constant, block broad UV rays	Optoelectronic and electronic devices, cosmetics, sunscreens	Singh et al. 2007. Huang et al. 2008
Titanium dioxide	Large energy band gap energy, photocatalytic	Sunscreens, photo catalytic and photovoltaic devices	Reinjnders 2008, Zhu et al. 2005
Cerium oxide	Catalytic and electrical properties	Oxygen sensors, fuel additive	Lin et al. 2006
Gold	High stability, inertness, tunable magnetic and electrical properties	Electronics, medical applications	Haick 2007
Aluminum oxide	High flexural and tensile strength, mechanical, electrical and magnetic properties	Cement, ceramic	Ke-long et al. 2007
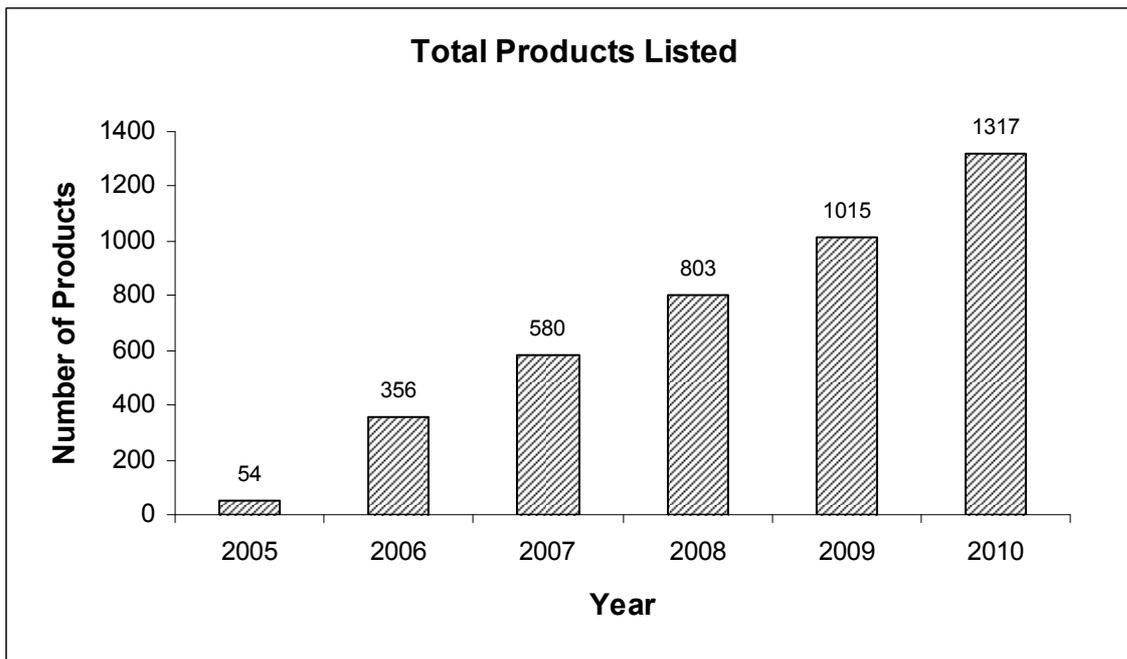
Figure 1 Number of total nanoproducts listed by year.

This signifies the relative increase of the nanomaterials throughout the recent past (Source: PEN 2011).

Properties such as high surface area to weight/volume ratio and small size that create NPs that are more useful are also linked with the creation of environmental and health issues (Kirchner et al. 2005). Engineered nanomaterials are likely to enter to the environment during any stage including manufacturing, processing, packaging, transporting, consumer handling, washing, and disposal (Oberdorster et al. 2005). Released compounds may introduce a variety of hazards into the environment and human life by physical and chemical means. Recognized hazards of engineered nanomaterials basically include toxicity to humans and other biological organisms as well as nano-waste management issues related with environmental pollution. Inhibitory effects of engineered NPs have been only partially recognized under laboratory experimental levels and few real world contaminations have been reported on airborne work place emissions (Oberdorster et al. 2005, Handy et al. 2008).

Toxicity studies had been conducted for different groups of organisms such as bacteria, aquatic organisms and also for cultured human cells with different nanomaterials (Handy et al. 2008, Piotrowska et al. 2009). In certain NPs biocidal and antimicrobial properties are prominent. As an example, nano Ag is incorporated in wound dressings, water treatment plants, washing machines, cleaning agents, disinfectants, cosmetics, swimming pool water, etc. (Bhattacharya and Mukherjee 2008, Senjen 2009). Like nano Ag, nano Cu/CuO (Chen et al. 2006) and nano ZnO (Blinova et al. 2010) have also been reported as toxic NPs to microorganisms.

The Woodrow Wilson International Center claimed that 20% of the nanoproducts produced in year 2007 contained nano Ag (Breggin and Pandergrass 2007). AgNPs receive the second highest funding for risk related research based on the inventory compiled by the Project on Emerging Nanotechnologies (Maynard et al. 2006). AgNPs are also one of the engineered NPs that have been employed in the widest range of applications (Rajeski and Lekas 2008). Antibacterial, antifungal, photocatalytic, and electrocatalytic properties are prominent among AgNPs. Therefore, application potentials have been created in catalysis, biological, and chemical sensing, nonlinear optics, electronics, and surface enhanced Raman spectroscopy (Kelly et al. 2003). Figure 2 represents the potential increase and the number of products associated with commonly found nanomaterials in the year of 2011. This implies the importance of investigating nano Ag in terms of characterization, exposure, fate and transport, and toxicity effects. Thus nano Ag related products are commonly found in nano commerce and 313 out of 1317 nanoproducts have been reported to contain nano Ag (PEN 2011).
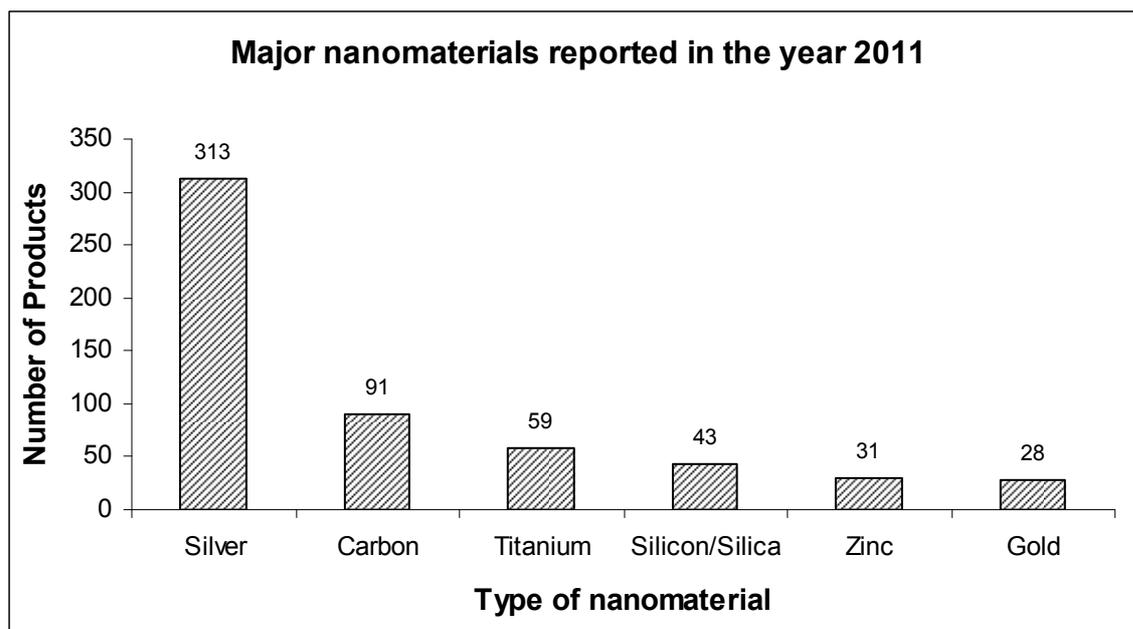
Figure 2 Number of products associated with major types of nanomaterials in the year of 2011 (Source: PEN 2011)

Recent literature demonstrates various synthesis methodologies for nano Ag such as chemical, electrochemical, γ radiation, photochemical, and laser ablation (Guzman et al. 2008). Synthesis of nano Ag is primarily based on the reduction of Ag ions using a specific reductant in order to form a zero valent stage of Ag (the resultants are the NPs because specific conditions are applied to gain nano size simultaneously). The majority of the reported studies on synthesis have used silver nitrate as the salt precursor. The chemical reducing agent may be a plant extract, chemical agent, or biological agent (Tolaymat et al. 2009). Modifications are employed with regard to the specific primary synthesis method during and/or after the preparation of NPs that would give rise to particles of variable sizes and shapes with different characteristics. For example, variations in temperature, pH, and agitation may influence inherent properties of NPs significantly in the chemically synthesized method. The incorporation of "capping /coating/ stabilizing agents" during synthesis

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contribute to the stabilization of colloids by hindering aggregation and controlling the size and shape of the synthesized product (Olenin et al. 2008). The colloidal stabilization mechanism is obtained through electrostatic and/or electrosteric stabilization for a particular NP. Electrostatic stabilization is functionalized via the formation of an electrical double layer using the surface charge of the particles. Electrosteric stabilization is functionalized via absorption of the stabilizing agent onto the particle surface as a protective layer (Sun and Luo 2005, Hassell et al. 2007). Thus, a specific coating material could give rise to differently characterized NPs.

Increased manufacture and use of nanoproducts enhance the opportunity of NPs to enter the environment during any stage from cradle to grave. Thus NPs tend to end up in the soil, air, and natural waters such as rivers, lakes, streams, estuaries, and coastal waters. NP uptake and toxicity to bio-organisms are major concerns and challenges for toxicologists today. NPs have a large potential for generating adverse effects to both eukaryotes and prokaryotes. The study of aquatic toxicity of NPs will be a main contribution to nanotechnology, which will determine the propagation, use, and advancement, towards a safe environment for both human and other life forms of earth. Among the different types of nanomaterials nano Ag is a significant type of material with regard to its abundance in the market place and its relative toxic effects. Thus, there is a relatively high demand for the research on AgNPs in the newly emerged field of nanotechnology.

<u>Hypotheses and Research Objectives</u>

In this master's research thesis it was hypothesized that AgNPs exhibit coating material dependent toxicity. Various coating agents have been used to synthesize AgNPs both in the laboratory and in the commercial level. Capping agents facilitate the stabilization of the NPs, rendering extended life by maintaining specific chemical and physical properties. Toxicity

mediated by AgNPs may be influenced by the capping agent used during the synthesis. The main objective of this study was to determine AgNP toxicity as a function of the coating material, using 2 common aquatic organisms, *Daphnia magna* and *Escherichia coli.*

Recent discoveries suggest that the properties of NPs are greatly influenced by size and shape (Pal et al. 2007). Therefore, an upsurge in research interest remains for various synthesis methodologies that control the size and shape of NPs to gain the desired specialized characteristics (Zhou et al. 1999, Jana et al. 2001) by using different capping agents. Published literature suggests use of various capping agents (Citrate, Polyvinylpyrrolidone-PVP, Sodium dodecyl sulphate-SDS, etc.) during the synthesis process, which enhances the stabilization of the AgNPs in the suspension (Tolaymat et al. 2009). Coating materials were selected specifically considering their ionic/non-ionic nature and mechanism of NP stabilization. Citrate is a low molecular weight anionic coating material (molecular weight, 189 g/mol) and NP stabilization is induced by electrostatic forces. PVP (molecular weight, 40 kg/mol) is a well-known, non-ionic, and hydrophobic polymeric coating material, where the AgNPs are stabilized by electrosteric forces (Garrec et al. 2004). BPEI (molecular weight 1.2 kg/mol) is composed of protonated amine groups providing a positive charge to the molecule that renders electrostatic stabilization. The hydrophobic character rendered by alkyl group, enhances the electrosteric stabilization of BPEI-AgNPs (Signori et al. 2010). Chemical structures of the coating materials that were used in the present study are displayed in the Appendix A. Thus, this study addresses the coating material specific toxicity effects of AgNPs, considering their mechanism of stabilization and inherent surface properties, gained during synthesis.

Numerous studies have taken place to investigate the toxicity of AgNPs as a function of synthesis and modification methods. However, few of the studies have explained the coating material specific toxicity based on their inherent properties or/and stabilization mechanism.

Therefore, this study has investigated the toxicity of citrate-AgNPs, PVP-AgNPs, and BPEI-Ag NPs to common aquatic dwellers, *D. magna* and *Escherichia coli*. The toxicity effects were evaluated and explained based on the characteristics of the NPs in the dilutions. Further, toxicity effects exhibited by the 2 organisms have been compared based on available literature.

<u>Research Approach</u>

This research focused on 3 commonly used capping agents in AgNP synthesis (citrate, PVP, and BPEI) in order to investigate the capping agent dependent toxicity. Static, 48-hour *Daphnia magna* acute toxicity tests (USEPA 2002[a]) and MetPLATE™ toxicity assays were performed to accomplish the task. The 48-hour *D. magna* test is an acute toxicity test generally performed to understand the toxicity level of any novel compound by regulatory agencies. The median lethal concentration (LC_{50}) was calculated based on the mortality of the organisms after 48 hours of exposure, and then compared among the types of NPs. The MetPLATE™ toxicity assay is traditionally a heavy metal toxicity test and is based on a kit with a strain of *Escherichia coli* (Bitton et al. 1994). The median effective concentration (EC_{50}) was calculated after each assay to determine the toxicity levels rendered by each type of NP.

CHAPTER 2

COATING MATERIAL DEPENDENT TOXICITY OF SILVER NANOPARTICLES

USING *DAPHNIA MAGNA* AS THE TEST ORGANISM

<u>Introduction</u>

Nanotechnology today is a major scientific research area as well as an enormous

industrial and economic growth area. Engineered NPs are generally defined as particles less

than 100 nm. The small size and characteristic surface properties of NPs furnish numerous

advantageous properties. These properties permit NPs to be used in a variety of applications

(Moore 2006). NPs may be released during any stage, from cradle to grave, and contaminate

the environmental compartments (Oberdorster et al. 2005). In addition to the beneficial

properties displayed by the NPs, scientists and researchers have directed their attention

towards the potential environmental and health problems of NPs. Examples of potential

problems from NPs would be the toxicity to organisms upon exposure and issues that might

arise from the management and disposal of nanowaste.

