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Fate and Behavior of Silver Nanoparticles to the Estuarine Organisms, *Prorocentrum minimum* and *Crassostrea virginica*

by

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Coastal Carolina University, 2013

Submitted in Partial Fulfillment of the Requirements

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ABSTRACT

Nanoparticles are known to be entering the environment and are shown to have adverse effects on aquatic organisms (Fabrega et.al. 2011, Lapresta-Fernandez et.al. 2012, Matranga and Corsi 2012, Moore 2006). The purpose of this study was to quantify the uptake of AgNPs by two estuarine organisms and determine the associated toxic effects of accumulation. There are multiple procedures for nanoparticle (NP) synthesis that involve various core and surface materials, which result in various characteristics such as size, shape, and surface coating. In this study silver nanoparticles (AgNPs) were synthesized by a chemical reduction of silver salts and characterized in stock solution and saltwater algal growth media (L1-Si) using a multi-methodological approach. Dynamic light scattering (DLS) and ultraviolet visible spectrometry (UV-vis) NP characterization resulted in an average size of 11.2 nm for citrate coated AgNPs (cit-AgNPs) and 17.6 nm for polyvinylpyrrolidone (pvp) coated AgNPs (pvp-AgNPs). Transmission electron microscopy (TEM) determined NP size of 14.0 ± 1.7 nm and 14.8 ± 5.7 nm (mean ± 1 s.d.), for cit-AgNPs and pvp-AgNPs respectively. DLS and dissolution analysis showed that cit-AgNPs aggregated in the saltwater media, while pvp-AgNPs remained mono-dispersed in the media as indicated by a polydispersity index (pdi) value of 0.2-0.4.

When exposed to the concentrations of 50-100 ppb, cell abundance of *Prorocentrum minimum* decreased, with observed LC_{50} values of 61.9 ppb (cit-AgNPs)

and 59.1 ppb (pvp-AgNPs). *Crassostrea virginica* exposures showed concentration and tissue dependent responses for silver (Ag) accumulation, with a direct relationship between exposure concentration and Ag accumulation. Lipid peroxidation assays indicated a significant difference from the control in the 15 and 50 ppb exposures for cit-AgNP and $AgNO_3$ treatments and no toxic effect was observed in the 1 and 10 ppb exposures of all treatments. Examining AgNP toxicity on the level of a primary producer and primary consumer are extremely important in that these trophic levels are the base of multiple food webs in the marine and estuarine environment. An inhibition of abundance decreases the population size of the exposed or affected species.

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LIST OF ABBREVIATIONS

Ag ..... Silver

AgCl ..... Silver chloride

$AgNO_3$ ..... Silver nitrate

AgNP(s) ..... Silver Nanoparticle(s)

BFSW ..... Baruch filtered seawater

Cd ..... Cadmium

Cit-AgNP(s) ..... Citrate coated silver nanoparticles(s)

DI ..... De-ionized water

DLS ..... Dynamic light scatters

DLVO Theory ..... Derjaguin and Landau, Verwey and Overbeek Theory

EC_{50} ..... Half-Maximum effective concentration

HCl ..... Hydrochloric acid

ICP-MS ..... Inductively coupled plasma mass spectrometry

ICP-OES ..... Inductively coupled plasma optical emission spectrometry

K_2PO_4 ..... Potassium phosphate

LPx ..... Lipid peroxidation

MDA ..... Malondialdehyde Tetraethylacetal

Na^+ ..... Sodium

$NaBH_4$ ..... Sodium borohydride

NP(s) .....Nanoparticle(s)

Pb .....Lead

PEG .....Polyethylene glycol

PEI.....Polyethylene imine

pdi .....Polydispersity index

pvp..... Polyvinylpyrrolidone

pvp-AgNP(s) ..... Polyvinylpyrrolidone coated silver nanoparticle(s)

TBA.....Thiobarbituric Acid

UV-vis .....Ultraviolet visible spectrometry

Zn ..... Zinc

INTRODUCTION

Nanoparticles (NPs) are particles within the 1-100 nm size range which exhibit novel size dependent properties. NPs possess electron spatial constraints, a large specific surface area, small size, and high surface energy. These properties give rise to unique environmental behaviors, such as toxicity increasing with surface area and dramatic changes in crystalline structure that enhances surface reactivity (Aufann et al. 2009; Croteau et al. 2011). Nanomaterials are currently used in electronic, biomedical, and pharmaceutical drug design, cosmetics, nutrition supplements, energy applications, and clothing manufacturing, (Nowack and Bucheli 2007; Piccinno et al. 2012). NPs are known to be introduced into the environment through runoff. Direct inputs come from the use of zero valent NPs for groundwater remediation of organic pollutants and metals (Cho et al. 2015) as well as applications for water treatment and disinfection purposes (Yang et al. 2013; Kakavandi et al. 2014). As a result of increased use and introduction to the environment, the U.S. National Nanotechnology Initiative (NNI) directed 5% of their yearly budget (over $800 million) to research on nanoscale processes in the environment as well as the environmental and social implications and consequences of nanotechnologies (Wiesner et al. 2006; Lin et al. 2012).

Generally NPs are synthesized by physical and chemical methods including chemical and photochemical reductions, or hydrothermal and electrochemical techniques (Frattini 2005). Each synthesis method involves various core materials such as gold, silver, zinc,

iron, or cerium as well as capping agents such as polyethylene glycol (PEG), oleic acid, sodium citrate, polyvinylpyrrolidone (pvp), or amino acids (Singh et al. 2009; Tejamaya et al. 2012; Maruyama et al. 2014). For example zinc oxide NPs have been synthesized by an alcohol based hydrothermal stability method that can use up to three different capping agents including triethanolamine, oleic acid, and thioglycerol (Singh et al. 2009). Another method combines crystalline zinc with citric acid and pvp simultaneously as capping agents in a low heat chemical process (Yogamalar et al. 2009). Iron oxide NPs can be synthesized by thermal decomposition of iron (III) using PEG, pvp, or polyethylene imine (PEI) (Zhang et al. 2013). The most widely used method for gold NP synthesis is the Turkevich reduction method, which uses $AuCl_4$ as a core material and sodium citrate as the capping agent (Turkevich et al. 1951). Other methods include the use of amino acids, peptides, and proteins as capping agents and are classified as "green" synthesis methods, as they satisfy the global efforts to reduce hazardous waste (Sharma et al. 2009; Maruyama et al. 2015).

Capping agents aid in surface chemistry alteration by binding to the core of the NP to form the surface material and therefore controls NP size and shape, as well as stabilize the highly reactive surface to prevent dissolution or aggregation. Depending on the material used, the bound molecules can be stabilized through electrostatic repulsion, van der Waals attraction, or steric stabilization (Lead 2009). The small size, high specific surface area, and high surface energy of NPs cause instability that promotes a tendency to aggregate for stabilization (Lowry et al. 2012). The addition of a reducing agent, which caps the particle during synthesis, can help to reduce the affinity to aggregate or dissolve in solution. When using a capping compound that creates a sterically stable particle such

as pvp the NPs are more stable and remain mono-dispersed in saline solution. Conversely, when using a compound that creates a charge stable particle, such as sodium citrate, the particles tend to aggregate in saline solutions thus causing them to fall out of suspension or be taken up by the organism in agglomerates outside of the nano-size range. The difference in aggregation affinity is controlled by the Derjaguin and Landau, Verwey and Overbeek (DLVO) theory and Brownian processes.

The DLVO theory investigates the repulsive and attractive forces between particles and predicts their stability in suspension. This theory is based on the assumption that the total interaction energy between two particles is composed of only electrostatic double-layer interactions (repulsive) and the van der Waals interaction (attractive) (Bargozin et al. 2015). The balance of these two forces determines the stability of NPs. Van der Waals interactions are dependent on the size and material of the NP while electrostatic forces are related to the charge of the particle in media (Israelachvili 2011). If the charge is strong enough to overcome attractive forces the NPs will repel each other, if the charge is weak or ions of the opposite charge are present in media then aggregation will occur (Levard et al. 2012). Cit-AgNPs, being charge stable are more likely to aggregate in saline solutions. Salts screen the electron double-layer, allowing particles to get closer and the attractive van der Waals interactions to take over, resulting in agglomeration (Israelachvili 1997). Pvp-AgNPs being sterically stable are thermodynamically unfavorable to aggregate. In order for agglomeration to occur the H_2O molecules and polymer (pvp) would have to be separated, from an energy standpoint it is not beneficial to remove the polymer chain.

The random motion of particles in solution (Brownian motion) and collisions with also enhance aggregation. Solutions with higher ionic strength (i.e., more salts and particles) result in higher aggregation or formation of Cl^- species, than solutions with lower ionic strength (Li et al. 2010). Strong ionic solutions in the environment will increase aggregation of AgNPs through destabilization and increased collisions between particles (Chinnapongse et al. 2011; Huynh and Chen 2011). The different effects of charge and sterically stable NPs are important aspects and must be investigated in order to better understand the environmental impact of NPs.

Silver nanoparticles (AgNPs) are currently one of the main types of NPs used in consumer products due to their strong antimicrobial properties. According to the Woodrow Wilson Center on Emerging Nanotechnologies (2014) inventory, roughly 24% of the 1628 commercially available nanomaterial-based products contain AgNPs (Sondi et al. 2004; Guo et al. 2015). Products include clothing, blankets and bed sheets, cleaning products, paints and coatings, health and fitness supplements, antibacterial wound dressings, food packaging, refrigerator linings, and cosmetics (Ntim et al. 2015; Benn et al. 2010; Klaine et al. 2008; Morones et al. 2005; Project on Emerging Nanotechnologies, 2014; Sun et al. 2005; Wijnhoven et al. 2009; Weir et al. 2008; Yoon et al. 2008; Tulve et al. 2015). The increased and frequent use of AgNPs in commercial products increases the potential for these particles to enter the environment and therefore may become an environmental hazard. These particles could be introduced to the environment through a point source such as industrial effluent pipes or a non-point source such as terrestrial runoff. Coastal zones, inlets, and estuaries are at the highest risk of point and non-point sources of input due to their immediate adjacent proximity to the land. As previously

stated the novel properties of NPs, and the use of various capping agents, increase the likelihood of aggregation or dissolution. In recent studies capped AgNPs have been shown to be generally more stable than naked (un-capped) AgNPs in environmental conditions and sterically stable particles have been observed to be most resistant to aggregation (El Badawy et al. 2010). However, studies that produce AgNPs with different capping agents do not stress the respective specific effects of each of these particle types.

Various organisms including algae, *Daphnia magna* (water flea), *Danio rerio* (zebra fish), and *Oncorhynchus mykiss* (rainbow trout*)* have been used to determine the fate and effect of manufactured AgNPs. Although former studies have addressed the impact of NPs on algae, there is little information regarding the actual mechanism that causes toxicity to estuarine algae species. Algae possess a complex structure of carbohydrates and proteins that make up a semi-permeable cell wall (Heredia et al. 1993; Knox 1995). The cell wall could act as an ideal site for NP interaction and entrance, or as a barrier to prevent NP penetration into the cell. Examining AgNP uptake and toxicity on a primary producer level is beneficial and will highlight possible implications for biomagnification and trophic transfer within an ecosystem.