Apparent cytotoxic and genotoxic effects of several nanomaterials to aquatic organisms

have been reported in many scientific works (Pal et al. 2007, Kennedy et al. 2010). In aquatic

environments, nanomaterials rest as colloids (Klaine et al. 2008), and the colloidal behavior

of the specific nanocompound should be well recognized to explain the mechanisms of

hazardous effects. Among the toxic nanomaterials, nano Ag is considered to be one of the

most biocidal nanocompounds based on the studies performed in the recent past. A range of

stabilizing agents are in use for the synthesis of nano Ag such as, Polyvinyl pyrrolidone

(PVP), Sodium dodecyl sulphate (SDS), Poly(N-isopropyl acrylamide) like surfactants,

alkylthiols, and carboxyl like groups and citrate, branched Polyethyleneimine (BPEI) like

reducing agents (Guo et al. 2007, Si and Mandal 2007, Vasiliev et al. 2009). AgNPs are also

one of the NPs that have been employed in the widest range of applications (Rajeski and Lekas 2008). The antibacterial, antifungal, photocatalytic, and electrocatalytic properties of nano Ag have led to the creation of application potentials in catalysis, biological and chemical sensing, nonlinear optics, electronics, and surface enhanced Raman spectroscopy. These potentials have drawn the attention of scientists and industrial persons for the generation of novel products using nano Ag (Kelly et al. 2003). Nano Ag related products are one of the commonly found NPs in nano-commerce (Maynard and Michelson 2006). AgNPs receive the second highest funding for risk-related research based on the inventory compiled by the Project on Emerging Nanotechnologies (Maynard et al. 2006).

Due to its associated antibacterial properties, Ag and Ag-incorporated compounds have been used for the treatment of wounds and burns for centuries (Rai et al. 2009). Huge controversy exists on the toxicity mediating component in the nano Ag suspension, whether it is mediated by nano Ag or Ag ions. Published literature has shown the equal toxicity of nano Ag as well as Ag ions. Most evidence supports higher inhibitory levels associated with the fraction of Ag ions than the fraction of AgNPs. Numerous methods are in use to fractionate Ag ions from AgNPs such as filtration, diafiltration (Rinzler et al. 1998, Sweeney et al. 2005), ultrafiltration (Liu and Hurt 2010), centrifugation (Fabrega et al. 2009), use of radioactively labeled AgNPs (Zhao and Wang 2011), and use of ligands such as amino acids (cystein) (Navarro et al. 2008, Kawata et al. 2009), which bind with Ag ions to form conjugations exposing AgNPs to the test organisms. Diafiltration is considered to be an efficient, convenient, and rapid method compared to the traditional methods such as ultrafiltration and filtration. This allows purification of a sample through a continuous flow process over a specific pore sized filter/membrane via a passive way under a particular hydrostatic pressure (Sweeney et al. 2006).

Discoveries related to nano Ag toxicity have demonstrated noticeable inhibitory levels on bacterial species (Pal et al. 2007), algae (Miao et al. 2009), Daphnids, fish (Oberdorster 2004), and cultured human cells (AshaRani et al. 2009, Kawata et al. 2009). Based on the published literature so far, little is known about the toxicity mechanisms and it has yet to be discovered in relation to specific characteristics and the type of NP (AshaRani et al. 2009). Few toxicity mechanisms have been suggested and experimentally proven. Those can be listed as creation of free electron formation in bacterial and chlorophyll-containing organisms and the formation of redox reactive molecules and reactive oxygen species in large mouth bass (Oberdorster et al. 2005).

Aquatic organisms are widely used and recommended by regulatory agencies (e.g., United States Environmental Protection Agency-USEPA) in investigating the toxicity of the chemical components for many reasons. Aquatic environments are the ultimate destination for many types of ecologically significant chemicals (Van der Oost et al. 2003). It provides more precise detectability of toxicant levels than available chemical methods. Current risk assessment methods are in need of the use of organisms for hazard detection. Toxicity tests are used to recognize the "safe" and "no effect" levels of a contaminant in receiving waters, based on the observed toxic effects of the selected organism (USEPA 2002[a]). Existence of original nature of NPs depends on the background electrolyte type, pH, and ionic strength of the aquatic environment (Badawy et al. 2010).

Daphnia magna, a cladoceran used in the present study, is a well-known zooplanktonic (freshwater) dweller, an aquatic model species, and is a used as a bio-indicator by regulatory organizations including the USEPA (Lovern and Klaper 2007). There are mainly two types of tests performed; chronic and acute. In terms of aquatic organisms, performance of these tests can be done via static, static renewal, and flow – through systems. *Daphnia* interacts with large portions of an aquatic environment, filtering approximately 16.6 mL/h. Also *D. magna*

is a main component in the aquatic food chain and is consumed by many animals in high trophic levels such as fish and snails etc (Lovern and Kapler 2007).

The present chapter focuses on the coating material dependent toxicity of AgNPs using *D. magna* as the test organism. Studies on coating material dependent toxicity effects of aquatic organisms are scarce. The present study considers 3 types of AgNPs (citrate-AgNP, PVP-AgNP, and BPEI-AgNP) synthesized using 3 different types of coating materials (Citrate, PVP, and BPEI) that afford varying inherent properties and mechanisms of stabilization.

<div align="center">Materials and Methods</div>

Synthesis and Purification of AgNP

Detailed methods of synthesis of citrate-AgNP, PVP-AgNP, and BPEI-AgNP are presented in the Appendix A. All the methods have used $AgNO_3$ as the source of Ag, and different reductants and stabilization agents have used for different methods. A modified method of Turkevich et al. (1951) was used for the synthesis of Citrate-AgNP. For the PVP-AgNP synthesis a commercially available powder (Nanostructures and amorphous materials, Houston, TX, USA) form was obtained and stabilized in PVP stabilizing agent. BPEI-AgNP synthesis was followed by the method reported by Tan et al. (2007) (Badawy et al. 2010). Original AgNP stock solutions were purified using Kross Flow Research IIi TFF system (Spectrum Labs). Purified citrate-AgNPs, PVP-AgNPs, and BPEI-AgNPs were obtained from the National Risk Management Laboratory in Cincinnati affiliated with USEPA. The photographs (Appendix B, Figure 8) and a detailed explanation of the purification system are available in Appendix B. The system uses a membrane barrier (10 kD) to separate the molecules based on the size using the pressure as the driving force. This employs a tangential flow filtration process where fluid flows through the hollow fiber in a high velocity

(Appendix B, Figure 9). The molecules with a smaller diameter than the pore size build up as the permeate, and the remaining solution becomes the retentate, which consists of large sized particles.

Nano Ag Characterization

Original nano Ag suspensions and treatments were characterized using UV-Vis spectrophotometry (HACH[TM] DR 5000, HACH Company) in order to confirm the formation of AgNPs. The hydrodynamic diameter (HDD) and average zeta potential was measured using NICOMP[TM] 380 zeta sizer (particle sizing systems). Further conductivity and pH measurements (HI 9828 Multiparameter, Hanna Instruments) were performed for all originally synthesized solutions and dilutions. Total Ag was analyzed using Graphite Furnace Atomic absorption (GFAA) spectrometry (USEPA SW-846 Method 7010, Varian Spectra 220Z) following the acid digestion (USEPA SW-846 Method 3010A) of the AgNP suspensions. Thus, AgNP concentration was given as total Ag ion concentration. Detailed explanations of the USEPA methods 3010A and 7010 are given in the Appendix D and E respectively (USEPA, 2003).

Detection of Ag Ion Concentration

Ultracentrifugation (Thermoscientific Sorvall MX series) was used to separate nano silver from the ionic counterpart. Here the AgNP suspension (8mL) was ultracentrifuged at 100,000 xg for 60 min. A 1.5 mL portion of the supernatant was withdrawn and was acidified with nitric acid to analyze in GFAA (Varian spectra 220Z) following the USEPA SW-846 method 7010. Dissolved ionic fraction was expected to present in the supernatant, whereas, the nano Ag fraction was expected to settle down at the bottom of the centrifugation tube. The same procedure was used by Kennedy et al. (2010) to fractionate nano Ag.

Laboratory Culture and Maintenance of *Daphnia magna*

Daphnia starter cultures were obtained (Department of Biological and Environmental Sciences, University of Tennessee), scaled up, and maintained in 750 mL glass jars following the USEPA (2002[b]) guidelines. Standard synthetic moderately hard reconstituted water (MHRW) was used as the culture medium (Appendix J, Table 12). Cultures were maintained at 20 ± 1^0C temperature, with a photo period of 16 hours of light and 8 hours of dark. Alga, *Selenastrum capricornutum*, and trout chow, were provided as food. Algal starter cultures were obtained (Aquatic Biosystems, Colorado) and scaled up in the lab providing optimum culture conditions (Appendix J, Figure 12) (USEPA 2002[b]). Details of culture and maintenance of *D. magna* and algae are given in the Appendix J. Laboratory *D. magna* cultures were monitored regularly to ensure the favorable water quality conditions by monitoring parameters such as dissolved oxygen (DO), pH, conductivity (HI 9828 Multiparameter, Hanna instruments), total hardness (HACH, 8213), and Ammonia-Nitrogen (HACH method 8155). Table 2 displays the acceptability criteria of the physico-chemical characteristics that should be maintained in the *D. magna* cultures. Detailed descriptions of HACH methods 8213 and 8155 are given in Appendix H and I, respectively.

Table 2 Acceptable criteria maintained in the cultures and test media

Parameter	Acceptable level
Temperature (^{0}C)	20 ± 1
pH	7.4-8.2
Conductivity (μS/cm)	340-480
Hardness (mg/L $CaCO_3$)	75-90
Dissolved Oxygen (DO) (mg/L)	>5
Ammonium-nitrogen (mg/L)	<4.11

Sources: USEPA 2002[b], Heckmann and Cannon 2007.

D. magna Acute Toxicity Test Performance

Static, non-renewal, 48-hr acute toxicity tests were performed (USEPA 2002 [b]). Less than 24-hour old neonates from *D. magna* cultures were used as the test organisms. In order to obtain the specific aged neonates, egg bearing adults were prepared a day prior to the test performance (Appendix J, Figure 11). Plastic disposable cups (30 mL) were used as test chambers. A 30 mL of test solution was used in each test chamber. Triplicates were run for each dilution. Ten neonates were allocated to each replicate using block randomization procedure (blocks or distributes brood effects evenly among the treatments) from pre-arranged individual brood cultures. Copper was used as the reference chemical, and was tested at regular time intervals to ensure Quality Assurance/ Quality control (QA/QC) requirements. MHRW and copper were used respectively as negative and positive controls. Concentration respective to the LC_{50} value of Cu was used as the positive control in each test performance. Initially, range finding tests were performed to discover the minimum and maximum concentrations that cause 100% mortality and 100% survival of neonates respectively, for the 48-hr test period in order to narrow down the test concentration range. Final tests were carried out using those concentrations as the minimum and maximum concentrations in the array of dilution and using 3-4 dilutions between to obtain sufficient numbers of partial mortalities for statistical analysis.

Acute toxicity tests were conducted in $AgNO_3$, PVP-AgNP, Citrate-AgNP, and BPEI-AgNP suspensions. Possible existence of Ag ions in AgNP suspensions was suggested by use of $AgNO_3$ as a test candidate. Impact of the other probable interferences (tri-sodium citrate that was used for the synthesis of citrate-AgNP, PVP, and BPEI) for all selected types of NP suspensions were investigated, exposing *D. magna* to the expected concentration in the test solutions. The tested concentration of each interfering substance (Tri-sodium citrate, PVP,

and BPEI) was decided based on the nominal concentration of the substance at the highest total Ag concentration used in the array of dilution.

Organisms were not fed during the period of the experiment. Mortality was detected at the end of the 48-hour incubation period. Required physical measurements; pH, conductivity, temperature, hardness, and ammonia-nitrogen concentration were measured at the end of the each test to insure the maintenance of favorable environmental conditions during the incubation period. LC_{50} values (Probit analysis, Minitab version 14) were calculated based on the analytical results for each test as a measure of toxicity.

Analytical Procedures

All concentrations of the dilution series of all AgNP types were separately acid digested and analyzed using GFAA to quantify the recoverable and dissolved Ag (total Ag) at initial exposure. Every replicate of all AgNP types were also acid digested after the incubation period, and concentrations of total Ag were quantified using GFAA. The values of replicates were averaged to obtain the concentration of total Ag for a particular dilution at the end of the incubation period. Total Ag levels below the Method Detection Limit (MDL) of GFAA, in the series of dilution were derived based on the dilution factor and the total Ag level measured at the highest concentration of the series.

QA/QC Measures

MDL and quantitation limit (QL) GFAA (Appendix F) were calculated before sample analysis. Cu was used as the reference chemical. Positive (Cu) and negative (MHRW) controls were performed for each final test. For the GFAA Ag analysis, blanks, standards, spiked blanks, spiked samples, and duplicate samples were run after every 10 samples of analysis. All these were run for both acid-digested and undigested forms (Appendix G).

LC$_{50}$ values and associated 95% confidence intervals were calculated by employing

probit analysis. LC$_{50}$ values were compared using ANOVA (Minitab version 14). Probit

analysis is a specialized type of regression used to study binomial variables. This transforms

the sigmoid dose response curve into a straight line that allows for the determination of the

dose of a component at an interested point of response (Finney 1952).

<div align="center">Results</div>

Characterization of Original AgNP Suspensions and Dilutions

The summary of results of the characterization of purified AgNP suspensions are

presented in Table 3. Zeta potential is a measure of surface charge of the NP. It is established

by an electrical double layer comprised of charged NPs and oppositely charged surrounding

ions. PVP-AgNPs and citrate-AgNPs bear a negative surface charge while BPEI-AgNPs bear

a positive surface charge. HDD of all original stock suspensions were approximately 10 nm.

Detailed graphs of the HDD for each tested NP type are given in Figure 10 (Appendix C).

HDD and average zeta potential of all dilutions were not able to be measured via zeta sizer at

low concentrations of AgNPs. HDD and average zeta potential were measured at the first

highest concentration of the PVP-AgNP dilution series (HDD=11.0±0.9 nm, Average zeta

potential= -5.42 mV). Figure 3 displays the UV-vis spectra of each AgNP type used in the

study. This affirms the availability of AgNPs in the synthesized AgNP suspensions. The

range of conductivity for purified AgNP suspensions were 9-10 µS/cm. However, possible

residual ions might be present proportional to the observed conductivity in negligible levels.