Bivalves have also been used in recent studies as an indicator species for marine pollution monitoring (Anajjar et al.2008; Fukunaga and Anderson 2011; Sarkar et al. 2006). As sessile suspension feeding organisms, bivalves can accumulate high concentrations of pollutants in their tissues and internal organs. In addition bivalves indiscriminatively filter the surrounding water into their mantle cavity, over the gills, and their labial palps are used to sort and identify phytoplankton-sized particles within a 1-12

μm size range for consumption (Barrington 1979), thereby directly consuming NPs and NP aggregates. Once ingested the AgNPs could be rejected as pseudo feces, ingested, digested, and excreted as feces, or incorporated into tissues. If AgNPs are rejected as pseudo feces or excreted as feces they are reintroduced to the environment where they can interact with other material and organisms that inhabit an oyster reef. These particles can be directly taken up by another organism from the water column, or the particles could aggregate, fall out of suspension, and settle into sediments. Through bioturbation, currents, tides, and human recreational and commercial influences resuspension of AgNPs can also occur. This resuspension can lead to a higher amount of exposure to additional organisms include the oysters which are sessile and cannot depart from the contaminated waters.

The environmental risks associated with AgNPs will depend on levels of environmental exposure and the hazards produced by exposure concentration and duration. In order to fully understand the impact nanotechnology has on our environment as well as the associated hazards and risks, the characteristics of AgNPs as a function of surface coating and solvent should be documented. This study aims to create and define the uptake and fate of two different AgNPs, charge stable cit-AgNPs and sterically stable pvp-AgNPs. The ultimate goal is to provide insights to particle characteristics that lead to low hazard and low risk nanotechnologies for the benefit of human an environment health. Specifically, we aim to determine if the AgNP capping agent causes a significant difference in uptake and toxicity among estuarine phytoplankton and bivalves. We hypothesize that toxicity is dependent on the type of surface material coating the AgNP.

CHAPTER 1: SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES

1.1 INTRODUCTION

NPs are currently used in a wide range of consumer products such as scratch proof eyeglasses, crack resistant paints, self cleaning windows, and transparent sunscreens. Each type of particle used in these products has a unique synthesis procedure that uses various core and surface materials. These different synthesis processes result in various characteristics such as size, shape, and surface coating. The reduction of metal salts has become one of the most commonly used synthesizing techniques due to numerous applications of metal NPs in current manufactured products such as printer ink, electronic hardware, water repellent and automotive sealing sprays, and paints (Project on Emerging Nanotechnologies, 2014). AgNPs are currently one of the main types of NPs used in consumer products due to their strong antimicrobial properties (Sondi et al. 2004). Products include fabrics, cleaning products, paints, and food packaging (Ntim et al. 2015; Benn et al. 2010). The frequent and increasing use of AgNPs in commercial products promotes the potential for these particles to enter the environment and therefore may become an environmental hazard.

Due to different protocols and variability in NP production, the characterization of produced NPs is essential to properly investigate the chemical and physical composition and to better examine uptake, exposure, and associated effects. There are several instruments and techniques currently in use to test NP physical and chemical

characteristics, however, several studies that characterized commercial NPs have reported high variability in measured size (Domingos et al. 2009; MacCuspie et al. 2011). This variability could be explained by the use of commercial NPs and instrument limitations. Therefore we aim to produce AgNPs in-house and use a multi-methodological approach to define AgNPs with three commonly used analytical tools. Dynamic light scattering (DLS) results in an average size distribution based on the measurement of the diffusion coefficient and hydrodynamic diameter of NPs. Ultraviolet visible spectrometry (UV-vis) measures the absorption of visible wavelength (400-700 nm) and will help determine the stability and distribution of NPs over time. Transmission electron microscopy (TEM) uses high resolution and a beam of electrons to penetrate through a sample (e.g. NPs) preserved on a carbon fiber grid. When the beam passes through the grid and scatters, it changes wavelength and as a result the size of the particle is determined based off of the interference pattern.

AgNPs in this study will be formulated using silver nitrate ($AgNO_3$) as the soluble metal salt and sodium citrate and $NaBH_4$ as reducing and capping agents to control particle size. AgNPs exhibit specific novel physical and chemical properties that are controlled largely by the size of the particle. Size is controlled by a chemical reaction that reduces and stabilizes the metal salt. Sodium borohydride ($NaBH_4$) is an effective reducing agent used in the manufacturing of pharmaceuticals, electronics, and water treatment applications. Therefore $NaBH_4$ will be used as the reducing agent in the chemical reaction to reduce the $AgNO_3$ ions in solution to the desired size. Sodium citrate is an organic polymer that is typically used as a food additive to regulate acidity or stabilize an insoluble emulsion such as oil. These common applications make sodium

citrate an ideal compound to bind to a particle surface. Therefore sodium citrate will be used as the capping agent to stabilize and cap the $AgNO_3$ ions to prevent further growth or aggregation. Pvp will be used as the second type of capping agent, it is a synthetic disintegrant used to prevent denaturation over the expected shelf life in pharmaceuticals and as a thickener or binder in cosmetics (Senak et al. 1987; Robinson et al. 1990). Due to its inert behavior towards salts and insolubility in water (Robinson et al. 1990) pvp will be used to cap the Ag core and prevent dissolution during exposures.

The environmental risks potentially associated with AgNPs will depend on exposure, capping agents used during synthesis, and the hazards produced. In order to fully understand the hazards and therefore risks of AgNPs it is important to investigate the characteristics of AgNPs as function of the capping agent. In this study we aimed to produce and characterize AgNPs for the development of low hazard and low risk nanotechnologies for the benefit of human and environment health.

1.2 METHODS

1.2.1 NANOPARTICLE SYNTHESIS

AgNPs were synthesized by chemical reduction of silver salts in a method similar to that performed by Romer et al. 2011. This process reduced and stabilized silver ions via sodium borohydride and sodium citrate or pvp. Three solutions were created in three different volumetric flasks to synthesize AgNPs. The first with 4.2 mg of $AgNO_3$ in 100 ml of deionized water (DI), the second with 6.51 mg of sodium citrate in 100 ml DI, and the third with 1.8 mg of $NaBH_4$ in 50 ml of DI. The three solutions were then cooled at 4°C for 30 minutes. The sodium borohydride was diluted by adding a 1.5 ml aliquot of

the original solution to 4.5 ml of DI. $AgNO_3$ and sodium citrate solutions were then added to a 500 ml beaker and stirred. Immediately after mixing these two solutions, the diluted aliquot of $NaBH_4$ was added and stirred vigorously for 10 minutes. The temperature was raised to 110°C for another 90 minutes in order to speed up the chemical reaction within the beaker and to obtain the desired size of NP. After 90 minutes the particles were removed from the heat, covered, and stored at room temperature over night. This synthesis produced a 200 ml NP solution. After the cooling process the particles were washed by ultrafiltration to remove excess chemical species using a diafiltration approach to prevent drying and aggregation. This washing process was established by Romer et al. 2011 and was optimized to ensure the removal of greater than 90% of free citrate ions by using a citrate solution and continuous stirring. The particle stock solution was added to a pressure filtration cup, total volume 200 ml, with a 1 kDa cellulose membrane, and filtered down to a volume of 100 ml using positive pressure nitrogen gas. Once a 100 ml volume was reached, the cup was filled with 100 ml of fresh citrate solution to rinse the particles again. This was repeated a total of 3 times. The citrate solution was made by dissolving 3.25 g of sodium citrate in 100 ml of DI.

Half of the original 200 ml batch of cit-AgNPs was recapped with pvp using a ligand exchange process. The recapping material was prepared by adding 5 mg of a 10,000 molecular weight pvp powder to 10 ml of distilled water to make a 500 ppm solution. From there, 100 ml of AgNP stock solution was separated into a new beaker and 1.6 ml of the 500 ppm pvp solution was added and immediately stirred vigorously for 10 minutes. After the initial 10 minutes stirring was decreased to a gentle mixing for an

additional 60 minutes and left to sit overnight at room temperature. The other half of the stock solution remained unchanged and was stored at 4°C.

1.2.2 NANOPARTICLE CHARACTERIZATION

Inductively coupled optical emission spectrometry (ICP-OES) was used to determine the concentration of the cit- and pvp-AgNP stock solutions. The mean hydrodynamic size (z-average), polydispersity index (pdi), and zeta potential of the stock solutions were quantified by dynamic light scattering (DLS). At least three consecutive measurements were recorded to calculate the z-average size (nm) and pdi. DLS measures the size of the particles in suspension from Brownian motion. In general a laser incident on a solution containing particles scatters light in all directions. The interference of the scattered light produces a pattern that is formed by constructive and destructive interference of the laser. These patterns are collected every few milliseconds by the software. After a time t the particle positions have changed, due to Brownian motion (where D is the diffusion coefficient, k is the boltzman coefficient, T is temperature, b is liner drag), that they no longer correlate with the initial pattern. Smaller particles cause this process to be faster than larger particles, thus size of the particles in suspension can be measured.

$$D = kT/b \text{ Brownian motion}$$

The software then uses the Stokes-Einstine equation (where D is diffusion coefficient, η is liquid viscosity, k Boltzmann coefficient and T absolute temperature) to find the hydrodynamic diameter from the diffusion coefficient. It assumes all particles are spherical. This assumption results in an overestimation of size.

$$d\,(h) = \frac{kT}{3\pi\eta D} \qquad \textit{Stokes - Einstine}$$

The absorption spectra of both particle types were analyzed via UV-vis and recorded after 24, 48, and 72 hours to test for stability and aggregation within the stock solutions as well as a saltwater exposure media (L1-Si). UV-vis takes an average of the NP clusters present in solution rather than individual ones. In this study we used the Shimadzu UV spectrometer that takes absorption measurements with a wavelength resolution of 1nm, between 250 and 900 nm for all samples. For the measurements, 1 ml of NP containing solution was placed into a micro-cuvette. The machine was baselined with reference cuvett of miliQ water for stock NP suspensions characterization, L1-Si for phytoplankton exposures (Chapter 2), or Baruch filtered seawater (BFSW) for oyster exposures (Chapter 3). In this study we used the Shimadzu UV spectrometer that takes absorption measurements with a wavelength resolution of 1 nm, between 250 and 900 nm for all samples. There are two light sources contained within the machine, visible and UV spectrum portions, as well as an energy selector with blue and red filters, and a monochromator. All wavelengths are selected independently and recorded to give a full absorption spectrum. To achieve this, the monochromator ensures that only one wavelength is incident on the sample at one time. The beam is then split into two before entering the specimen chamber where the sample is located. One beam is passed through a reference cuvette while the second beam is passed through the sample cuvette. The reference cuvette contains an identical liquid to that the sample is being suspended, this allows any absorption from the suspension liquid to be removed from the final absorption spectrum. The beams are then passed as signals to the computer to analyze. The computer will remove the reference beam signal from that of the sample to leave a spectrum that

will only show the absorption of the actual specimen without the contribution from the suspension liquid.

Further examination of both cit- and pvp-AgNPs in stock solution and within L1-Si media was performed after 72 hours using transmission electron microscopy (TEM). Images were taken on a Hitachi H-8000 TEM, fitted with a field emission gun with an electron energy of 200 kV. The images were collected via SIA bottom mount CCD camera. Analysis was done using ImageJ to find the average diameter of particles. In brief, TEM uses a field of emission gun to produce a beam of electrons which passes through the center of the aperture. There are two condenser lenses which first de-magnify the beam and second focus it at the front focal plane of the objective lens. The second lens also controls image focusing. The beam of electrons passes through the sample and is deflected again into a set of imaging lenses before they are projected on the viewing screen and an image is seen.

1.3 RESULTS AND DISCUSSION

1.3.1 CHARACTERIZATION IN STOCK SOLUTION

For DLS analysis samples were filtered through a 1 μm syringe filter to remove particles above 1000 nm. The first characteristic tested was the zeta potential. When particles are dispersed into liquids there is an interface formed between the surface of the particle and the liquid. The zeta potential is the electrical potential at that shear plane between the two surfaces (Hunter et al. 1998, Ross and Morrison 2002, Greenwood 2003), thus zeta potential is a measure of the charge of the particle. Cit-AgNPs resulted in zeta potential of -42.5 mV while pvp-AgNPs resulted in a zeta potential of -30.9 mV

(Table 1.1). Zeta potential also indicates the degree of repulsion between adjacent, similarly charged particles. Particles on the nano-scale are small enough that a high zeta potential increases the chance of stability and resistance to aggregation.

On the other hand, when zeta potential is low the attraction forces exceed the repulsion forces and particles tend to aggregate. Cit-AgNPs being charge stable particles should have a more negative zeta potential than the sterically stable pvp-AgNPs. The negative charge of the cit-AgNP increases the chance of aggregation due to the negative charge of the particle creating an affinity to bond with the positive charge of water molecules (H^+ atoms). As expressed in the DLVO theory, this affinity increases even more in saltwater due to the presence of sodium (Na^+) and chloride (Cl^-). The negatively charged cit-AgNPs have the potential to bind to the Na^+ and H^+ cations and form aggregates. Conversely, pvp-AgNPs are sterically stable particles and should have a less negative zeta potential. The capping agent of pvp reduces the charge in the shear interface between the particle and the surrounding solution therefore reducing the affinity to aggregate for stability. The – 42.5 mV value recorded for cit-AgNPs indicates a zeta potential where aggregation is needed for stabilization, while the -30.9 mV of pvp-AgNPs indicates a particle that is more likely to remain stable over time.

To test this stability of each particle further analysis was conducted to determine the polydispersity index (pdi). Polydispersity index is a measure of the width of the size distribution compared to the median size (Rogosic et al. 1996), where a smaller pdi (0.3 and below) indicates a more uniform size distribution. Pdi values were determined to be 0.2 for cit-AgNPs and 0.3 for pvp-AgNPs (Table 1.1). The recorded pdi values for both

AgNPs indicate a mono-dispersed stock solution, showing that the majority of NPs within the solution are uniform.

The second parameter analyzed was the z-average. The z-average is a measurement via DLS of the average size of the particles in suspension determined from the light intensity that is scattered in all directions. The resulting measurement is determined by an autocorrelation based on a cumulative frequency size distribution. The DLS analysis of cit-AgNPs indicated a z- average of 11.2 nm with a single peak, 11.24 ± 1.9 nm (mean ± 1 s.d.) (Table 1.1). The pvp-AgNPs had a z-average of 17.6 nm, and two peaks, 23.9 ± 14.5 nm and 2.6 ± 0.6 nm (mean ± 1 s.d.). Small molecules and NPs in solution undergo Brownian motion which results in the constant changing of the distance between particles over time. Due to the forces acting on the NPs and the resulting size being an average measurement, this method cannot solely be used to determine the exact size of NPs in solution. For example if the sample has a majority of 10 nm sized particles but outliers of roughly 2 nm or larger aggregates, then the average will be skewed and not an accurate representation of the NP stock solution.

Although these are a good techniques to get a quick average size value DLS is not the most accurate measurement of size due to the fact that it is based on the hydrodynamic properties of the particle and often the shell of the NP is larger than the core. To account for this error in size measurement TEM was used to analyze the size of the particles. TEM uses a beam of electrons to penetrate through the NP to adequately measure the proper size value. This examination revealed a spherical particle shape for both cit- and pvp-AgNPs and an average particle size of 14.0 ± 1.7 nm and 14.8 ± 5.7 nm (mean ± 1 s.d.), respectively (Table 1.1).

1.3.2 CHARACTERIZATION IN SALT WATER MEDIA

Cit- and pvp-AgNPs were added to seawater L1-Si media for an exposure time of 72 hours to mimic the exposure conditions for future experiments (See Chapter 2). Cit-AgNPs were expected to aggregate immediately upon addition to L1-Si media, however when examining the UV-vis data (Figure 1.1-A) it can be seen that there is not observable peak at 400 nm wavelength which is characteristic of AgNPs. If the NPs were aggregating the UV-vis would still be able to detect the presence of Ag within the sample solution. The absence of the 400 nm peak suggests that cit-AgNPs are most likely dissolving in the L1-Si media and forming silver chloride (AgCl). When cit-AgNPs dissolve Cl^- binds to the free Ag ions, forming AgCl, this new compound does not have a detectable wavelength due to Cl^- masking the Ag signal (Figure 1.1-A). Pvp-AgNPs show a distinct peak at 400 nm (Figure 1.1-B) indicating that these particles were detectable in the saltwater media. Further analysis showed that the loss in peak size of pvp-AgNPs was less than 20% over the 72 hour exposure therefore showing that pvp-AgNPs barely changed in media over time. This was expected given that pvp has a strong interaction with the AgNP surface and is known to better stabilize AgNPs than other macromolecules (Huynh and Chen 2011) resulting in an average maximum peak loss of 12% after 21 days (Tejamaya et al. 2012). The claim that cit-AgNPs aggregate and fall out of suspension immediately upon addition to media is further supported by the lack of detection via DLS analysis. This loss of cit-AgNPs upon addition to environmentally relevant media (OECD Daphnia media of varying ionic strength of sulfate and nitrate) was also observed by Tejamaya et al. (2012) where they observed a 100% loss of cit-AgNP and 40% loss in 10 fold diluted OECD media. The loss of particles may be

attributed to the weak charge stable bond that holds sodium citrate ions to the AgNP core. Once added to salt water media (L1-Si) the negatively charged cit-AgNP tends to bind to the positively charged Na^+ and H^+ atoms.

Pvp-AgNPs remained fairly stable in media over time. After 24 hours a pdi value was recorded at 0.6 and a z-average of 49.6 nm, with peaks of 111.9 ± 48.6 nm and 14.5 ± 4.1nm (mean ± 1 s.d.) (Table 1.2). Both the pdi and the z-average decreased after 48 hours to 0.5 and 20.6 nm, with a solitary peak of 21.2 ± 9.5 nm (mean ± 1 s.d.). This indicates that as time increased the NPs became more stable and the size range narrowed. Stable mono-dispersed NPs will have a pdi of 0.2 where as values greater than 0.2 indicate poly-dispersed NPs. As pdi increased the NPs became more widely dispersed, showing a direct relationship. This is also seen in the z-average value; the z-average measures the average size of the particles within the solution and therefore as the particles become more mono-dispersed (i.e. uniform size), the z-average will decrease and better represent the actual size of the majority of particles in solution. At 72 hours of exposure the pdi decreased further to 0.4 and the z-average was recorded at 16.4 nm, with a single peak at 18.5 ± 6.9 nm (mean ± 1 s.d.) (Table 1.2). The decrease in the pdi value and z-average suggests that the pvp-AgNPs became more stable in media as time increased. Stabilization of pvp-AgNPs over time was also seen in a recent study where high resolution TEM images showed pvp-AgNPs mono-dispersed in solution with an apparent surface coating after 48 hour in exposure media (Newton et al. 2013). This stability can be attributed to pvp acting as an electron donor during synthesis, where pvp gives its lone pair of electrons to Ag creating a strong complex bond and strengthening the adsorption of pvp to the surface of the NP.

Both AgNPs (cit and pvp) were kept separately in L1-Si media for 72 hours and analyzed via TEM to account for the error in DLS analysis when determining hydrodynamic size. This analysis resulted in a spherical particle shape for both cit- and pvp-AgNPs and an average particle size of 20.7 ± 6.5 nm and 17.4 ±7.2 nm (mean ± 1 s.d.), respectively. At the end of the exposure period there were aggregates of both particle types visible on the TEM grid and calculated to be an average size of 38.5 ± 9.3 nm and 22 ± 7.0 nm (mean ± 1 s.d.), respectively (Table 1.2).

1.4 CONCLUSION

AgNPs enter the environment in large amounts from various sources (Mueller and Nowack 2008; Kakavandi et al. 2014; Cho et al. 2015). Due to the ionic complexity of seawater, the novel characteristics of NPs, and known toxic effects of Ag ions to organisms, these AgNPs create a chemical, physical, and toxicological hazard, that could potentially turn into a risk. These particles have been shown to behave differently based on the surface coating, where sterically stable pvp-AgNPs remained mono-dispersed in seawater while charge stable cit-AgNPs dissolve. We also show that duration in media contributes to the stability of NPs. Cit-AgNPs were not affected by the amount of time in media as they dissolved immediately and most likely formed AgCl. However, pvp-AgNPs size range was reduced as time increased. These and multiple other factors that have yet to be tested will only added to the uncertainty of how NPs will behave in the environment, therefore presenting various problems and hazards to surrounding environments and biota. The unknown nature and behavior of NPs as a function of capping agent could cause changes in the pH of water and redox reactions, as well as the

speciation of chemicals present in a body of water or a significant toxic and even fatal impact on surrounding biota. This study showed that the addition of a reducing agent that caps the particle during synthesis does have an effect on particle characteristics as well as how the particle behaves in exposure media as seen in the differences between cit-AgNPs and pvp-AgNPs.

Table 1.1: Characteristics of stock cit-AgNPs and pvp-AgNPs. Peaks above 1000 nm were filtered out with a 1μm syringe filter and were not included in this table. ND (no data) indicates only one peak was detected. TEM and peak sizes are reported as mean ± 1 s.d.

Particle	**Media**	**Batch conc. (ppm)**	**Zeta potential (mV)**	**z-average (nm)**	**pdi**	**TEM size (nm)**	**Peak Size (nm)**	
							1	2
cit-AgNP	Water	8.6	-42.5	11.2	0.2	14.0±1.7	11.24±1.9	ND
pvp-AgNP	Water	8.6	-30.9	17.6	0.3	14.8±5.7	23.9±14.5	2.6±0.6

Table 1.2: Characteristics of cit-AgNPs and pvp-AgNPs in L1-Si media over a 72 hour exposure period. Peaks above 1000 nm were filtered out with a 1μm syringe filter and were not included in this table. Note: Cit-AgNP results are not included due to immediate aggregation upon additional to media. ND (no data) indicates only one peak was detected and UD (undetected) indicates a parameter that was undetectable. Cit-AgNP results of 24 and 48 hours are not included in this table. TEM and peak sizes are reported as mean ± 1 s.d.