Table 3 Characterization of original AgNP types (Stock suspensions)

AgNP type	UV-vis wavelength at the highest absorbance (nm)	Characteristic	Reading
Citrate-AgNP	410	HDD (nm)	10.9±0.8
		Average zeta potential (mV)	-20.08
PVP-AgNP	405	HDD (nm)	11.0±0.7
		Average zeta potential (mV)	-7.49
BPEI-AgNP	410	HDD (nm)	10.9±0.8
		Average zeta potential (mV)	+28.8
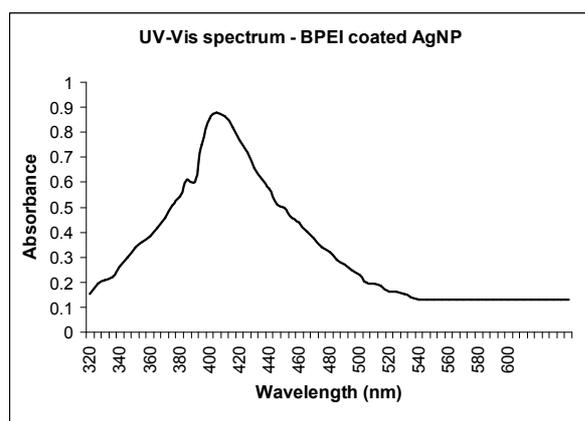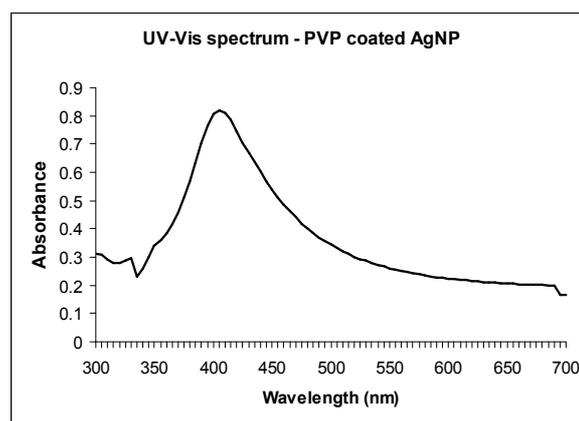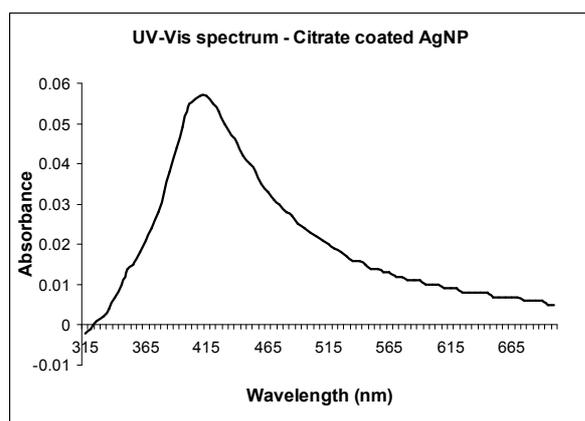
Figure 3 UV-Vis spectra of the selected types of AgNPs

<u>Ag Ion Levels</u>

Detected Ag ion levels in stock AgNP suspensions and at the highest concentrations used in the *D. magna* toxicity tests are given in Table 4. Ag ion levels observed in the stock solutions and in the highest concentrations used in the *D. magna* toxicity test, were in the range of 6.55-14.7% and 3.08- 19.2% respectively for all AgNP types. Ag ion level in PVP-AgNPs decreased (3.08%) with dilution in MHRW, while increased in citrate-AgNP and BPEI-AgNP (19.2 and 14.5% respectively). The order of Ag ion percent can be given as BPEI-AgNP>PVP-AgNP>citrate AgNP and Citrate AgNP> BPEI AgNP> PVP AgNP, respectively, for stock solutions and for the highest concentrations used in the test.

Table 4 Percentage Ag ion concentrations observed in the original stock AgNP suspensions and the highest concentrations used in the *D. magna* toxicity test (Ultracentrifugation technique)

Type of nano Ag	Total Ag concentration (mg/L)	Stock AgNP suspension		Highest concentrations* used for the *D. magna* test	
		% Ag ion level	Concentration of Ag ion (mg/L)	% Ag ion level	Concentration of Ag ion (mg/L)
Citrate-AgNP	14.9±2.94	6.55	0.98	19.2	2.86
PVP-AgNP	43.2±2.94	9.27	4	3.08	1.33
BPEI-AgNP	8.56±0.74	17.96	1.53	14.5	1.24

*Dilution was made using MHRW (Dilution water)

Physico-Chemical Characteristics

Physico-chemical characteristics (temperature, pH, conductivity, DO, Ammonia-Nitrogen level, and hardness) were within the acceptable range in all dilutions before and after the incubation period (Table 2). Therefore, standard and known physico-chemical stressors were completely eliminated from the test media that were unfavorable for the growth/survival of the organisms.

Analytical Measurements

MDL and QL of GFAA were detected as 0.89 and 2.96 µg/L respectively. Concentrations of originally synthesized NPs were 14.9±1.15, 43.2±2.94, and 8.56±0.74 mg/L for citrate-AgNP, PVP-AgNP, and BPEI-AgNPs respectively. No notable difference was exhibited between the total Ag concentrations observed before and after the 48-hr exposure period (Appendix J). Thus, the results indicate the total recovery of Ag during the exposure period and possible exposure to the relatively same concentration at the time of initial introduction. All observed concentrations of the dilutions were within ±20% of the nominal concentration based on the highest concentration of the dilution series for all AgNP types and also were within ±20% of nominal concentration for $AgNO_3$ and Cu concentrations. All 3 replicates of each dilution were within ±20% of the expected nominal concentration for all the tested components. QA/QC measures were within ±22% of the nominal standard concentrations (Appendix G).

Toxicity Effects

The highest toxic component among the tested species was the BPEI-AgNP (LC_{50}=0.53 µg/L) and the least toxic component was the PVP-AgNP (LC_{50}=10.74 µg/L) (Table 5). BPEI-AgNP is 4 times more toxic than $AgNO_3$ and PVP-AgNP is 5 times less

toxic than $AgNO_3$. $AgNO_3$ and Citrate-AgNPs showed an equal toxicity to *D. magna*. Toxicity exerted by all tested components were statistically significantly different (P=0.000). However, the toxicity of $AgNO_3$ and citrate-AgNPs were not reported as significantly different. Thus the order of toxicity can be given as $AgNO_3$ /BPEI-AgNP >Citrate-AgNP > PVP-AgNP. The toxicity exhibited for all tested components were concentration dependent. LC_{50} of reference chemical (Cu) was 6.17 µg/L. No mortality was shown at the highest expected concentration of each individual coating material (Tri sodium citrate, PVP, and BPEI) in the array of dilution.

Table 5 LC_{50} values of each tested component resulted from *D. magna* 48hr acute toxicity assay

Tested species	LC_{50} (µg/L)			
	1	2	3	Mean* ± SD
$AgNO_3$	2.14	2.05	2.44	2.21± 0.204 [a]
Citrate-AgNP	3.61	2.98	2.90	3.16 ± 0.39 [b]
PVP-AgNP	12.17	10.27	9.28	10.74 ± 1.44 [c]
BPEI-AgNP	0.58	0.46	0.56	0.53 ± 0.064 [a]

LC_{50} values have been calculated for each type of AgNP based on the mortalities of neonates. Means are given with the respective standard deviations (SD).

* Means with different superscripts are significantly different.

Discussion

One of the major concerns in NP research, "toxicity effects", is entirely dependent on the characteristics of the specific NP. Most studies done so far indicate size, shape (Pal et al. 2007), and surface charge, dependent (Badawy et al. 2011) toxicity effects. One of the common modes of achieving the desired characteristics in metal NPs, is the use of a

stabilizing agent in conjugation with a reducing agent (Rozenberg and Tenne 2008).

Stabilizing agents stabilize the synthesized thermodynamically less stable colloidal NPs, via electrostatic and/or electrosteric interactions. Further, incorporation of the stabilizing agent can change the colloidal weight, surface charge, chemical functionality, and hydrophilicity (surface chemistry of the AgNP), which can exhibit variable toxicity impacts to the organisms. Significant coating material dependent toxicity effects on *D. magna* were observed from the present study.

The study has used 3 commonly employed coating materials in AgNP synthesis; citrate, PVP, and BPEI. PVP (molecular weight, 40 kg/mol) is a water soluble and non-charged polymeric substance, providing steric stabilization for the coated AgNPs. Here the NPs are kept isolated by coating the core of the NP with a thick layer of PVP. During citrate-AgNP preparation, citrate molecule acts both as a reductant as well as a stabilizing agent. Stabilization of citrate-AgNPs is functionalized by electrostatic adsorption of the citrate molecule to the synthesized AgNP, and the NP cores are kept apart by forming an electrical double layer. BPEI (molecular weight 1.2 kg/mol) is a polyelectrolyte that behaves both as a reductant as well as a stabilizer during the synthesis process. Stabilization of coated NPs is provided by electrosteric and electrostatic forces respectively by hydroxyl moieties and alkyl groups (Rozenberg and Tenne 2008, Bae et al. 2010, Signori et al. 2010) of the BPEI molecule.

Published literature manifests purification techniques to eliminate the residual reactants and possible toxic ionic compounds (eg., Ag ions) from the synthesized AgNP suspension. Kros-flow purification system allows eluting particles smaller than pore size of the ultrafiltration membrane (10 kD) and retains AgNPs >1nm and coated AgNP in the retentate. This retentate was referred to as the purified AgNPs in the study, where a majority of the Ag ions (107 D) were eluted out (Spectrum laboratories 2010, Sweeney et al. 2005).

The size of the "Coated AgNP" may be higher than the individual coating material, or AgNP, because of the prevailing electrostatic and electrosteric interactions. Thus, the theoretical explanation verifies the existence of coated AgNPs in the retentate.

With subsequent dilution in MHRW, Ag ions may be released into the suspension at negligible levels (Navarro et al. 2008, Liu and Hurt 2010, Zhao and Wang 2011). Published studies have used ultracentrifugation and/or field flow fractionation to fractionate dissolved Ag ions from AgNP suspensions. Kennedy et al. (2010) has reported 6% and 3% fractions of Ag^+ respectively for the highest concentrations of citrate-AgNPs and PVP-AgNPs used in the dilution series. However, in the present study for citrate-AgNP and BPEI-AgNPs, elevated Ag ion levels resulted when diluted in MHRW, while for PVP-AgNP (3.08%) a similar value resulted as in Kennedy et al. (2010). No toxicity was observed when exposed to individual coating materials. Therefore, the exhibited toxicity was solely a representation of the impact of coated AgNP and was not due to the coating material used.

Relating the trend of toxicity and relevant ionic fractions of each type of AgNP, the study has shown a positive correlation that aids in understanding the toxicity effects. Ionic counterpart of Ag has been observed as the most toxic form from several acute toxicity studies conducted for AgNP types (Kennedy et al. 2010, Zhao and Wang 2011). Kennedy et al. (2010) have discovered comparable LC_{50} values for both fractionated Ag^+ and for Ag^+ as $AgNO_3$. Thus observed toxicity/LC_{50} values in the present study are due to a cumulative effect of the fraction of Ag ions and the fraction of AgNPs. Significantly different toxicity effects observed among the types of NPs are an indication of coating material dependent toxicity of AgNPs.

Surface properties inevitably play an important role in determining the toxicity effects that are associated with the penetrability into the cells (Bae et al. 2010). Impact of the pH, ionic strength, and electrolyte type of the AgNP suspensions on electrosterically stabilized

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NP (PVP-AgNPs), electrostatically stabilized NPs (NaBH$_4$ synthesized AgNPs and Citrate-AgNPs), and both electrostatically and electrosterically stabilized NPs (BPEI-AgNP) have been studied by Badawy et al. (2010). All AgNP dilutions of the current study had an ionic strength in the range of 3-4 mM and pH in the range of 7.4-7.8. At 10 mM ionic strength (NaNO$_3$ as the electrolyte, at neutral pH) HDD of the Citrate-AgNP, PVP-AgNP, and BPEI-AgNP were reported 10, 200, and 12 nm respectively. Gao et al. (2009) has been observed decreased toxicity effects of MetPLATE bacteria, while increasing the ionic strength. This affirms aggregation of NPs at high levels of ionic strength. Therefore the derived HDDs of the AgNPs in the current study can be given as <10, <200, and < 12 nm respectively for Citrate-AgNP, PVP-AgNP, and BPEI-AgNP. Size dependent toxicity is prominent among NPs and has been observed in many studies published so far (Pal et al. 2007, Choi et al. 2008). The higher toxicity of citrate-AgNPs and BPEI-AgNPs than PVP-AgNPs can be explained as a size induced toxicity phenomenon. Although the dilutions were done with MHRW (ionic strength 3-4 mM, conductivity 340-440 µS/cm, EPA 2002[b]) that contains a mixture of electrolytes (Appendix J, Table 12), the approximated HDD values can be changed significantly. Thus, the literal HDDs of the NPs in the dilutions are unpredictable with currently available literature. Theoretically, BPEI-AgNPs are more stable, based on the governing electrostatic and electrosteric forces, than PVP-AgNP and citrate AgNP. This validates the data observed in the present study in another way.

Kennedy et al. (2010) also has reported LC$_{50}$ values for citrate-AgNPs (HDD =70± 1 nm) and PVP-AgNPs (HDD= 96±0 nm), respectively in the range of 10.2-14.1 µg/L and 87.2-107.9 µg/L when performed *D. magna* 48-hour acute toxicity tests. Observed low toxicity of NP suspensions in the above study could be reasoned out by the larger NPs (Pal et al. 2007). However, the trend of toxicity dictated between citrate-AgNP and PVP-AgNP are comparable with the present study. A similar trend of toxicity has also been observed with

Pimephalas pomelas 48-hour acute toxicity tests by the same study. Zhao and Wang (2011) have performed a coating material dependent toxicity study. This study reported 28.7 µg/L of LC_{50} value for PVP-AgNPs, which was much higher than the LC_{50} (11.17 µg/L) of the present study. However, the Ag ion fraction observed was low (0.5%) and the zeta potential was high (-19.5 mV) in comparison with the present study, which may be the reason for observed lower toxicity for PVP-AgNPs. This research has also observed a coating material dependent toxicity effect among PVP, sodium dodecylbenzene sulfonate, and lactate coated AgNPs.

Various mechanisms of toxicity have been published in literature that are pertinent to ionic and NP fraction of Ag. Ion-regulatory disturbance has been seen during *D. magna* acute exposure to Ag ions, with a competitive inhibition of active sodium uptake from the environment. This has lead to a blockade of Na, K ion, and ATPase inhibition followed by ion accumulation (Bianchini and Wood 2002). Microscopic studies have shown accumulation of NP fraction in the gut of *D. magna* during acute exposure (Petersen et al. 2009, Rosenkranz et al. 2009). Accumulated AgNPs may inhibit food uptake, eventually causing growth retardation, reduced reproducibility, and death. However, no correlation has been observed between AgNP accumulation and the toxicity in the study done by Zhao and Wang (2011). Therefore, more studies are needed to determine the governing physiological effects that lead to toxicity and daphnid mortality.