Particle	**Media**	**Exposure time in media (hrs)**	**z-average (nm)**	**pdi**	**TEM size (nm)**	**Peak Size (nm)**	
						1	2
cit-AgNP	L1-Si	72	UD	UD	20.7±6.5	ND	ND
pvp-AgNP	L1-Si	24	49.6	0.6	NA	111.9±48.6	14.5±4.1
		48	20.6	0.5	NA	21.2 ± 9.5	0 ± 0
		72	16.4	0.4	17.4±7.2	18.5 ± 6.9	ND

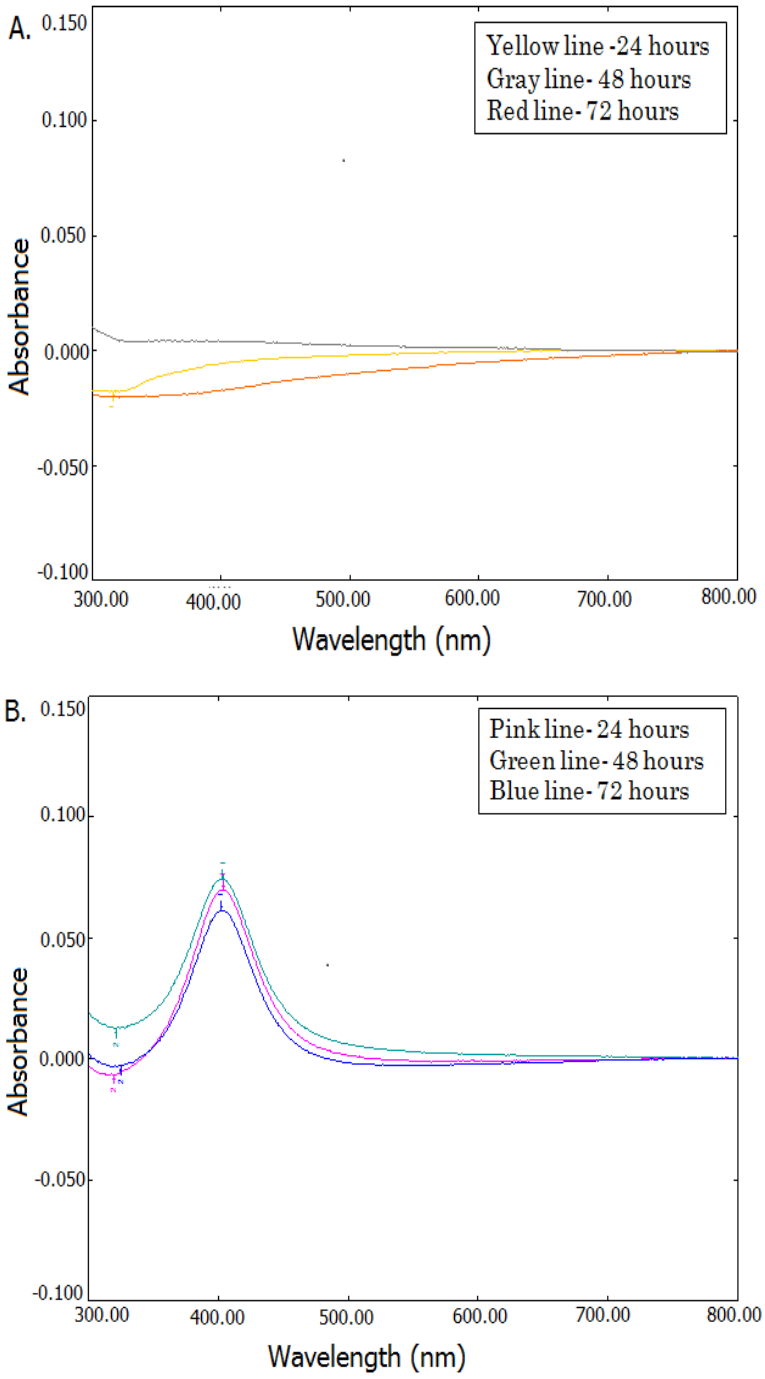

Figure 1.1 A-B: Stability results from UV-vis analysis of AgNPs in L1-Si medi over a 72 hour exposure time. A) Stability of cit-AgNPs in L1-Si media over 72 hours. (Yellow (middle) = 24 hours, Gray (top) =48hours, Orange (bottom) =72 hours). B) Stability of pvp-AgNPs in L1-Si media over 72 hours. (Pink (top) = 24 hours, Green (middle) =72hours, Blue (bottom) =72 hours).

CHAPTER 2: SILVER NANOPARTICLE EXPOSURE AND TOXICITY TO AN ESTUARINE DIONFLAGELLATE

2.1 INTRODUCTION

NPs can be introduced to the environment in multiple ways through various sources (see Chapter 1). Coastal zones such as inlets and estuaries are at the highest risk of contamination from terrestrial run-off and effluent pipes due to their immediate proximity to land. Once introduced to the environment NPs may behave differently than expected due to chemical and physical interactions. This increases the chance of exposure to organisms that occupy the surrounding area and could cause a significant toxic and even fatal impact on surrounding biota. However, the addition of a capping agent adds stability to NPs and potentially decreases the chance of aggregation, dissolution, and other transformations in environmentally relevant media.

Recently there has been an emphasis to better understand the impact of nanotechnology on our environment. There have been multiple studies to characterize this potential toxic effect using estuarine organisms as models to determine the fate and toxicity of manufactured AgNPs (Navarro et al. 2008; Ali et al. 2011; Canesi et al. 2012; Newton et al. 2013). Although algae have been the organism of interest in these studies, there is a lack of detailed information about the cause of toxicity in relation to NP type, especially capping agent, to estuarine algal species.

Algae possess a complex structure of carbohydrates and proteins that make up a semi-permeable cell wall (Heredia et al. 1993; Knox 1995). This could act as an ideal site for NP interaction as an entrance or as a barrier to prevent NP penetration into the cell. If the NP is taken across the membrane and enters the cell this could cause a higher toxic effect than if it were to just adhere to the outside of the cell wall. Alternatively, the particle could induce changes in the cell wall structure by attaching externally, thus increasing the chances of entrance into the cell and therefore internalization of the NPs (Navarro et al. 2008).

Prorocentrum minimum is a mixotrophic flagellated species of phytoplankton therefore it is an ideal organism for toxicity determination due to its ability to obtain energy photosynthetically and take up nutrients and organic matter from the surrounding water by diffusion or endocytosis. Also, being a flagellated species it is able to migrate vertically and horizontally in the water column increasing mobility and therefore potential exposure to present toxicants.

Increased use of nanomaterial and technology is widely thought to have adverse effects on marine organisms (Fabrega et al. 2011; Lapresta-Fernandez et al. 2012; Matranga and Corsi 2012; Moore 2006). In this study we used previously characterized AgNPs (see Chapter 1) in exposures to estuarine dinoflagellates and quantified the internalization of Ag within the organism and the associated impact on population growth. It was hypothesized that Ag will accumulate and internalize within the dinoflagellates in large quantities and a small amount will remain loosely bound to the theca especially when exposed to higher concentrations of AgNPs. It was also hypothesized that there will be a significant change in the population growth of

P.minimum over time due to a dose response exposure of AgNPs. Furthermore, exposure to higher concentrations of cit- and pvp-AgNPs will be more toxic to phytoplankton causing a negative impact or decrease in population growth. The overall objective of this study is to determine how concentration impacts NP toxicity and inhibition of dinoflagellate growth. Furthermore, examining AgNP toxicity on the level of a primary producer is important because primary producers are the foundation of multiple food chains and webs in various ecosystems. If the level at which multiple other species feed upon is disrupted then the entire ecosystem is at risk for changes in structure and dynamics.

2.2 METHODS

2.2.1 SILVER NANOPARTICLES

Cit-AgNPs and pvp-AgNPs that were synthesized in Chapter 1 were used in these dinoflagellate exposure studies.

2.2.2 ORGANISMS

The estuarine dinoflagellate *Prorocentrum minimum* (CCMP# 695) was obtained from the National Center for Marine Algae and Microbiota (NCMA) and grown in 0.2 μm filtered L1-Si (Guillard and Ryther 1962; Guillard 1975) enriched seawater media, in 23°C, on a 12 hour light and dark cycle, with a light intensity range of 90-100 μmol quanta $m^{-2}s^{-1}$. The dinoflagellate culture was allowed to grow for four days prior to transfer to individual exposure bottles to ensure there would be enough cells in each exposure bottle for proper measurements.

2.2.3 EXPOSURES

Exposures were carried out for 72 hours at the concentrations of 0.1– 1000 μg l^{-1} (ppb) in 500 ml glass pyrex bottles. This length of time was chosen for exposures based on standard protocol for proper bio-assessment and to allow sufficient time for dinoflagellate communities to respond to the added treatment (Munawar and Munawar 1987; Schafer et al. 1994). For each exposure, 200 ml of L1-Si media and 25 ml of cultured dinoflagellates were added to individual glass bottles along with the $AgNO_3$, cit-AgNP, pvp-AgNP, citrate solution, or pvp solution. Citrate and pvp solutions were formulated by adding the maximum amount of appropriate capping agent that would be present in solution if none were to adhere to the Ag core. These treatments were used as controls to determine if there was an adverse effect solely from the capping agent. Exposures were run in triplicate and were kept at 23°C on a 12 hour light and 12 dark cycle with a light intensity range of 90-100 μmol quanta $m^{-2}s^{-1}$ to mimic incubation conditions. To insure consistent light exposure to each bottle, a ultraviolet spherical quantum probe from Biospherical Instruments Inc. (model:QSI2101) was used along with the Logger 2100 program to measure the exposure irradiances and thus the desired distance from the light source. A 2 ml sample was taken every 24 hours from each bottle for fluorometry to determine chlorophyll *a* concentrations by fluorescence in raw fluorescence units (RFUs). Fluorescence was measured using a Turner Trilogy Fluorometer with the Chl *a-in vivo* module. For determination of abundance by direct cell counts, a 100 ml sample was taken every 24 hours and preserved in 1% Lugol's solution. Samples were then enumerated number of cells using inverted light microscopy on an Olympus IMT-2 microscope and a Burker counting chamber (10 μl sample per slide). A

linear regression was then performed to relate the number of cells in a sample to the amount of fluorescence yield. This was done to ensure that fluorescence measurements provided an accurate relative measure of the number of cells in a sample. Fluorescence values were then used to calculate the percent inhibition for each of the exposures using the following equation:

$$\%relative\ inhibition = \frac{control - treatment}{control}\ x\ 100$$

Final inhibition for each treatment relative to the control cultures were calculated and plotted against the exposure concentration. Sigmoidal concentration-response curves were derived using the following equation: (See results for treatment specific equations)

$$y = bottom + (top - bottom)/(1 + EC_{50}/x)^{hillslope}$$

Where y is the observed value of % inhibition, bottom is the lowest value of % inhibition observed, top is the highest value of % inhibition observed, EC_{50} is the half-maximum effective concentration, x is the treatment concentration, and hillslope is the largest absolute value of slope of the curve. From this equation the concentration that resulted in a 50 % decrease of dinoflagellate abundance (EC_{50}) was determined for each treatment in IBM SPSS v. 21 (Motulsky and Christopoulous 2004).