Conclusions

BPEI-AgNPs were the most toxic AgNP type for *D. magna* among the types of AgNPs tested in the study. Coating material dependent toxicity was prominent for the tested AgNP types. However, the toxicity effects exerted were a cumulative effect of ionic counterpart and the AgNP counterpart functionalized with different coating materials. The *D.*

magna 48-hour acute toxicity test is recommended as a suitable test to evaluate the toxicity of

AgNPs/metal NPs in environmental samples.

CHAPTER 3

COATING MATERIAL DEPENDENT TOXICITY OF SILVER NANOPARTICLES TO
METPLATE^{TM} *ESCHERICHIA COLI*

Introduction

In the field of nanotechnology, applications of manufactured nano Ag are
significantly increasing over the other manufactured NPs due to its incredible antimicrobial
and conductive properties. Therefore, manufactured nano Ag has been frequently used in the
medical field, wastewater treatment facilities, and in the textile industry (Rai et al. 2009).
Inevitably, these nano compounds will be released into air, soil, and water (Oberdorster et al.
2005). Thus, these NPs may exert toxicity to biota in the environment based on its
bioavailability to the organisms. Discoveries related to nano Ag toxicity studies have
demonstrated noticeable inhibitory levels on bacterial species (Pal et al. 2007), algae (Miao et
al. 2009), fish (Oberdorster 2004), and mammalian species (AshaRani et al. 2009, Kawata et
al. 2009).

Based on the published toxicity studies, the bactericidal effect of nano Ag is well-
known. Inhibitory effects of the bacterium *Escherichia coli* have been well studied due to its
relative high abundance in the environment and its consideration as a typical gram negative
bacterium. Gram negative bacteria (*Escherichia coli*) have shown a higher inhibitory level
than gram positive (*Staphylococcus aureus*) bacteria on exposure to nano Ag (Shrivastava et
al. 2007). Peptidoglycan layer of gram positive bacteria is reported to be thicker than gram
negative bacteria. Therefore, the peptidoglycan layer of gram negative bacteria provides
relatively easy access to NPs than gram positive bacteria (Kim et al. 2007). Mechanisms of
toxicity have been partially identified within bacterial species. Recent studies have indicated
the following mechanisms of toxicity that govern among bacterial species; loss of DNA

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replication, physical damage to the microbial cell wall by direct contact (Zhang et al. 2005), and reactive oxygen species formation (Oberdorster et al. 2005).

The present study uses MetPLATE[TM] toxicity assay to determine the toxicity of nano Ag to a strain of *E. coli* (gram negative) using a kit. Traditionally, MetPLATE[TM] assay has been used to explore the heavy metal toxicity (Huang et al. 1999). This test examines the activity of β-Galactosidase enzyme expressed by *E. coli* in the presence of test material/contaminant, and the toxicity is evaluated by a colorimetric method. The kit contains lyophilized bacterial reagent, chlorophenol red galactopyronoside (CPRG), which acts as the substrate for the intra bacterial β-galactosidase enzyme and diluent, moderately hard water. In the reaction CPRG (yellow) is cleaved in the presence of the β-Galactosidase enzyme producing, galactopyranose and chlorophenol red (purple) (Figure 4) (Dagan 2003).
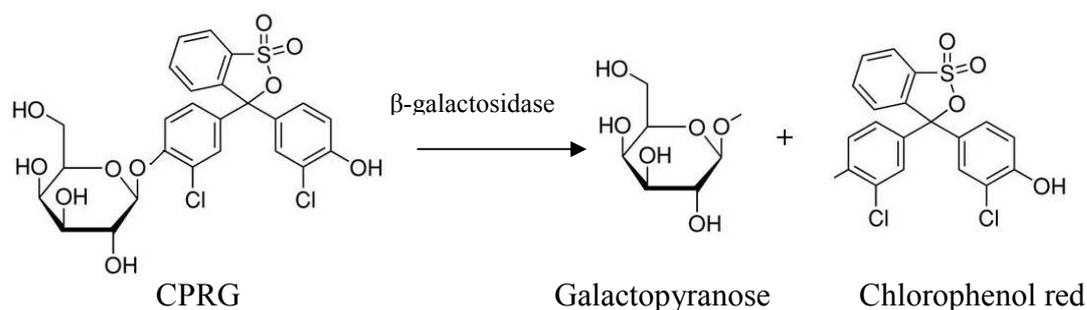
Figure 4 Reaction of CPRG in the presence of β-galactosidase enzyme (Dagon 2003)

Gao et al. (2009) have performed MetPLATE toxicity test to study the acute toxicity of nano Cu and nano Ag in natural water samples. They have not revealed toxicity for nano Cu in environmental concentrations. However, they have observed toxicity for nano Ag suspensions. Silver, copper, and fullerene NPs have shown sensitivity towards Metplate bacteria in the study done by Gao (2008). MetPlate toxicity test is considered as a rapid screening test compared to the use of other traditional toxicity tests (Stook et al. 2004, Dubey et al. 2007).

The goal of the present study was to determine and explain the coating material dependent toxicity effects of AgNPs to a strain of *E. coli* using MetPLATE™ toxicity assay. To accomplish the task, the same coating materials, citrate, PVP, and BPEI, were used in the AgNP synthesis that were also used in the *Daphnia* test (Chapter 2). At the same time, the test applicability for toxicity assessment in aquatic bodies was evaluated. Different toxicity responses of various AgNPs were explained based on mechanisms of stabilization, inherent properties, and the size.

<p style="text-align:center">Materials and Methods</p>

Synthesis, Purification, and Characterization of AgNPs

The Same AgNP types that were used in Chapter 2 (Citrate-AgNP, PVP-AgNP, and BPEI-AgNP) were used to perform the MetPLATE toxicity test.
Synthesis, purification, and characterization methods were also similar to the corresponding section in the Chapter 2.

MetPLATE™ Toxicity Test

The pH of the test solutions was measured and ensured to be in the range of 6-7.5. Steps followed during the test procedure are illustrated in Figure 5. A detailed explanation of the test is provided in the Appendix K. Copper sulphate and Evian water (Moderately hard water, conductivity 560 μS/cm) were used as the positive and negative controls respectively, according to the protocol. Triplicates were tested for each dilution and controls. Absorbance values were read from the microplate reader at 575 nm wavelength. Dilutions were acid digested (USEPA SW-846 method 3010A, USEPA 2003), and concentrations of total Ag were quantified for total recoverable and dissolved Ag (total Ag) using the GFAA (Varian

Spectra 220Z) (USEPA SW-846 method 7010). EC_{50} (median effective concentration) was calculated for each test component using the graphical method (Appendix K). To determine the influence of the individual coating materials, MetPLATE test was also performed for the highest expected concentration of each coating material used in the study (tri-sodium citrate, PVP, and BPEI).

```
┌─────────────────────┐  ┌─────────────────────┐  ┌─────────────────────┐
│ 0.1 mL bacterial    │  │ 0.1 mL of bacterial │  │ 0.1 mL bacterial    │
│ reagent + 0.9 mL    │  │ reagent + 0.9 mL    │  │ reagent + 0.9 mL    │
│ test sample         │  │ positive control    │  │ diluent             │
│                     │  │                     │  │ (negative control)  │
└─────────────────────┘  └─────────────────────┘  └─────────────────────┘
                                    │
                                    ▼
          ┌──────────────────────────────────────────────┐
          │   Incubate tubes at 35⁰C for 90 minutes.       │
          └──────────────────────────────────────────────┘
                                    │
                                    ▼
          ┌──────────────────────────────────────────────┐
          │   Add 0.2 mL aliquots from each test tube to   │
          │   the microplate.                              │
          └──────────────────────────────────────────────┘
                                    │
                                    ▼
          ┌──────────────────────────────────────────────┐
          │   Add 0.1 mL of reconstituted CPRG to each     │
          │   microplate well.                             │
          └──────────────────────────────────────────────┘
                                    │
                                    ▼
          ┌──────────────────────────────────────────────┐
          │   Incubate the microplate at 35⁰C for about    │
          │   0.5-2 hrs.                                    │
          └──────────────────────────────────────────────┘
                                    │
                                    ▼
          ┌──────────────────────────────────────────────┐
          │   Determine the color intensity by measuring   │
          │   the absorbance at 575 nm in a microplate     │
          │   reader.                                       │
          └──────────────────────────────────────────────┘
                                    │
                                    ▼
                    ┌──────────────────────────┐
                    │   EC₅₀ determination.      │
                    └──────────────────────────┘
```

Boxes, in sequence:

- 0.1 mL bacterial reagent + 0.9 mL test sample
- 0.1 mL of bacterial reagent + 0.9 mL positive control
- 0.1 mL bacterial reagent + 0.9 mL diluent (negative control)
- Incubate tubes at 35^0C for 90 minutes.
- Add 0.2 mL aliquots from each test tube to the microplate.
- Add 0.1 mL of reconstituted CPRG to each microplate well.
- Incubate the microplate at 35^0C for about 0.5-2 hrs.
- Determine the color intensity by measuring the absorbance at 575 nm in a microplate reader.
- EC_{50} determination.

Figure 5 Flow chart of the steps performed in the MetPLATE^{TM} assay

QA/QC Measures

Positive (CuSO$_4$,1 mg/L) and negative controls (Evian water) were run for each test. For the GFAA blanks, standards, spiked blanks, spiked samples, and duplicate samples were performed after every 10 samples of analysis. These were performed for both acid-digested and undigested forms.

Statistical Analysis

EC$_{50}$ values were calculated separately for each replicate using graphical method (Microsoft Excel) and then were averaged. Normality of EC$_{50}$ values were tested using normal probability plot. Homogeneity of variances was tested via Bartlett's test and Levene's test. Statistical significance among EC$_{50}$ values were tested using one-way ANOVA, followed by Tukey's pair wise comparisons. All statistical tests were carried out using Minitab version 14.

Results

Characterization of Original and Diluted AgNP Suspensions

A detailed explanation of characterization of stock AgNP suspensions has been provided in the results section of the Chapter 2. HDD and average zeta potential of the fourth highest concentration of PVP-AgNPs in the dilution series (2.2 mg/L) were reported as, 11.8 nm and 0.47 mV respectively. At 1.38 mg/L total Ag concentration of citrate-AgNPs, the HDD and average zeta potential were detected as 10.9±0.8 (100%) nm and -1.91 mV respectively. At 1.21 mg/L of total Ag BPEI-AgNPs, HDD and average zeta potential were detected at 25±2.7 (100%) nm and 6.05 mV, respectively. Any concentration higher than mentioned above displayed higher levels of stability than the stability of the above mentioned

concentrations. For all tested types of AgNPs Ag ion concentrations observed in the stock solutions and the highest concentrations used in the series of dilution are displayed in Table 7. The overall Ag ion concentration varied from 6.55-14.7% and 2.39-14.7% respectively for stock solutions and highest concentrations used in the study. For both Citrate-AgNP and BPEI-AgNPs stock solutions itself were used as the highest concentrations of the dilution series. Therefore, to understand the concentration of Ag ion upon dilution, citrate-AgNP and BPEI-AgNPs were diluted in Evian water. The resultant Ag ion concentrations were 0.064% and 5.86% for citrate-AgNP and BPEI-AgNPs respectively, indicating a decline of Ag ions than those observed in stock AgNP suspensions. Thus, the order of Ag ion concentrations resulted for the suspensions which were used in the test can be given as BPEI-AgNPs>Citrate-AgNPs> PVP-AgNPs.

Toxicity Effects

The percentage mean inhibition values observed throughout the series of dilution for each AgNP type are displayed in Figure 6. Photographs of the MetPLATEs with resulted color intensities obtained for each type of AgNP and $AgNO_3$ are shown in Figure 13 (Appendix K). The results demonstrated the presence of 2- or 3-phase dose-response relationships in MetPLATE *E. coli* on exposure to AgNP suspensions. The 3 phases can be referred to as, the first inhibitory phase, induction phase, and second inhibitory phase, following the descending order of total Ag concentration. PVP-AgNP and BPEI-AgNPs displayed a 3-phase inhibitory scenario, while citrate-AgNPs displayed only the 2-phase inhibitory scenario (first inhibitory phase and induction phase, with decrease of total Ag concentration) (Figure 6). The EC_{50} values were calculated considering only the first inhibitory phase for all types of AgNPs. The Calculated EC_{50} values and statistically significant pairs are displayed in Table 6.

Table 6 EC$_{50}$ values reported from MetPLATE toxicity assay

Tested species	EC$_{50}$ (µg/L)			
	1	2	3	Mean* ± SD
AgNO$_3$	214	235	314	254 ± 52 [a]
Citrate-AgNP	1340	1350	1140	1270 ± 110 [b]
PVP-AgNP	1800	1650	1731	1727 ± 75 [c]
BPEI-AgNP	3700	210	360	310 ± 88 [a]

EC$_{50}$ values have been calculated for each type of AgNP based on the inhibition values observed in the 1st inhibitory phase (Figure 6). Means are given with the respective standard deviations (SD).

*Means with different superscripts are significantly different and vise versa.

Table 7 Percentage Ag ion concentrations observed in the original stock AgNP suspensions and the highest concentrations used in the MetPLATE toxicity test (Ultracentrifugation technique)

Type of nano Ag	Total Ag concentration (µg/L)	Stock AgNP suspension		Highest concentration used in the MetPLATE toxicity test	
		% Ag ion	Concentration of Ag ions (mg/L)	% Ag ion	Concentration of Ag ions (mg/L)
Citrate-AgNP	14.9±1.15	6.55	0.98	6.55 [c]	0.98
PVP-AgNP	43.2±2.94	9.27	4	2.39*	1.03
BPEI-AgNP	8.56±0.74	14.73	1.53	14.73 [c]	1.53

*Dilution was made using Evian water

[c] Stock solution itself used as the highest concentration of the dilution series.
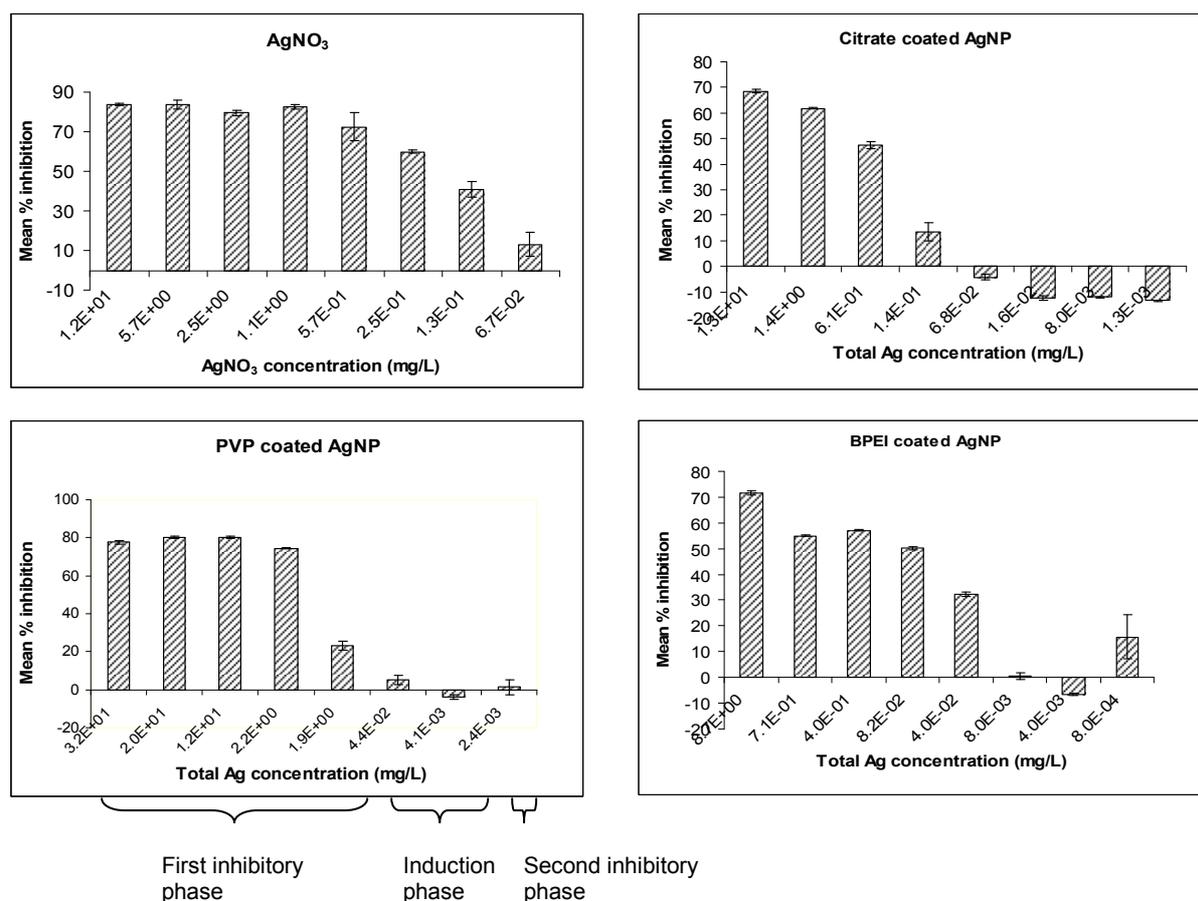
Figure 6 Mean percentage inhibition of NPs with respect to the concentration used

The BPEI-AgNP and PVP-AgNPs were recorded as the highest (310 ± 90 μg/L) and least toxic (1720 ± 110 μg/L) compounds, respectively, among the tested components to MetPLATE *E. coli* bacteria. The EC_{50} value of $AgNO_3$ was recorded as 250 ± 50 μg/L, indicating higher toxicity than PVP-AgNP and citrate-AgNPs. Considering the statistical significant toxicity effects among the 3 types of NPs, citrate-AgNP and PVP-AgNPs were did not exhibit significant difference. However, significant high toxicity effects were observed in BPEI-AgNPs when compared with PVP-AgNP and citrate-AgNPs. The percentage mean inhibition displayed by tri-sodium citrate (1×10^{-2} M), PVP (0.25%), and BPEI (0.5 mM) (calculated concentration of coating material relevant to the highest concentration of AgNP suspension in the array of dilutions) were recorded respectively as -0.35%, 0.4%, and 2%

indicating no notable level of inhibition. Therefore, the toxicity effects explained for the rest of this chapter will not address the effects of individual coating materials.

<div align="center">Discussion</div>

Initial Inhibitory Phase

This study clearly demonstrates the presence of 2 inhibitory phases and an induction phase at non-cytotoxic levels, demonstrating hormesis effect. $AgNO_3$ has shown the highest toxicity among the species tested in the present study and was given the order of toxicity; $AgNO_3$= BPEI-AgNPs> citrate-AgNPs > PVP-AgNPs. So far, no studies have reported on the toxicity of BPEI-AgNP on *E. coli* or any other organism. Kennedy et al. (2010) have performed 48-hour acute toxicity tests to *D. magna* and *Pimephalas pomelas* for citrate-AgNP and PVP-AgNP. They have observed the same trend of toxicity between citrate-AgNPs and PVP-AgNPs that was observed by the present study.

As mentioned in Chapter 2, Ag ions play a significant role in determining toxicity in the MetPLATE test as well. The trend of toxicity has a positive correlation with the Ag ionic fraction. However, the significant difference of observed toxicities among AgNPs and explanation of hormesis followed by second inhibitory phase (described later in this chapter) indicates presence of AgNPs at the concentrations relevant to the first inhibitory phase.

The pH of the citrate-AgNP and PVP-AgNPs throughout the treatments remained in the neutral pH range (6.80-7.44). In BPEI-AgNPs at the first highest concentration, the pH was 8.2 and in other dilutions remained in the range of neutral pH (7.38-7.56). Ionic strengths of the treatments were approximately 5 mM, except for the first 2 highest concentrations of citrate-AgNPs and PVP-AgNPs (0.02 mM) and the first highest concentration in BPEI-AgNPs (0.02 mM). HDD of the first 2 highest concentrations for both PVP-AgNP and citrate-AgNPs and the first highest concentration of the BPEI-AgNPs were detected between

<div align="center">48</div>

10-11 nm. Mean inhibition values decreased in a concentration dependent manner in all types of AgNPs. High mean inhibition values resulted at higher concentrations may be due to the effect of the small size of the particles (Figure 6) (Pal et al. 2007). Badawy et al. (2010) have shown HDD variations of the coated AgNPs at 10 mM and 100 mM ionic strengths. A decrease of toxicity has been reported with an increase of ionic strength for MetPLATE bacteria during exposure to nano Ag (Gao et al. 2009). Regardless of the electrolyte type, the sizes of the citrate-AgNP, PVP-AgNP, and BPEI-AgNPs for other dilutions can be derived respectively as ≤ 10, ≤ 100, and ≤ 10 nm. Thus, the high toxicity observed in the BPEI-AgNP and citrate-AgNPs is directly correlated with the size of the AgNP at the particular ionic strength (Pal et al. 2007, Badawy et al., 2010). Smaller sized NPs have a high surface area that allows the particle to reach or penetrate the bacterium easily. This would enhance the enzymatic inhibition (Pal et al. 2007, Choi et al. 2008) with the eventual death of the bacterium, exhibiting higher toxicity.

Evian water (Table 15) that was used in the test consists of a mixture of electrolytes (Ca^{2+}, Mg^{2+}, Na^+, K^+, HCO_3^-, Cl^-, SO_4^{2-}, NO_3^-, SiO_2) (Mineral waters of the world 2010). AgNPs suspended in the $Ca(NO_3)_2$ electrolyte have shown higher HDD than those suspended in the electrolyte $NaNO_3$ with the same ionic strength. Therefore, the sizes derived above have a huge possibility to deviate, considering the multi-electrolytic composition of Evian water. Further Ag ion concentrations have been reduced when diluting in Evian water, which was not observed when diluting in MHRW (Chapter 2). This may be due to the stabilization of NPs, with the aid of specific types of electrolytes in electrostatically stabilized NPs. However, the phenomenon could not be explained with the existing literature for the electrosterically stabilized NPs.

A high level of interaction exists between positively charged (+28 mV) BPEI-AgNPs and gram negative *E. coli* bacteria (zeta potential -9.86 mV) due to prevailing attractive

electrostatic forces. This may be the cause for the potentially higher toxicity of BPEI-AgNPs than the other 2 types of NPs. In comparison of the citrate-AgNPs with BPEI-AgNPs, citrate-AgNPs (-20 mV) do not tend to interact with *E.coli* bacterium to a similar extent as BPEI-AgNPs, due to the repulsive forces mediated by the opposite charges. The same conditions also apply during PVP-AgNPs and bacterium interaction. Badawy et al. (2011) have clearly demonstrated the charge dependent toxicity of differentially coated AgNPs towards negatively charged *Bacillus* species, revealing the BPEI-AgNPs as the most toxic type among the tested components. Thus, the present study suggests the toxicity induction of BPEI-AgNPs is predominantly charge dependent rather than size.

Choi et al. (2008) have shown cell wall pitting of nitrifying bacteria, enhancing the cell instability. Sondi and Sondi (2004) have demonstrated penetration of nano Ag through outer and inner membranes of the *E. coli* cells via scanning electron micrograph images. Proteomic analyses have also suggested an accumulation of envelope protein precursors, indicating AgNP approach to the cell membrane (Lok et al. 2005).

Hormesis Followed by Secondary Inhibitory Effects

Interestingly, this study demonstrates enzymatic induction of *E. coli* bacteria at non-cytotoxic doses for all AgNP types tested. This may be due to hormesis effect characterized by stimulation of organisms on exposure to low doses of environmental toxic agents. The phenomenon is evident on exposure to metal ions. Low levels of Cu^{2+}, Cd^{2+}, and Cr^{3+} demonstrated the hormetic effect on many types of freshwater luminescent bacteria (Shen et al. 2009). Evidence exists specifically on Ag ion hormesis effect. As an example, Onuki et al. (2007) have observed enhanced growth of human leukemic cells upon exposure to low doses of Ag ions. Kawata et al. (2009) have observed an induction of human hepatoma cell proliferation at low concentrations of polyethyleneimine stabilized AgNP exposure. Fabrega

et al. (2009) have shown an induction of *Pseudomonas fluorescens* cultures on exposure to low concentrations (at 20 and 200 ppb total Ag concentrations) of citrate-AgNPs at pH 9 and 6 at 3 hours after exposure.

Percentage induction exhibits a negative correlation with the total Ag concentration (Figure 6). This may be due to enhanced induction at very low concentrations of total Ag (AgNP and Ag ion counterparts) resembling the hormesis effect. At very low concentrations the exact concentration of stabilizing agent may significantly reduce to a level where the colloidal disaggregation is favored (Daniel and Astruc 2004, Rozenberg and Tenne 2008). At this point induction followed by inhibitory effects can be expected with the subsequent release of Ag ions due to electrostatic and electrosteric instability of AgNPs. However, observed percent inhibition may be a cumulative effect of both ions and the NPs, where the majority is the ionic counterpart.

Serial release of Ag ions beyond the induction phase may result in an accumulation of Ag ions higher than the threshold concentration, exhibiting toxic effects or inhibiting the bacterial cells (second inhibitory phase). This phenomenon was observed in the BPEI-AgNP and PVP-AgNPs. At the concentrations used in the Citrate AgNPs, the second inhibitory phase was not observed. No correlation or coating specific relationship could be derived based on the induction and second inhibitory phase. Therefore, it is obvious that the effects of the second inhibitory phase solely account for Ag ions released out from disaggregation of AgNPs. Fabrega et al. (2009) have shown similar results; an induction phase followed by a second inhibitory phase (second inhibitory phase) at very low concentrations of AgNP exposure (2 ppb, pH 9). Both the induction and second inhibitory phases indirectly confirms the presence of AgNPs in the dilutions relevant to the first inhibitory phase, as well as the presence of AgNPs in the induction phase (see the explanation of induction and 2nd inhibitory

phases). The study also suggests non-persistence of AgNPs in natural waters due to release of Ag ions or formation of ionic complexes with facilitated dilution.

Thus, the study affords a new area of research on AgNPs in relation to their behavior and fate in natural waters. Further studies are needed to confirm the low concentration of AgNP induced effects with *E. coli* and other types of natural organisms in various environmental conditions and with use of other types of tests to better understand the toxicity. These would inevitably contribute to recognize the behavior of NPs and the governing mechanisms of toxicity.

The protocol of MetPLATE toxicity test uses Evian water (moderately hard water). This provides a favorable electrolytic environment for the growth and propagation of the particular strain of *E. coli* bacterium. As mentioned in the results section of the present chapter, NPs did not remain stabilized throughout the dilution series for any of the tested type of NP. This is a main drawback of the use of the MetPLATE assay in NP toxicity assessments.

Calculation of EC_{50} Using Best Fit Regression

Linear regression was used to calculate EC_{50} values as per the MetPLATE protocol. However, the inhibition data appeared well fitted to polynomial or exponential regressions than the linear regression. Thus, an attempt was made to see the difference of the EC_{50} values with best fit regressions and linear regression (i.e., MetPlate protocol). For $AgNO_3$, Citrate-AgNP, and BPEI-AgNP, polynomial equation and for PVP-AgNP exponential equation was found to be the best fit. The EC_{50} values (Table 8) appeared to be much less using the best fit regression than the values obtained from linear regression. Significant difference was observed only between PVP-AgNP and other test components as a group. The trend of the toxicity observed was as, $AgNO_3$ = Citrate-AgNP = BPEI-AgNP > PVP-

AgNP. This different trend of toxicity was observed with the 2 different approaches of calculation, linear and best fit regressions.

Conclusions

In conclusion, coating material dependent toxicity is prominent for AgNPs tested, considering the microbial enzymatic inhibitory effects investigated in the study. Triphasic dose response relationship was observed for the tested organism during AgNP exposure. BPEI-AgNPs was reported as the most toxic type of AgNP to MetPLATE *E.coli* from the types of coated AgNPs tested. Variations of the inhibitory effects are mainly due to the modifications of the surface properties of each type of NP induced during synthesis procedure, which is entirely based on the specific coating material in combination with the size of the NP. Overall high EC_{50} values suggest inapplicability of the MetPLATE toxicity test to assess the aquatic toxicity in the environmental compartments.