2.2.4 AG ACCUMULATION

At the end of the 72 hour exposure period, a 50 ml sample was taken from each bottle and centrifuged at 2000 rpm, at 4°C, for 10 minutes to precipitate the suspended phytoplankton. A 10 ml sample was taken from the supernatant and placed into a new bottle with 100 µl of nitric acid. The remaining water was carefully pipetted out as to not disturb the phytoplankton pellet at the bottom of the centrifuge tube. With the pellet

remaining in the bottom of the bottle, 10 ml of fresh L1-Si media was added and centrifuged again for 10 minutes at the same conditions to wash the pellet of any silver that was loosely bound to the theca of the organisms. The 10 ml wash was then removed from the centrifuge tube and placed into a new tube with 100 µl of nitric acid. The pellet remaining in the original tube was then digested in 100 µl of nitric acid and made up to a 1% acid solution for ICP-OES analysis. The ICP-OES analysis was conducted to determine the amount of Ag within each sample and provide a measure of internalized and loosely bound Ag.

2.3 RESULTS AND DISCUSSION

2.3.1 AG ACCUMULATION AND INTERNALIZATION

Internalization analysis was performed on the 1, 40 and 100 ppb exposures for cit-AgNPs, pvp-AgNPs and $AgNO_3$. However, only the results of the 40 and 100 ppb exposures are presented in this paper since the samples from the 1 ppb exposures were undetectable via ICP-OES analysis. It is inferred that the amount of Ag internalized at this level is below the detection limits of ICP-OES and accurate readings were not determined. Future studies will look at these samples via inductively coupled plasma mass spectrometry (ICP-MS) analysis to determine the amount of silver present within the dinoflagellates at this low exposure concentration.

Ag was taken up and internalized within dinoflagellate cells (Figure 2.1). It cannot be determined if internalization was dependent on AgNP type for the reason that in the 40 ppb exposure cit-AgNP internalized more Ag (ca. 30%) than pvp-AgNPs (ca. 13%) and the opposite was seen in the 100 ppb exposures; cit-AgNP (ca. 30%) and pvp-AgNP (ca.

7%) (Figure 2.1). Although there was not a capping agent effect seen in this study there was an inverse relationship between the accumulation of Ag and exposure concentration. Of the total Ag added a larger percentage internalized was seen in the 40 ppb exposures than the 100 ppb exposures regardless of capping agent. The same trend of lower concentrations accumulating more Ag was also seen in the $AgNO_3$ exposures (Figure 2.1). This could be due to the dinoflagellates dying at a faster rate in the 100 ppb bottles and not having the opportunity to accumulate as much as the 40 ppb bottles.

When examining the results in more detail the accumulation from cit-AgNP exposures mimic those of the $AgNO_3$ exposures. In the 40 ppb cit-AgNP exposures nearly 30% of total Ag was accumulated while the 40 ppb $AgNO_3$ roughly 27% of total Ag (Figure 2.1). The similarity in these results indicated that the cit-AgNPs are behaving like the $AgNO_3$ exposures, therefore suggesting that the cit-AgNPs are dissolving in solution and the dinoflagellates are being exposed to free Ag ions. As discussed in Chapter 1 it is also possible that the cit-AgNPs are forming AgCl compounds after dissolving and the dinoflagellates are then exposed to these newly formed compounds. The same reactions are likely occurring in the $AgNO_3$ exposures, causing the dinoflagellates to be exposed to AgCl as well. In the pvp-AgNP 40 and 100 ppb exposures accumulation of Ag remained low (<15% of total Ag) and accumulated less than half the amount that was accumulated in the cit-AgNP and $AgNO_3$ treatments (ca. 27-30% of total Ag). These results indicate that dinoflagellates more readily take up cit-AgNPs than pvp-AgNPs, however, this is most likely due to dissolution of cit-AgNPs and the resultant uptake of free Ag ions or AgCl by the dinoflagellate.

Prorocentrum minimum is a thecate species, thus it is covered with cellulose plates creating and extra boundary for these AgNPs to penetrate. While a portion of the AgNPs work their way into the organisms others may adhere to the cellulose plates causing the hard exterior shell to act as a binding site (Navarro et al. 2008). If the theca is indeed acting as such then this may create another pathway for dinoflagellates to be exposed to AgNPs. In all treatments there was significantly less Ag loosely bound to the surface of the dinoflagellate than internalized ($p < 0.05$). This indicates that the organism is taking up AgNPs across its cell membrane and the negative effect is caused by internalization of Ag. However, mass balance calculations indicate that the internalized and loosely bound portions account for a maximum of ca. 45% of total Ag added. Therefore, the total amount of Ag added cannot be accounted for by these two portions alone, suggesting that the majority of Ag was not taken up into the cell but instead remained in the media. Portions of Ag also could have been lost to the glassware and plastic tubes by absorbing to the sides. The low percentage of total Ag accounted for in the pvp-AgNP exposures (<15%) (Figure 2.1) may be due to pvp-AgNPs remaining mono-dispersed and stable in salt water media over time. It is also likely that pvp coating helps retain the novel NP characteristics such as surface charge and large specific surface area. The surface characteristics and sterically stable nature of the particles could deter the dinoflagellates from taking the NP up across their membranes for ingestion, resulting in low internalization of Ag.

In the 100 ppb $AgNO_3$ exposures there is little silver internalized (<10%) over the 72 hour exposure time (Figure 2.1) and a larger amount (ca. 24%) was internalized in the 40 ppb exposure. It was expected that more Ag would be internalized when exposed to 100

ppb than 40 ppb. The opposite result than what was expected can be due to the dinoflagellates quickly dying from the immense, initial, addition of $AgNO_3$, therefore the dinoflagellates would not have enough time to take up and internalize Ag before dying, resulting in the majority of Ag remaining in the media. Similar to the internalized portion, Ag detected on the theca (loosely bound portion) of the dinoflagellate in larger amounts in the 40 ppb exposure (roughly 7%) than in the 100 ppb exposure (<5%). The possibility of dinoflagellate death before Ag accumulation resulting in low percentages can also be applied to this result.

2.3.2 INHIBITION AND TOXICITY TO *P. MINIMUM*

A linear regression analysis was performed to relate the parameters of fluorescence (RFU) and cell abundance (cells ml^{-1}). The linear regression, performed with fluorescence as the independent variable and cell count as the dependent variable, was determined to be statistically significant (adj. R^2= 0.781, $p<0.05$) (Figure 2.2). Once this relationship was determined fluorescence was used to calculate the change in cell abundance (growth) of *P. minimum* during the remaining exposure trials. Data were normalized to the respective control to better visualize deviation from normal expected abundance. In the capping agent exposures, there were no differences in growth of the exposure bottles in relation to the control bottles. This indicates that the capping agent on its own does not negatively or positively influence cell abundance (Figure 2.3).

As expected higher exposure concentrations (100-1000 ppb) generally resulted in lower abundance values and low exposure concentrations (0.1-10 ppb) resulted in higher abundance, while the intermediate concentration (40 ppb) showed moderate abundance

values over time (Table 2.1). Results are further supported by a statistical analysis of variance (ANOVA) and REGWF test with abundance as the dependent variable and exposure concentration as the fixed factor ($p<0.05$ for all treatments).

For cit-AgNP treatments when exposed to low concentrations of 0.1 and 1 ppb the dinoflagellate population decreased over the first 24 hours. After the first 24 hours, abundance began to increase steadily for the remainder of the exposure. In the moderate concentration of 10 ppb abundance initially decreased, but began to increase after 48 hours and in the high concentrations (40-1000 ppb) dinoflagellate abundance continuously decreased over 72 hours (Figure 2.4 A).

In pvp-AgNP treatments dinoflagellate abundance initially decreased, then began to increase after 24 hours in the low concentrations (0.1-10 ppb). When exposed to the concentrations of 40-100 ppb a similar response was seen in dinoflagellate abundance where the population initially decreased and then began to increase after 48 hours. In the highest concentration of 1000 ppb dinoflagellate abundance consistently decreased over the 72 hour exposure period (Figure 2.4 B). This shows that the pvp-AgNPs are detrimental to dinoflagellate abundance and the population is able to recover after initial addition of these particles.

The $AgNO_3$ treatment resulted in an initial decrease in abundance and an increase after 48 hours in the concentrations of 0.1-10 ppb, and a continuous decrease in the 40 and 100 ppb exposures (Figure 2.4 C). This further supports previous research stating that $AgNO_3$ is highly toxic to aquatic marine organisms (Anajjar et. al 2008; Fukunaga and Anderson 2011). As in the cit-AgNP and pvp-AgNP treatments a top concentration of 1000 ppb was not used in the $AgNO_3$ treatment due to the fact that this amount of $AgNO_3$

significantly changed the pH of the exposure media from 7.10 to 6.2. This change in pH would not have been favorable for the dinoflagellates and we would not have been able to differentiate between cell death caused by NP exposure or due to change in pH.

It was originally thought that cit-AgNPs were aggregating immediately upon addition to salt water media, however, UV-vis analysis from Chapter 1 suggested that cit-AgNPs are dissolving in solution and/or forming AgCl. This data of dinoflagellate abundance further supports the idea that cit-AgNPs dissolve in salt water media and the dinoflagellates are then exposed to free Ag ions. When comparing the results of cit-AgNP and $AgNO_3$ exposures, similarities in abundance increases and decreases based on concentration and time can be seen. This suggests that cit-AgNPs are dissolving and the dinoflagellates are exposed to Ag ions which then follow the response of the $AgNO_3$ results. In both treatments the lower concentrations show an initial decrease in abundance followed by an increase after 24-48 hours. In the $AgNO_3$ exposures it takes longer for the dinoflagellates population to grow after addition of NPs which could be due to a larger amount of Ag ions present in the exposure bottle. Although the cit-AgNPs are dissolving that does not indicate that the same amount of Ag present in the $AgNO_3$ exposures is present in the cit-AgNP exposures after dissolution. The cit-AgNPs could be dissolving quickly or slowly in media releasing a varied amount of Ag into the media, which could be the cause of the earlier increase in abundance in the cit-AgNPs as compared to the $AgNO_3$ treatments. On the other hand, the pvp-AgNP treatments do not follow the pattern seen in the $AgNO_3$ treatments indicating that these particles are not dissolving in media. The same general trend of initial decrease followed by an increase in abundance can been seen pvp-AgNP treatments, yet, in all concentrations except for the 1000 ppb the

dinoflagellate population increases after 24-48 hours unlike those in the cit-AgNP and $AgNO_3$ treatments.