Table 8 Comparison of EC_{50} values resulted from best fit regressions and linear regression

Test component	Linear regression		Best fit regression*	
	$R^2 \pm SD$	$EC_{50} \pm SD$ (µg/L)	$R^2 \pm SD$	$EC_{50} \pm SD$ (µg/L)
AgNO$_3$	0.74 ± 0.02	254 ± 52	0.97 ± 0.01	25 ± 5
Citrate-AgNP	0.80 ± 0.01	1270 ± 110	0.99 ± 0.004	9 ± 8
PVP-AgNP	0.75 ± 0.02	1727 ± 75	0.87 ± 0.02	4590 ± 40
BPEI-AgNP	0.86 ± 0.03	310 ± 88	0.94 ± 0.03	3 ± 2

*For AgNO$_3$, citrate-AgNP, and BPEI-AgNP polynomial regression and for PVP-AgNP exponential regression were used as the best fit regressions in EC_{50} calculations.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Summary

Coating material dependent acute toxicity was investigated using 2 aquatic toxicity tests for 3 selected types of AgNPs based on their mechanism of stabilization and surface properties. The study used 48-hr *D. magna* acute toxicity test and MetPLATE test based on a strain of *E. coli*. Both *D. magna* and MetPLATE toxicity tests have shown significantly different toxicity effects for the tested AgNPs following the sequence, BPEI-AgNPs > Citrate AgNPs> PVP AgNPs. Thus, no significant difference regarding the trend of toxicity was detected between multicellular *D. magna* and single cell prokaryote, *E. coli*. However, the cellular arrangements of *D. magna* and *E. coli* are extremely different. *D. magna* bears a eukaryotic structural significance, whereas *E. coli* bears a prokaryotic structure. Various mechanisms have been depicted in literature to understand the toxicity mechanisms governing each tested species.

Kros-flow purification system was used with the 10 kD membrane to purify the AgNP suspension. Therefore, theoretical size of accumulated AgNP in the retentate is considered to be >1 nm. Enhanced solubility has been observed in small sized particles by Terwilliger and Chiang (1995) and Clarke et al. (2001). Therefore, supernatant used after the ultracentrifugation might exist as a fraction of AgNPs, which are small and increased solubility. Therefore, the analyzed Ag ion fraction could be overestimated via the technique used in the present study. Also, when particles reaches the size of 1-10 nm their melting point and sintering temperature decrease significantly, increasing the surface energy of the NP than their bulk counterpart (Ogura et al. 2011). This fraction of NPs may exhibit more toxicity than the >10 nm particles when an organism is exposed. Thus, the relative percentage of

AgNP size within the range of 1-10 nm may significantly impact the overall toxicity of a particular type of NP.

Ag ions were detected via ultracentrifugation technique in the range of 3.08-19.2% and 2.39-14.73% for *D magna* and MetPlate toxicity test respectively. A positive correlation between the exerted toxicity and the resulted Ag ion concentrations was observed. Thus, Ag ions have contributed significantly to the resulted toxicity impacts. Absorbance spectra suggest the presence of AgNPs in the suspensions. Also, the derived HDDs of NPs (dilutions) at test pH, ionic strength, and the stabilization mechanism, had an influence on the toxicity effects. Therefore, the presented toxicity effects can be considered as induced by both Ag ion as well as NP counterparts. Understanding of the relative percentages of the ionic and NP counterparts may aid in interpreting the data successfully and also in identifying the predominant mechanisms of toxicity. Even though there are few acceptable toxicity mechanisms available for bacterial species, no such mechanism is currently recognized to explain the *D. magna* toxicity effects. A 3-phase dose-response relationship was exhibited by MetPLATE *E.coli* bacteria on AgNP exposure. This phenomenon may explain the mechanisms governing disaggregation of AgNPs with serial dilution of the stock suspension that is related to the fate and behavior of the colloidal NPs.

The order of toxicity observed in *D. magna* acute toxicity tests was comparable with published literature for citrate-AgNP (electrostatically functionalized) and PVP-AgNPs (electrosterically functionalized). However, no toxicity studies have been done so far for BPEI-AgNPs (represents both electrostatically and electrosterically functionalized NPs) using both tests. Also, no MetPLATE toxicity test has been performed for any of the 3 types of NPs tested here. The high EC_{50} values derived in MetPLATE toxicity test for *E. coli* affirms the inapplicability of the test for NP-related aquatic toxicity assessment in the environmental compartments. On the other hand, LC_{50} values obtained for *D. magna* toxicity test renders for

an excellent understanding of the toxicity effects in the real world environmental compartments.

The ionic fraction resulted in the stock suspensions decreased when diluted in Evian water for all types of AgNPs. There were 0.097, 25.7, and 39.78% reduction when compared with the stock suspensions for citrate-AgNPs, PVP-AgNPs, and BPEI-AgNPs, respectively. However, when diluted in MHRW the release of Ag ions increased significantly for citrate-AgNPs (293%) and decreased for PVP-AgNPs. For BPEI-AgNPs the percentage remained close to the same fraction that was seen in the stock suspension (Table 9). Badawy et al. (2010) have shown the impact of pH, electrolyte, and ionic strength on NP aggregation mechanisms. The study reported varied Ag ion concentrations at different pH, ionic strength, and electrolyte conditions. This may be resulted due to disaggregation, aggregation mechanisms, or adsorption of Ag ions to AgNP colloids (Liu and Hurt, 2010). When Evian water was used as the diluent, Ag ion concentration were reduced significantly, which can be a result of ion adsorption into colloidal surfaces, whereas the opposite resulted in MHRW, based on the type of NP. Evian water and MHRW have different levels of ionic strengths and different electrolytes in varying levels. These varying conditions in water chemistry combined with the NP specific characteristics might be prevailing in disaggregation and/or aggregation mechanisms of NPs in different diluents. A clear explanation on the mechanisms of release of Ag ions in different dilutents was impossible due to the lack of scientific studies in the particular area.

Conclusions

Coating material dependent toxicity was significant for the *D. magna* and *E. coli* species tested in the present study. Both electrostatically and electrosterically functionalized NPs (BPEI-AgNP) showed a higher toxicity for both organisms than electrostatically (citrate-

AgNP) or electrosterically (PVP-AgNP) functionalized NPs. *D. magna* toxicity test is an excellent test whereas MetPLATE toxicity test is a less sensitive test to perform toxicity assessment of NPs in natural environmental compartments. However, MetPLATE assay can be concluded as a quick, short-term screening test for toxicity evaluation of AgNPs.

Future Work

Coating material specific studies with emphasis on mechanisms of stabilization provide a better understanding of the behavior of NPs in natural environmental conditions. Thus, more studies are needed to explore their aggregation and disaggregation mechanisms in natural environments pertinent to different ionic strengths, pH, and individual and multiple electrolytic conditions. This may contribute to understanding of the aquatic chemistry, fate, and behavior of NPs, which in turn may lead to understanding the toxicity mechanisms. It is crucial to characterize the NP sizes not simply as an averaged value, but also as relative percentages of specific size ranges, because of the size specific toxicity nature of NPs, in order to explain the toxicity mechanisms. Investigation of concentrations of ionic and nano counterparts separately throughout the series of dilution is also imperative to understand the toxicity effects. Also, it is important to investigate long-term (chronic) toxicity effects at low levels of exposure which are evident in the real world environmental compartments in order to investigate the toxicity effects and mechanisms that govern in the real world scenarios.

Table 9 Ag ion concentrations in stock suspension and MHRW, and Evian water diluted scenarios

Type of nano Ag	Total Ag concentration (µg/L)	% Ag ion		
		Stock suspension	Diluted in MHRW	Diluted in Evian water
Citrate-AgNP	14.9±1.15	6.55	19.2	0.064
PVP-AgNP	43.2±2.94	9.27	3.08	2.39
BPEI-AgNP	8.56±0.74	14.73	14.5	5.86

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APPENDICES

APPENDIX A: SILVER NP SYNTHESIS

Citrate Coated AgNP Preparation (Badawy et al. 2010)

Bluish green color colloidal solution was prepared (The HDD of the AgNP=10 nm). $AgNO_3$ solution of 1×10^{-3} M was mixed with 1×10^{-2} M $Na_3C_6H_5O_7.2H_2O$ (99%) in a volume ratio of 2:1 respectively. Mixture was heated for 4 hrs at 70^0C in water bath.

$$4Ag^+ + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^0 + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2\uparrow$$

PVP Coated AgNP Preparation (Badawy et al. 2011)

A 5×10^{-3} M solution of $AgNO_3$ was added drop wisely (1 drop per second) to a vigorously stirred ice cold 2×10^{-3} M $NaBH_4$ solution, dissolved in 1% PVP solution to give a final volume ratio 1:3 respectively.

$$AgNO_3 + NaBH_4 \rightarrow Ag + 1/2\ H_2 + 1/2\ B_2H_6 + NaNO_3$$
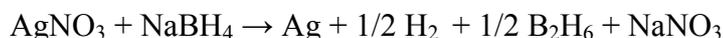
BPEI coated AgNP Preparation (Badawy et al. 2011)

$AgNO_3$ and Branched polyethyleneimine (99%, molecular weight 1.2kg/mol) was dissolved separately with a solution of N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonicacid (HEPES). Both solutions were mixed in a volume ratio of 1:1, for a molar ratio of 0.5:1:0.1, $BPEI:AgNO_3:HEPES$, respectively. The solution was exposed to UV radiation for 2 hours using a standard low pressure Hg arc lamp.
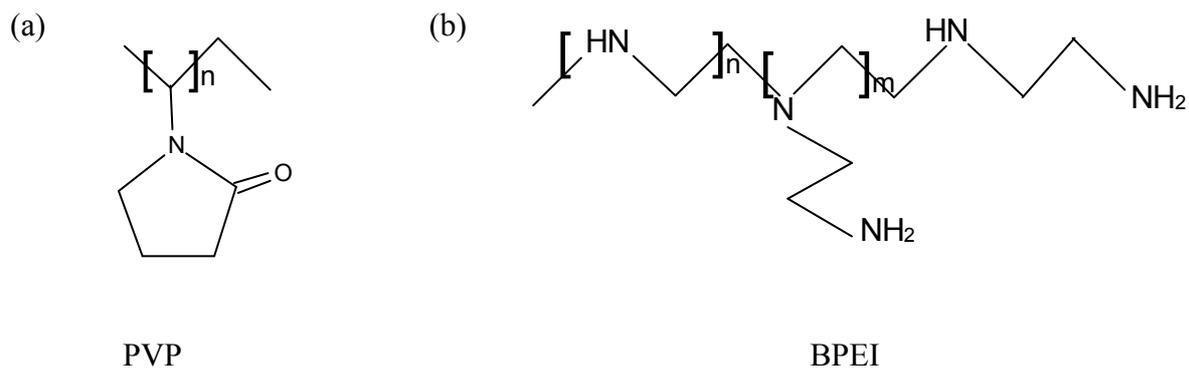
(a)   (b)

PVP   BPEI

Figure 7 Chemical structures of the polymers used in the study

BPEI molecule (Figure 7b) is a polymer, which consists of 'n' and number of –[NH-CH$_2$]-monomer units (Secondary amines) and 'm' number of –[N-CH$_2$]- (tertiary amines) monomer units. Thus, the molecule consists of primary, secondary, and tertiary amine groups providing a positive charge to the molecule. The alkyl moieties (CH$_2$) provide a negative charge to the molecule.
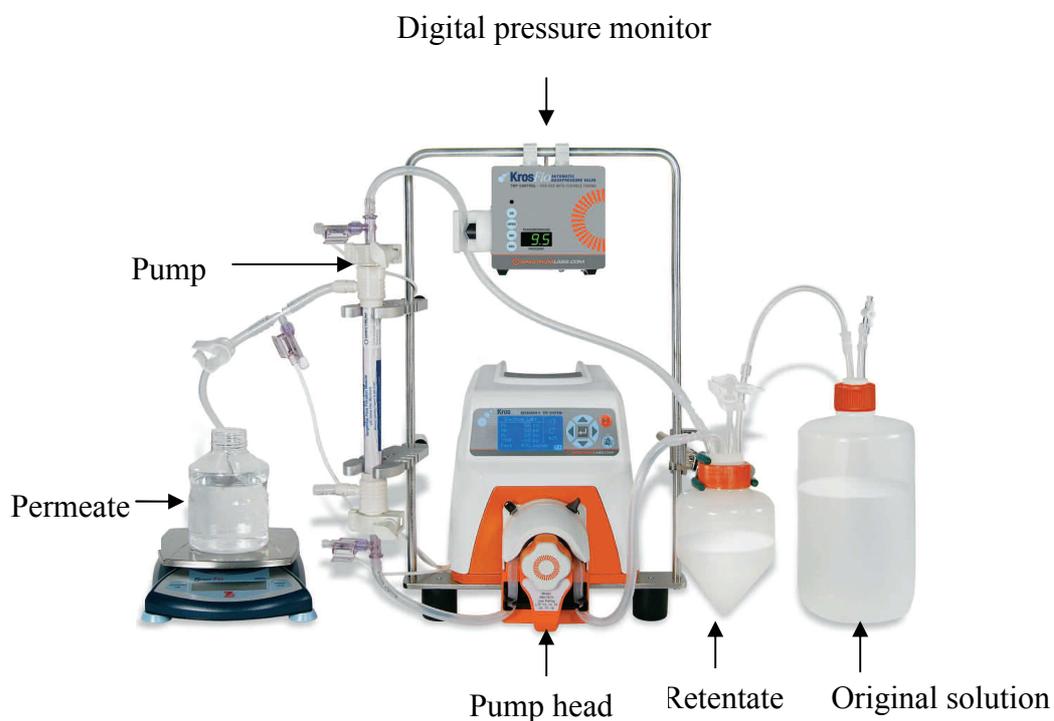
Figure 8 Diagram of Kross flow research IIi TFF system (Source: NICOMP 380 ZLS zeta sizer, 2010)

The Kross Flow Research IIi TFF system consists of pump, pump head, digital pressure monitor, and a disposable flow path (Figure 8). Disposable flow path includes filter, tubing, and pressure transducers. Disposable flow path allows samples to concentrate in the retentate, filtering based on the pore size of the of the ultracentrifuge membrane.
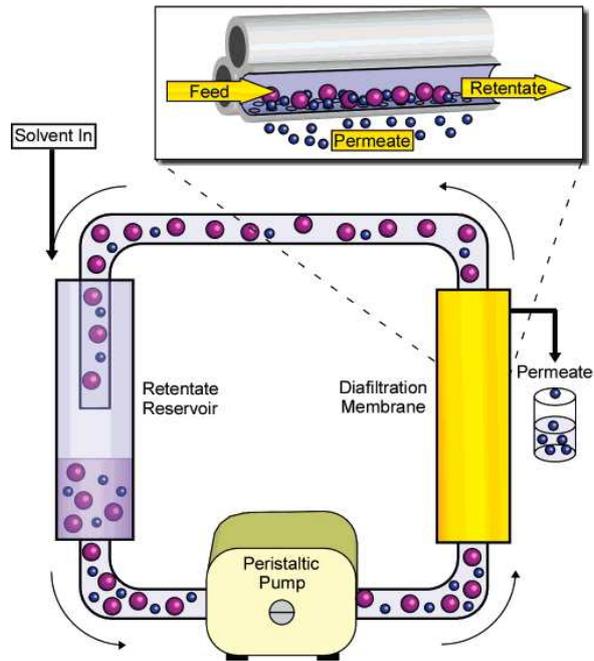
Figure 9 Schematic representation of the flow path of the Krossflow system.
(Source: Sweeney et al. 2006)

Diafiltration allows purification and size separation of the materials in the mother suspension from a single step procedure. Figure 9 illustrates the schematic of the flow path of the solution in a Krossflow system. The sample is introduced and pumped under a constant peristaltic pump rate through the diafiltration membrane. Smaller molecules (blue) than the pore size of the filter are eluted out to the permeate. Larger particles are retained in the retentate. This allows to fractionate the nanoparticles (>1 nm) from the smaller components in the suspension to the retentate.