Navarro et al. (2008) showed that interactions with manufactured NPs make the pores in a phytoplankton's cell wall bigger in relation to the NP size, allowing more NPs through the membrane and into the internal structure of the cell. Once inside the organisms the NP is free to interact with the cell plasma or organelles, causing a toxic or fatal impact. This can be used to explain what is occurring in the organisms observed in this study where population abundance decreases and it can be implied that the entry of NPs into the cell membrane is causing cell death. In the low concentrations of cit-AgNP and $AgNO_3$ treatments (0.1-10 ppb) accumulation and internalization is occurring however it is thought that the dinoflagellates are able to take up all of the Ag within suspension. When all of the Ag or AgNPs within the media are removed the dinoflagellates are then able take up nutrients without the hindrance of a present toxin and are able to grow and increase in abundance. Another theory is that the dinoflagellates are able to sense the AgNPs or Ag as a toxin and rapidly take up nutrients to accommodate for and fight against the toxin being taken up across their cell wall. Once the dinoflagellates that have taken up all of the Ag or AgNPs within solution there is an excess of nutrients available to the remaining dinoflagellates and as they continue to rapidly take up nutrients a bloom-like situation occurs resulting in an increase in abundance.

Dinoflagellate abundance values were additionally used to the find half-maximum effective concentrations (EC_{50}) of each treatment. Data points were fit with a sigmoidal concentration-response curve for each treatment (Figure 2.5):

cit-AgNPs: $y = 0 + (100 - 0)/\ (1 + 61.044/x)^{5.994}$

pvp-AgNPs: $y = 0 + (100 - 0)/\ (1 + 67.501/x)^{4.459}$

$AgNO_3$: $y = 0 + (100 - 0)/\ (1 + 30.293/x)^{2.199}$

From these equations, EC_{50} values of 61.9, 59.1, and 27.9 ppb were determined for cit-AgNP, pvp-AgNP, and $AgNO_3$ exposures, respectively, and adjusted R^2 values of 0.98, 0.999, and 1 respectively. Based on the dose response curve fit to our data we were able to determine there was not a significant difference between the toxic effects observed by particle type (i.e. capping agent) although it is widely accepted that cit-AgNPs are slightly more toxic to *P. minimum*. Also, it can be seen that $AgNO_3$ is the most toxic in having the lowest concentration of the three that causes death of 50% of the population.

2.4 CONCLUSION

AgNPs are potentially hazardous and capable of causing a toxic effect on dinoflagellate population growth resulting in a decrease in abundance. Although this change in abundance of a single species of dinoflagellates is a small scale impact, it has the potential to change population dynamics and community composition of primary producers. Pvp-AgNPs remained stable and mono-dispersed in media resulting in an EC_{50} of 59.1 ppb, but results indicate that pvp-AgNPs are not extremely toxic to *P.minimum* and populations are able to recover after initial exposures. Cit-AgNPs follow the same pattern of $AgNO_3$ results showing that these NPs dissolve in salt water media and resulted in an EC_{50} of 61.9 ppb. Results suggest that $AgNO_3$ is detrimental to dinoflagellate population growth at moderate and high concentrations causing complete death for nearly all treatments after 48 hours of

exposure with a reported EC_{50} of 27.9 ppb. For both NP types the amount of the Ag found internalized within the dinoflagellates was dependent on the exposure concentration. At high exposure concentrations (100 ppb) there was less Ag internalized than when exposed to moderate concentrations (40 ppb) of AgNPs. We infer that there would be little to no internalization of Ag in the low exposure concentrations (1-10 ppb). The results from this study provide valuable information on the potential lethal effects that AgNPs may have on an estuarine primary producer and suggests there may be possible impacts on estuarine food web structure if dinoflagellate populations decrease or are eliminated due to AgNP exposure, Ag accumulation, and death. By removing the primary producer from a food chain or web the structural support is disconnected and the web can no longer support itself. Therefore, this loss must be accommodated for and the dynamics and structure must change or the web could possibly crash.

Table 2.1: Univariate analysis of variance results for all treatments (cit-AgNPs, pvp-AgNPs, and $AgNO_3$). Homogeneous subsets are indicated by a continuous underline. A double underline indicates that the concentration is included in more than one homogeneous group.

Treatment	**Exposure concentrations**
cit-AgNP	1000 70 100 40 0.1 10 1
pvp-AgNP	1000 70 100 40 0.1 10 1
$AgNO_3$	100 40 1 10 0.1

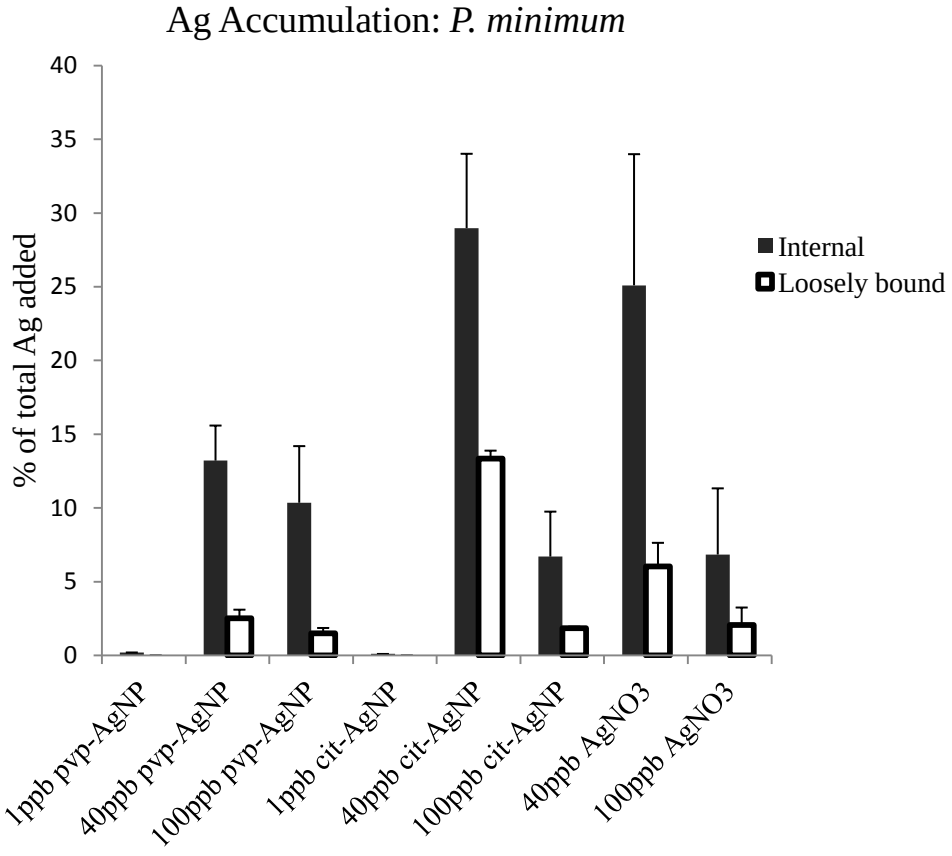

Figure 2.1: Results of the internalization of silver within *P.minimum* cells. (A) cit-AgNPs, (B) pvp-AgNPs, (C) $AgNO_3$ exposures. Internalization analysis was performed on 1, 40 and 100 ppb for each exposure. Each graph shows the concentrations of 40 and 100 ppb. 1ppb exposures were undetectable via ICP-OES analysis due to detection limitations. ($p<0.05$).

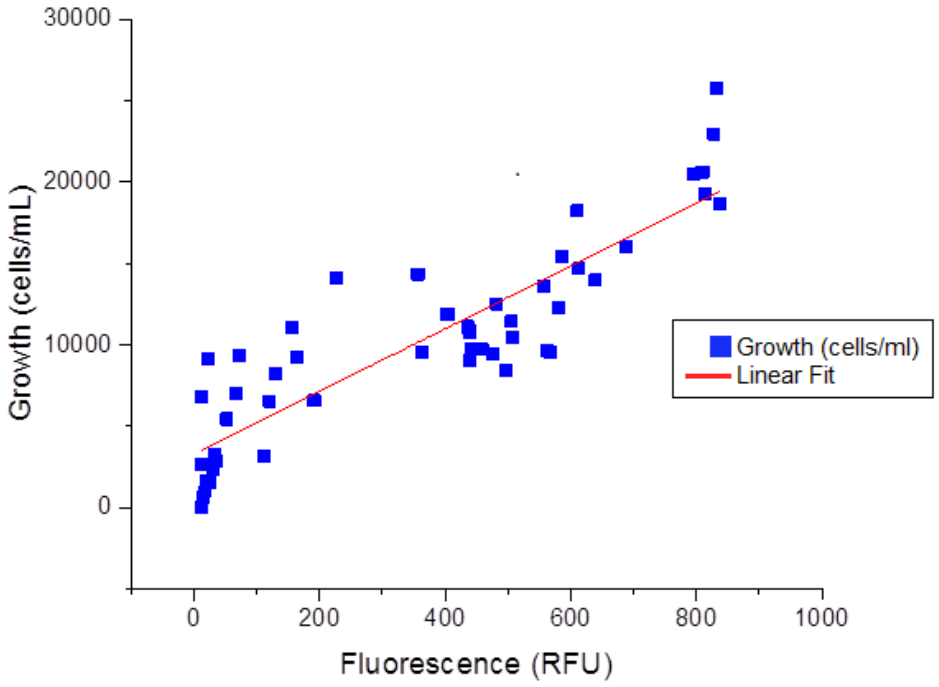

Figure 2.2: Linear regression performed with fluorescence as the independent variable and growth as the dependent variable to relate fluorescence as a function of cell numbers for remaining exposures. It was determined to be statistically significant (adj. R^2= 0.781, $p < 0.05$).

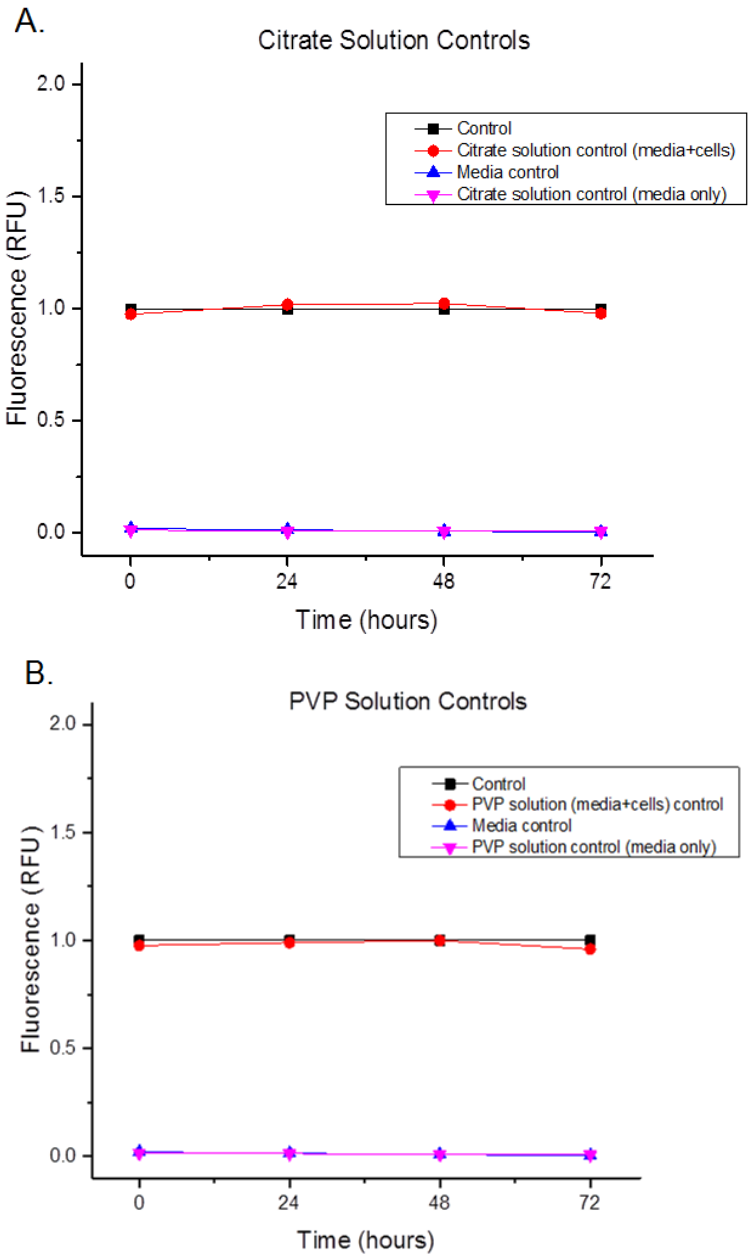

Figure 2.3: Citrate and pvp capping agents in L1-Si media. These solutions were tested in media and media with dinoflagellates to determine if the solutions interfered with fluorescence readings and to determine if there was a negative impact on cell growth due to the capping agent singularly. A.) Citrate solution: No observed difference from the control in either condition. B.) pvp solution: No observed difference from the control was seen in either of the conditions.

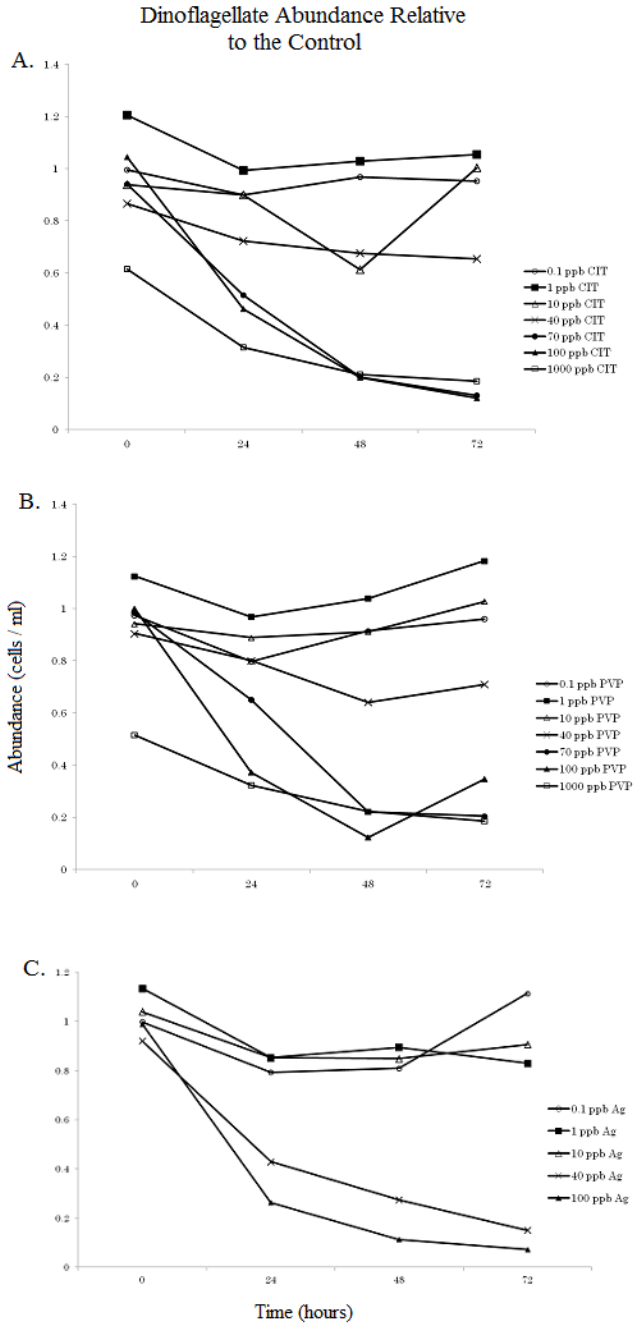

Figure 2.4 A-C: Graphed abundance results of a 72 hour exposure of various AgNPs to *P. minimum* cultures. 0 hour time point was taken immediately after addition of respective AgNP concentration. All results presented were normalized to the respective control. Each panel shows toxicity to *P. minimum* over a 72 hour exposure period A.) citrate-coated AgNPs B.) pvp-coated AgNP C.) $AgNO_3$ control representing a free ion exposure.

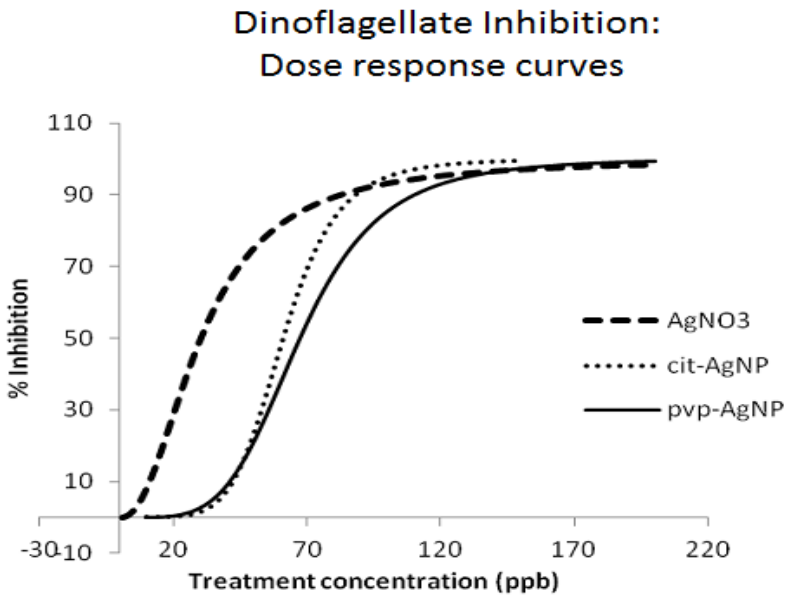

Figure 2.5: Graphed results of a regression ran with calculated percent inhibition at 72 hours as the dependent variable and treatment concentration as the independent variable fit to the (sigmodal) dose response curve. Curves for all three treatments are shown corresponding R^2 values are: cit-AgNP 0.928, pvp-AgNPs 0.921, and $AgNO_3$ 0.954.

CHAPTER 3: UPTAKE AND ACCUMULATION OF SILVER NANOPARTICLES BY AN ESTUARINE BIVALVE

3.1 INTRODUCTION

Due to an elevation in the amount of NPs introduced to the environment, it is becoming increasingly important to identify and better understand the impact nanotechnology has on aquatic habitats and organisms. In recent studies, bivalves have been used as an indicator species for marine pollution monitoring (Sarkar et al. 2006; Anajjar et al. 2008; Fukunaga and Anderson 2011). Being sessile, suspension feeding organisms, bivalves can accumulate high concentrations of pollutants in tissues and internal organs. Thus bivalves have the ability to integrate and indicate physiochemical and biological variations in their surrounding environment (Bryan and Langston 1992; Chapman et al 2009).

Crassostrea virginica is an intertidal reef forming bivalve and a valuable keystone species in south-eastern estuaries where salinities range from 5 to 30 ppt (Dame 1993). *C. virginica* is considered an ecosystem engineer in that their reefs provide multiple benefits for the ecosystem and increase biodiversity (Newell 2004; Grabowski and Peterson 2007; Hoellein and Zarnoch 2014). This species indiscriminately filters water and particles into its mantle cavity from the surrounding water column. Through the use of cilia oysters are able to pump large amounts of water across their gills with filtration rates as high as l l $hr^{-1}g^{-1}$-tissue weight (average of 50 gallons/day) (Jorgensen 1966). Although oysters are unable to decide what particles enter in to their mantle cavity, they are able to sort and

select suspended particles based on size and chemical composition (Shumway et al. 1985). *C. virginica* specifically is able to distinguish between organic and inorganic particles and has shown a preference for nitrogen rich particles over carbon rich particles (Newell and Jordan 1983). The process of sorting is carried out by specialized organs surrounding the mouth, called labial palps. Once particles are selected they are then moved by cilia to the mouth for ingestion (Ward et al. 1994). They use extracellular and intracellular digestion to breakdown and digest the desired component of filtered particles and release unabsorbed material as feces while non-nutritious or undesired particles are rejected as pseudofeces (Beninger et al. 199; Newell and Langdon 1996; Cranford et al. 2011). *C. virginica* is an ideal bioindicator species due to the constant gaping of its valves and continuous beating of cilia which allows for the pumping of water through the mantle cavity and across the gills as a continuous flow which increases exposure to the surrounding water (Jorgensen 1975).

According to research dating back to the late 1800s the United States has lost 88% of oyster reef biomass and in the Chesapeake Bay alone only 1% of the oyster population is remaining (Beck et al. 2011; Zu Ermgassen et al. 2012). Due to this reef decline it is becoming pertinent to determine the effects that the introduction of hazardous materials has on current bivalve populations. Since oysters couple benthic and pelagic components of ecosystems through filtration and excretion, the net loss of reefs could lead to ecological damage, biodiversity decreases, and ecosystem shifts. The main objective of this study was to couple the topic of AgNPs with a valuable estuarine organism, and specifically to determine the fate and uptake of these particles by *C. virginica.* This research identifies the location and quantity of Ag accumulation and the associated toxic

effects within oyster tissues due to exposure to two different types of AgNPs, cit-AgNP and pvp-AgNP.

3.2 METHODS

3.2.1 SILVER NANOPARTICLES

Cit-AgNPs and pvp-AgNPs that were synthesized in Chapter 1 were used in these exposure studies.

3.2.2 OYSTER COLLECTION

Oysters 6-10 cm in shell length were collected from Oyster Landing at North Inlet, Georgetown, South Carolina, at the Belle W. Baruch Institute for Marine and Coastal Sciences from August - October 2014. These oysters were exposed to varying concentrations of the cit-AgNPs and pvp-AgNPs previously synthesized (see Chapter 1) as well as $AgNO_3$, sacrificed, and dissected.