APPENDIX C: HDD FIGURES OF SILVER NANOPARTICLE TYPES (NICOMP 380 ZLS ZETA SIZER)

Citrate-AgNP

.574	
Diameter(nm) :	#1—10.9 nm
S.Dev.(nm/%) :	0.8 nm (7.0%)
Percent :	100.0 %

PVP-AgNP

.305	
Diameter(nm) :	#1—11.0 nm
S.Dev.(nm/%) :	0.7 nm (6.5%)
Percent :	100.0 %

BPEI-AgNP

REL.   VOLUME-WT NICOMP DISTRIBUTION

t.563

Diameter(nm) :   #1—10.9 nm
S.Dev.(nm/%) :   0.8 nm (7.0%)
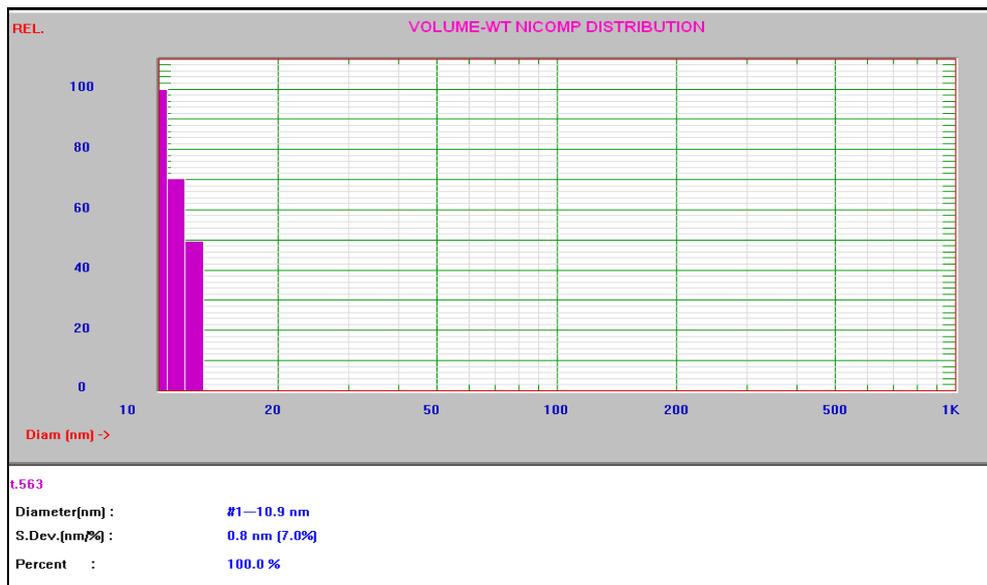Percent   :   100.0 %
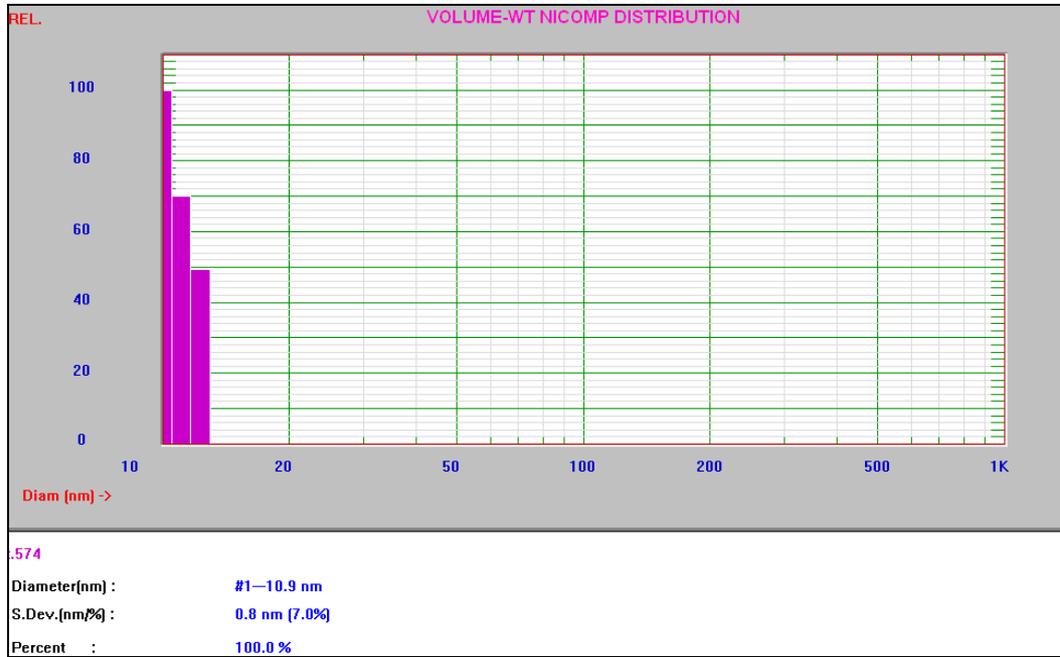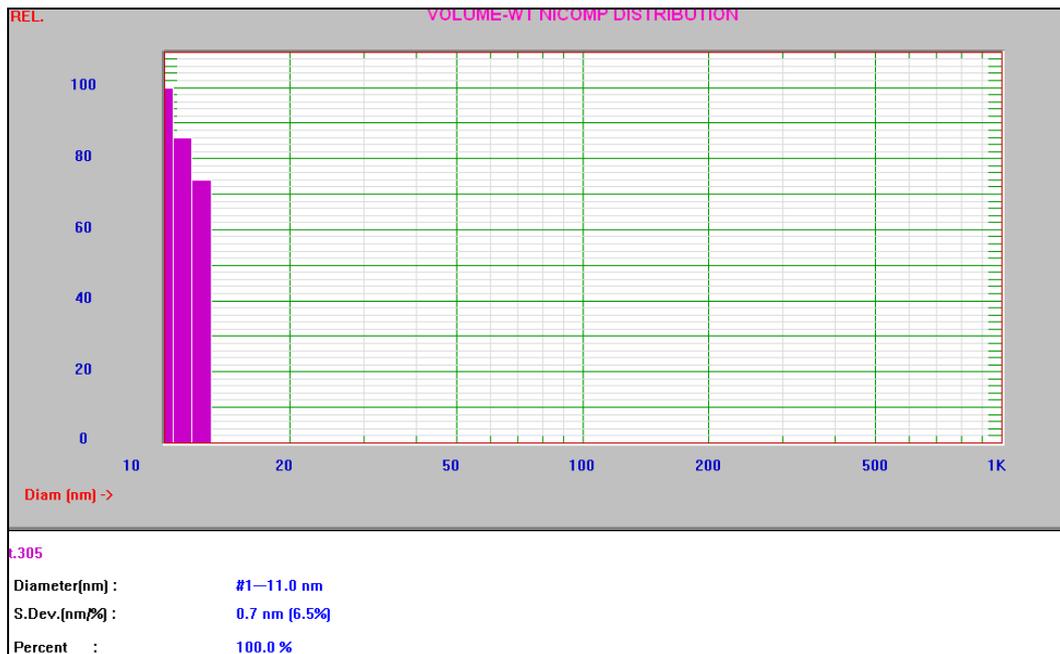
Figure 10 Figures for HDDs of AgNP types tested.

APPENDIX D: ACID DIGESTION (USEPA SW-846 METHOD 3010A)

Apparatus and materials:

1. Griffin beakers

2. Watch glasses

3. Qualitative filter paper/filter funnels

4. Graduated cylinder

5. Hotplate

Reagents:

1. Reagent water

2. Concentrated nitric acid

Procedure:

1. A 100 mL aliquot from the sample was transferred to a beaker.

2. Samples, standards, and properly spiked samples were placed on the hotplates under the hood.

3. A 3 mL of concentrated HNO_3 was added to each beaker and was covered with ribbed watch glasses. The solution was heated to 90^0C-95^0C until the volume was reduced to about 5 mL.

4. A 3 mL of concentrated HNO_3 was added again to the flasks after removing all samples from the hot plate but remained under the hood.

5. Beakers were placed again on the hotplate.

6. Samples were heated until the volumes reduce back to 5mL without allowing the bottom of the beakers become dry.

7. Steps 4-6 were repeated if a brown color or brown fumes are still emitting from the samples.

8. HCl acid was added (10 mL) into each beaker.

9. Beakers were returned back to the hotplate and were heated for 15 min.

10. Digestates of leachates and others were filtered.

11. Digestates were poured into 100 mL volumetric flasks and were brought up to 100 mL using DI water.

APPENDIX E: METAL ANALYSIS USING GFAA (USEPA SW-846 METHOD 7010)

Apparatus and Equipment:

1. Spectra AA 220Z

2. Auto sampler

3. GTA

4. Computer with AA program

5. Glass pipets

6. Sample vials

7. Nanopure water

8. Argon tank

9. Element lamp

10. Volumetric flasks

Calibration procedure:

1. Graphite tube was checked before beginning.

   If the autosampler is connected to the AA, then it was detached. The furnace unit

   proceeded to pull and was tilted. The latch was turned on at the top of the furnace to

   the right to unlock the graphite tube chamber and was opened.

   The graphite tube was carefully removed from the chamber and was checked if it

   needed to be replaced.

   The Tube with the capillary was reinserted to the insertion hole on the graphite tube,

   in the center of the circle of the furnace once you lock the chamber again.

   The autosampler unit was replaced.

2. The proper bulb was installed.

3. The AA, GTA, and the autosampler were turned on.

4. The worksheet was started, and the method was developed based on the metal of interest.

5. "Optimize" under "instruments" was clicked in the menu and clicked "ok" on the message.

6. The lamp was adjusted to get the highest possible reading of the green meter.

7. Necessary adjustments were made to ensure capillary tube on the autosampler can correctly move into designated sample vial and then into the graphite tube insertion hole.

8. "Align" was hit and watched the capillary arm rotate around to the graphite tube opening. The capillary tube was adjusted necessarily.

Chemicals and reagents:

1. Element standard

2. Element standard solutions for the calibration

3. 0.1% nitric acid-used as the blank material for the AA.

Procedure:

1. Spectra AA program was started. The worksheet was opened and then the method was developed.

2. GTA and AA were turned on and made sure the specific lamps for the analytes measuring are correctly installed.

3. Analysis tab was clicked and then was clicked the instrument menu in the top toolbar of the program. "Furnace facilities" was clicked. It was proceeded to do a rinse and tube condition to remove contaminants from the graphite tube. Window was closed.

4. Labels were renamed in the "Labels" section corresponding to the samples have.

5. "Analysis" was clicked and then "Start" was clicked to begin running samples. After all samples were analyzed, the furnace was turned off by turning off the water, gases, and finally the AA itself.

6. Worksheet was saved.

APPENDIX F: QL AND MDL DETERMINATION

Graphite Furnace Atomic Absorption Spectroscopy

GFAA was calibrated using 5 standards: 0, 10, 20, 30, and 40 µg/L.

A 5µg/L Ag standard was separately prepared and the reading was taken 8 times for the same standard.

Table 10 Concentrations obtained for the 5 µg/L standard at 8 different times of the same run.

Sample	Results for 5 µg/L nominal concentration (µg/L)
1	5.04
2	4.89
3	5.18
4	5.68
5	5.12
6	4.9
7	4.81
8	5.55
Mean	5.15
Standard deviation (s)	0.296

MDL and QL were calculated as follows.

t value $= t_{0.01, n-1}$ $= t_{0.01, 7}$ $= 2.998$ (at 99% confidence)

MDL $= s * t$ value $= 0.8876$ µg/L

QL $= 10 * s$ $= 2.9607$ µg/L

Sample	GFAA reading (µg/L)	spike % recovery
Undigested Blank	Under MDL Under MDL Under MDL	
Undigested Ag standard 10µg/L	9.056 9.695 9.899	
Undigested Sample AgNO$_3$ 5µg/L	1.866 1.791 1.477	
Undigested Spiked Blank (Blank 25mL+10ug/L Ag standard 25mL)	4.603 4.317 4.576	92.06 86.34 91.52
Undigested Sample spiked (AgNO$_3$ 5µg/L 5mL+ 45mL Ag standard 10ug/L	7.451 7.083 7.282	82.79 78.70 80.91
Digested Blank	Under MDL Under MDL Under MDL	
Digested Ag 10 µg/L standard	9.574 10.735 10.937	
Digested sample AgNO$_3$ 5 µg/L	1.748 1.926 1.362	
Digested spiked blank (Blank 25 mL+10 µg/L Ag standard 25mL)	4.562 4.277 4.651	91.24 85.54 93.02
Digested sample spiked (AgNO$_3$ 5 µg/L 5mL+ 45mL Ag 10ug/L standard)	7.636 7.309 7.133	84.84 81.21 79.26
Undigested sample citrate coated AgNP dilution 10^{-3}	2.832 2.979 2.518	
Undigested Sample citrate coated (total Ag 14.9 µg/L) (1mL) + 10 µg/L Ag standard (1mL)	6.007 6.354 6.646	79.48 84.98 89.62
Dige. Sample citrate AgNP dil. 10^{-3} (total Ag 14.9 µg/L)	18.598 18.635 17.291	
Digested Sample citrate AgNP dil. 10^{-3} (total Ag 14.9 µg/L) (1mL) + 10µg/L Ag standard (1mL)	14.157 14.956 14.489	113.71 120.13 116.38

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APPENDIX H: MEASUREMENT OF TOTAL HARDNESS (HACH 8213)

Materials and Apparatus

1. Erlenmeyer flasks

2. Magnetic stirrer

3. Graduated cylinder (100mL)

4. Digital titrator

5. Delivery tube for the digital titrator

Reagents (Provided by HACH Company):

1. ManVer 2 Hardness indicator powder pillow

2. Hardeness 1 buffer solution

3. EDTA titration cartridge

Procedure:

1. A 100mL of the sample was added to the Erlenmeyer flask.

2. A Clean delivery tube was inserted into the titration cartridge and the cartridge was attached to the titrator.

3. The Delivery knob of the titrator was turned to eject air and few drops of EDTA and was reset to zero.

4. Two mL of hardness 1 buffer solution was added and swirled to mix.

5. Contents of the ManVer 2 hardness indicator was added and mixed.

6. Delivery tube was placed in the solution and the knob of the titrator was turned on to add the titrant drop wisely into the solution.

7. Solution was swirled via a magnetic stirrer until the color changes from red to purple.

8. The number on the titrator was noted down. If used 100 mL of the sample the hardness (mg/L $CaCO_3$) equals to the number displayed on the counter.

APPENDIX I: MEASUREMENT OF AMMONIA-NITROGEN LEVEL (HACH 8155)

Materials and apparatus:

1. Sample cells and stoppers

2. DR 5000 UV-vis spectrophotometer

Reagents (Provided by HACH Company):

1. Ammonia cyanurate reagent pillows

2. Ammonia salicylate reagent pillows

Procedure:

1. Sample was added up to the 10 mL mark of the cell.

2. For the blank sample preparation, 10 mL of deionized water was added to another sample cell.

3. Contents of Ammonia salicylate powder was added to each cell.

4. Stopper was inserted to the cell and was shaken to dissolve. This was kept for 3 min until the reaction occurs.

5. Contents of the powder pillow Ammonia cyanurate was added to the cell after 3 min incubation to each cell.

6. Cells were capped and shaken to dissolve.

7. Cells were kept for 15 min until the reaction occurs.

8. At the end of the 15 min zero the UV-vis spectrophotometer (stored program – salicylate method) using the blank cell.

9. Sample cell was placed in the cell holder and was read the results in mg/L NH_3-N.

APPENDIX J: *DAPHNIA MAGNA* TOXICITY TEST

<u>*Daphnia magna* Culture Maintenance and Food Preparation</u>

<u>Culture Medium</u>

MHRW (total hardness of 80-100 mg/L as $CaCO_3$) was used for *D. magna* culturing,

Illumination

- Minimum illumination of 16 h/day was provided

Culture Vessels

- Each of 750 mL volume clear glass vessels/beakers were used to culture *Daphnia.*

- A batch of 20-30 organisms was stocked in each culture vessel.

- After the culture was established, each chamber was cleaned 3 times per week with deionized water.

Figure 11 Preparation of brood chambers day prior the test performance.

Moderately Hard Reconstituted Water (MHRW) preparation

Standard synthetic water was prepared using de-ionized (DI) water by adding the chemicals as mentioned in the Table 12.

Table 11 Preparation of moderately hard reconstituted water using chemicals.

Reagent added mg/L				Approximate final water quality		
$NaHCO_3$	$CaSO_4.2H_2O$	$MgSO_4$	KCl	pH	Hardness	Alkalinity
96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64

Feed preparation

Digested trout chow (or flake food):

1. A 5.0 g of trout chow pellets or flake food was added to 1 L of DI water and was stirred overnight.

2. Supernatant was filtered through a mesh screen and used as the feed.

Algal (*Selenastrum*) Food Culture

Algal Culture Medium

1. Five stock nutrient solutions were prepared using chemicals as described in Table 13.

2. From each stock solution, 1 mL was added to approximately 900 mL of DI water and was mixed well after the addition of each solution. Then it was diluted up to 1 L.

3. The medium was sterilized by autoclaving after it was placed in the culture vessels if sterile conditions were not applied during preparation.

Table 12 Nutrient stock solutions for maintaining algal stock cultures.

Stock solution	Compound	Amount dissolved in 500 ml DI water
1. Macronutrients		
A	$MgCl_2.6H_2O$	6.08 g
	$CaCl_2.2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B	$MgSO_4.7H_2O$	7.35 g
C	K_2HPO_4	0.522 g
D	$NaHCO_3$	7.50 g
2. Micronutrients	H_3BO_3	92.8 mg
	$MnCl_2.4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg
	$FeCl_3.6H_2O$	79.9 mg
	$CoCl_2.6H_2O$	0.714 mg
	$Na_2MoO_4.2H_2O$	3.63 mg
	$CuCl_2.2H_2O$	0.006 mg
	$Na_2EDTA.2H_2O$	150.0 mg
	Na_2SeO_4	1.196 mg

Algal Cultures

Two types of algal cultures were maintained: (1) stock cultures (2) "food" cultures.

All handling and maintaining procedures of algal cultures were carried out aseptically.

Establishing and Maintaining Stock Cultures of Algae:

1. Starter culture (3 mL) was aseptically transferred to 3 L of culture medium that contains in algal culture bags.

2. The remainder of the starter culture can be held in reserve for up to 6 months in a refrigerator (in the dark) at 4°C.

3. Cultures were maintained with the illumination of approximately 86 ± 8.6 µE/m2/s, or 400 ft-c (continuous "cool-white" fluorescent lighting).

4. Seven-day old algal stock cultures (about 1-3 mL) were transferred (approximately 1.5×10^6 cells/mL) to 100 mL of fresh culture media. The inoculum should be in an initial cell density of 10,000-30,000 cells/mL in the new stock cultures.

5. Cultures were tested microscopically weekly, at transfer, for microbial contamination.

6. Upon reaching the expected level cell density, cultures were refrigerated until use.

7. Algal suspension (1-2 mL) was fed daily for 750 mL of the *Daphnia* medium.

Figure 12 Algal cultures maintained in the laboratory.

Analytical Results of *D. magna* Toxicity Tests

Table 13 Analytical Concentrations obtained for the concentrations* used in the final tests of *D. magna* test.

Component	Dilution	Concentration (μg/L)			
		Before	After		
			Replicate concentration	Mean	Standard deviation
PVP -AgNP	1:100	407.3	402.89	395.60	8.43
			397.55		
			386.37		
	1:1000	39.12	39.40	40.29	1.18
			39.83		
			41.64		
	1:2000	22.06	20.14	21.15	1.02
			21.12		
			22.19		
	1:10^4	4.39	3.98	4.32	0.82

			5.26		
			3.72		
Citrate -AgNP	1:100	140.96	135.62	138.92	3.10
			141.76		
			139.38		
	1:1000	14.71	14.75	14.06	0.64
			13.51		
			13.90		
	1:2000	8.26	7.40	7.22	0.27
			6.90		
			7.35		
	1:4000	3.02	3.06	2.98	0.41
			3.34		
			2.54		
	1:10^5	1.33	1.27	1.29	0.04
			1.26		
			1.34		
BPEI- AgNP	1:1000	7.54	8.08	8.558	0.699
			8.23		
			9.36		
	1:2000	3.72	4.18	4.25	0.07
			4.24		
			4.32		
AgNO$_3$ (Nominal concentration)	10µg/L	5.81	5.75	5.85	0.24
			5.68		
			6.13		
	5µg/L	2.85	3.21	3.26	0.08
			3.35		
			3.21		

*Only the concentrations above the MDL of GFAA have shown here. For the concentration below the MDL, nominal concentrations were used based on the maximum concentration of the dilution series.

APPENDIX K: METPLATE TOXICITY TEST

MetPLATE Test - Materials and Procedure

Metplate kit contains,

- bacterial reagent, freeze dried chromogenic substrate, diluent, buffer, positive control

Apparatus and Materials

- 96 well microplate

- Test tubes with caps

- Pipettors with tips

- Incubator

- Microplate reader

- pH meter

- Nanoparticle solution

Procedure

Reagent Preparation

Preparation of Chromogenic Substrate

- Buffer (10 mL) was added to the chromogenic substrate vial.

- The Chromogenic substrate was then hand-shaken or vortexed for approximately 10 seconds.

Preparation of Bacterial Reagent

- Diluent (5 mL) was added to the vial containing the Bacterial reagent.

- The bacterial reagent was hand-shaken or vortexed for approximately 30 seconds to obtain a uniform suspension.

- Reconstituted bacterial reagent was incubated at room temperature (18°C -25°C) for 15 minutes prior the performance of the toxicity assay.

MetPLATE toxicity assay:

The pH of the sample was tested.

Samples with pH value beyond the range 5.0-7.5 were adjusted by adding NaOH or HCl.

A complete toxicity test includes the undiluted sample in triplicate, 4-5 sample dilutions in triplicate, and controls in triplicate (positive, negative, and blank).

1. Reconstituted bacterial reagent (0.1 mL) was added to tubes containing 0.9 ml of the tested sample.

2. Each test sample was capped and was vortexed for 10 seconds.

3. To prepare the negative controls, 0.1 mL of reconstituted bacterial reagent was added to 0.9 ml of diluent. Tubes were labeled, capped, and vortexed for 10 seconds.

4. To prepare the positive control, 0.1 mL of reconstituted bacterial reagent was added to 0.9 mL of positive control to clean glass tubes. Tubes were labeled, capped, and vortexed for 10 seconds.

5. All the tubes were incubated at 35°C for 90 minutes in an incubator.

6. After incubation, 0.2 mL aliquots from each test tube were pipetted onto the microplate and 0.1 mL of the reconstituted chromogenic substrate was added to each test well.

7. Microplate was incubated at 35°C until color develops in the negative control wells which should read an absorbance of approximately one within 0.5 - 2 hours incubation period.

8. After the incubation period, intensity of the red/purple color was observed, which indicates the level of enzyme activity. The sample will remain yellow in the positive

control or in the presence of a very toxic sample. The sample will turn red/purple in

the negative control and in the non-toxic or slightly toxic samples.

9. Color Intensity was determined by measuring the absorbance at 575 nm using a

   microplate reader.

10. EC_{50} was calculated using graphical method.
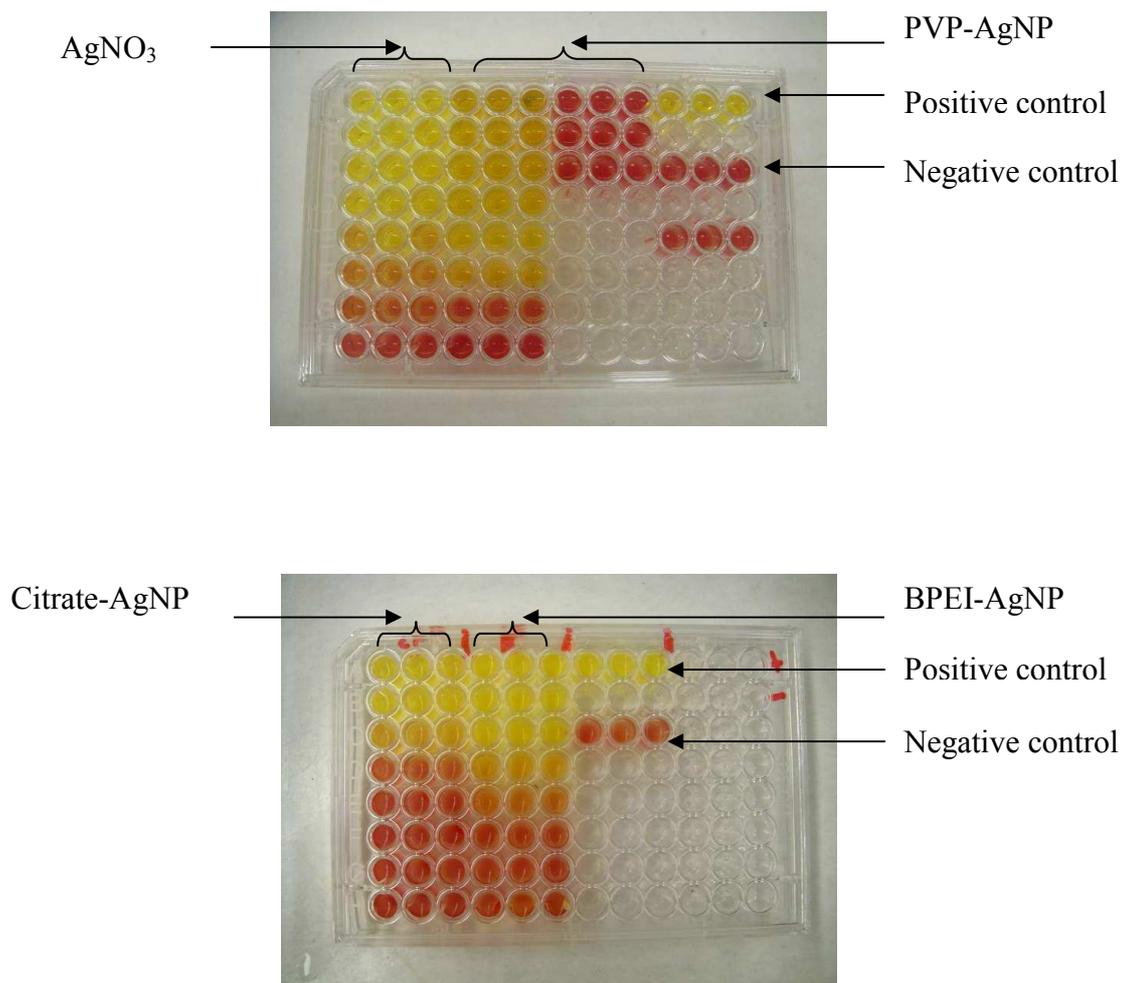

Figure 13 Microplate color intensities resulted for the test components.

Composition of Evian Water

Table 14 Major components and several important characteristics of Evian water.

(Source: Mineral waters of the world, 2010)

Parameter/ electrolyte	Quantity
Gas (CO_2)	N/A
pH	7.2
Conductivity	560 µS/cm
Total dissolved solids	309 mg/L
Ca^{2+}	78 mg/L
Mg^{2+}	13.5 mg/L
Na^+	5 mg/L
K^+	1 mg/L
HCO_3^-	357 mg/L
Cl^-	4 mg/L
SO_4^{2-}	10 mg/L
NO_3^-	3.8 mg/L
SiO_2	13.5 mg/L

EC_{50} Calculation Using Graphical Method (Linear Regression)

1. Percent inhibition level (derived using the absorbance values of negative control and the test samples obtained from the microplate reader) was plotted against the log concentrations of the total Ag analyzed (ICPMS) to obtain a linear relationship.

2. EC_{50} was calculated based on the following equation.

   $EC_{50} = (\log^\wedge(\log 50 - Intercept)) \div Slope$

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