3.2.3 30 MINUTE EXPOSURES

This preliminary study was designed to test which tissues of the oyster would be desirable for Ag accumulation analysis and to show that oysters collected from Oyster Landing were pristine organisms and therefore were not exposed to any pre-existing toxins or metals. First, two unexposed oysters were sacrificed and dissected. The gills (G), labial palps (LP), digestive tract (DT), and hepatopancreas (HP) were extracted and acid digested in trace metal nitric acid. This acidified tissue was diluted to 1% acid with DI and analyzed for elemental composition via ICP-OES to determine if there was any

pre-existing Ag, cadmium (Cd), lead (Pb), or zinc (Zn), within the tissues. These elements were chosen because in this study AgNPs are used and we wanted to confirm that detected Ag was from the addition of AgNPs and the other elements are known environmental contaminants. Pre-existing levels of contaminants would indicate the collection site is not pristine and could cause complications during analysis. Subsequent exposures were administered in 2 liter glass Pyrex beakers. All beakers were acid washed in HCl^-, for 24 hours and rinsed in DI before and between exposures. Each beaker contained a single oyster and 1800 ml of Baruch filtered sea water (BFSW, 0.2 μm filter) from the oyster collection site. Trials were conducted for 30 minutes at ambient salinity and pH (34-35 ppt, 7.6 - 8 .0 pH).

Oysters were exposed to 200 ppb (μg l^{-1}) of, cit-AgNP, pvp-AgNP, or $AgNO_3$. Each condition was repeated twice and all parts were dissected (G, LP, DT, and HP), acid digested, diluted, and analyzed via ICP-OES.

3.2.4 4 HOURS EXPOSURES

Exposures were administered in 2 liter glass beakers, all beakers were acid washed in HCl^-, for 24 hours and rinsed in DI before and between exposures. Each beaker contained a single oyster and 1800 ml of BFSW. All trials were carried out for 4 hours at ambient salinity and pH (34-35 ppt, 7.6 - 8 .0 pH). This study consisted of two experimental treatments, one control, and one comparison beaker; each was replicated five times.

Within the experimental treatments oysters were exposed to a range of four concentrations 1, 10, 15, and 50 ppb (μg l^{-1}). These trials consisted of two different AgNPs varying by the type of capping agent used during synthesis which resulted in a

specific surface material, either citrate or pvp (See Chapter 1). The comparison beaker tested the effect the same concentrations of $AgNO_3$ had on the oyster while the control beaker had no NP or Ag addition. Water samples were taken at time points of 0 and 4 hours. Once the exposure time elapsed the oyster was removed from the beaker, sacrificed, and the gills (G) and hepatopancreas (HP) were dissected and weighed. Both of the tissues were then cut in half and weigh separately. One half was placed in a plastic cryogenic elution tube (1.5 ml), wrapped in tin foil to reduce light exposure, and stored in -80°C. The other half was placed into a plastic 15 ml centrifuge tube with 1 ml of trace metal free nitric acid and placed in an incubator shaker (New Brunswick Scientific Innova 44 Incubator Shaker Series) at 300 rpm and 20°C, for 24 hours. The frozen half was saved for future lipid peroxidation assays; the acid digested half was used for Ag accumulation analysis via ICP-MS.

3.2.5 LIPID PEROXIDATION

Lipid peroxidation was quantified using malondialdehyde (MDA) concentration as an indicator of tissue damage from reactive oxygen species (Kelly et al. 1998; Ringwood et al. 1999). Tissues were homogenized in 50 nM potassium phosphate buffer (pH 7.0) and centrifuged for 15 minutes at 13,000 rpm, 4°C. The supernatants were extracted and combined with butylated hydroxytoluene (BHT), and trichloroacetic acid (TCA) containing thiobarbituric acid (TBA). A stock solution of 10 nM MDA was prepared and heated in a warm water bath (50°C) for 60 minutes. Standards of known MDA concentrations were prepared from this stock solution in concentrations ranging from 25 to 800 μM. Standards and samples were boiled in a water bath for 15 minutes, then

centrifuged for 5 minutes at room temperature (25°C), 13000 rpm. Supernatants were analyzed via spectrophotometry at 532 nm wavelength (μQuant Platereader, Biotek Instruments, Inc.).

3.3 RESULTS AND DISCUSSION

3.3.1 TISSUE ELEMENT ANALYSIS

Results are presented in ppb (μg l^{-1}) and not in μg-Ag g-tissue^{-1} because this was a quick preliminary study to identify antecedent levels of Ag and other contaminants. There were large amounts of Zn detected in all tissues, ranging from the highest value observed in the G (8.87 μg l^{-1}) and the lowest in the DT (1.01 μg l^{-1}) (Figure 3.1-A). Concentrations less than 0.02 μg l^{-1} of Cd and Pb were detected in all tested tissue (Figure 3.1-A). No preexisting Ag was seen in the LP, and below 0.09 μg l^{-1} was detected in the G, DT, and HP, therefore preexisting Ag concentrations are negligible (Figure 3.1B). Results between individual oysters were not significantly different. It is known that metal composition of oyster soft tissue seasonally fluctuates with elevated levels in the in the summer followed by depressed levels in the winter (Galtsoff 1964; Pringle 1968; Kopfler and Mayer 1969; Roosenburg 1969). Due to this phenomenon it is important to predetermine the amount of background trace elements in oyster tissues to create a baseline to be able to determine the source of accumulated Ag.

3.3.2 3O MINUTES EXPOSURES

For all tissues, accumulated Ag was detected in the largest quantities after exposure to $AgNO_3$ treatments (max=1.80 μg-Ag). Cit-AgNP treatments caused the second highest Ag accumulation (max=1.375 μg-Ag), and pvp-AgNP treatments accumulated at the

lowest quantities (max=0.75 μg-Ag) (Figure 3.2). Each of these highest accumulation values for each treatment were detected in the HP. The gills accumulated the second highest concentration over all treatments and accumulated relatively the same concentration for cit-AgNP and $AgNO_3$ treatments (1.33 μg-Ag and 1.42 μg-Ag) (Figure 3.2). The similarities in Ag accumulation between cit-AgNP and $AgNO_3$ treatments could indicate that cit-AgNPs are dissolving and the oyster is taking up Ag ions. The LP and DT accumulated the least amount of Ag in all treatments (>0.375 μg-Ag), however there were higher amounts of accumulated Ag detected in the DT when exposed to $AgNO_3$. The higher amount seen here could indicate that Ag ions may be taken up, ingested, and digested more readily than AgNPs, as shown by its presence in the DT after digestion occurs in the HP.

Ag accumulation after exposure to pvp-AgNPs was consistently the lowest across all of the tissues (Figure 3.2). This could be due to the sterically stable nature of these NPs. The oyster may have an aversion to the surface properties or presence of pvp once filtered into the mantle cavity and are therefore sorted by labial palps and rejected as pseudo feces. As discussed earlier it is known that *C. virginica* prefers organic particles rather than inorganic particles, this preference could be the reason why pvp-AgNPs are not selected for ingestion and therefore do not accumulate as much Ag as cit-AgNPs.

The LP and DT have almost identical patterns in the NP treatments, showing less than 0.25 μg-Ag. The LP results show low accumulation in all treatments with roughly 0.23 μg-Ag detected (Figure 3.2). Since the function of the LP is to sort particles, it is logical to observe low accumulation in this location. Due to the quick movement of particles through the LP the NPs are not being retained and do not have the chance to absorb into

the LP tissue. The DT varies from the LP results in that when exposed to $AgNO_3$ accumulation was slightly higher, ca. 0.30 μg-Ag. This is expected since it is known that metals, including Ag, accumulate in bivalves. The function of the DT is to transport waste from the HP after digestion. It is suspected that along with nutrients being absorbed, Ag is also absorbed in the HP before reaching the DT, therefore low values are logical since there is little Ag for the wall of the DT to absorb. The HP accumulated nearly 2 μg-Ag in the $AgNO_3$ treatment, this was the highest amount accumulated within any of the tissues. Accumulation in the gills and HP for the cit-AgNP (1.33 μg-Ag, 1.375 μg-Ag) and pvp-AgNP treatments (0.73 μg-Ag, 0.75 μg-Ag) were shown not to be significantly different (Figure 3.2).

Based on these results the gills and HP were selected for the 4 hour exposure study for the reason that they showed a peculiar similarity in accumulation patterns and values for all treatments and higher accumulation values than the LP and DT.

3.3.3 4 HOUR EXPOSURES

3.3.3.1 AG ACCUMULATION

A larger quantity of Ag was accumulated in the HP than in the gills. This can be attributed to the fact that oysters are filter feeders and the main function of the gills is to trap and transport food and particles. Water is pumped over the gills by the use of cilia, suspended particles are then trapped in mucus secreted by the gills and transported for ingestion or rejection as pseudo feces (Haven and Morales-Alamo 1970). It is logical to assume that the AgNPs would be passed quickly through the gills, decreasing the chance

for accumulation, and be retained, therefore concentrated, in the HP where digestion occurs.

A statistically significant increase in Ag accumulation was seen in the gills in the pvp-AgNP treatment of 10 ppb and in the $AgNO_3$ treatment of 15 and 50 ppb (Figure 3.3 A-C). There was no significant increase or difference between the concentrations in the cit-AgNP treatments ($p=0.073$). For HP tissues, a significant increase was not seen in the HP in any of the treatments (pvp-AgNP $p=0.479$, cit-AgNP $p=0.472$, $AgNO_3$ $p=0.188$) (Figure 3.4 A-C). Pvp-AgNPs accumulated the most in the moderate concentration of 10 ppb while cit-AgNPs accumulated the most in the high concentrations of 15 and 50 ppb. This could be due to the oyster sensing a difference in the charge of the cit-AgNPs or the capping agent of the pvp-AgNPs and rejecting them as pseudofeces therefore not incorporating it into its tissues.

There are similarities in accumulation patterns of cit-AgNP and $AgNO_3$ treatments. Both accumulated large quantities of Ag at the higher concentration exposures (15 and 50 ppb), with the largest amount accumulating in the HP, and $AgNO_3$ accumulating nearly 60 fold higher than cit-AgNPs. These mirrored results indicate and support our claim that cit-AgNPs dissolve in salt water media. These AgNPs are dissolving when added to the media and Ag is being taken up and accumulated as free Ag ions as it is in the $AgNO_3$ treatments. The Ag ions in both the cit-AgNP and $AgNO_3$ treatments could also be forming $AgCl^-$ after dissolution in the salt water media, as seen in Chapter 2 when exposed to dinoflagellates.

Although there were some differences in the accumulation of Ag due to capping agent, it cannot be determined if cit-AgNPs or pvp-AgNPs accumulated in larger

quantities. At exposure concentrations of 1 and 10 ppb cit-AgNPs showed larger quantities of accumulated Ag in both the gills and HP, and at concentration of 15 and 50 ppb pvp-AgNPs showed greater accumulation in both tissues. Therefore, the hypothesis that capping agent has an effect on NP uptake cannot be fully supported.

The degree in variability in accumulation between treatment exposures can also be attributed to the physiological characteristics of the individual oyster. Although filtration rates were not directly measured each individual was monitored for the duration of the exposure to ensure the oyster filtered for the entire 4 hours. Individual oysters could have different feeding habits or filtration rates and patterns which could cause an individual to filter faster or slower than another and as a result would have been exposed to a variable amount of AgNPs. The difference in accumulation may also be due in part to the preferential selection of organic particles for ingestion. It was stated previously that oysters will ingest organic over inorganic particles. Sodium citrate is an organic molecule and the oyster may show preference in selecting cit-AgNPs for ingestion rather than the pvp-AgNPs that are capped with an inorganic substance. Other considerations when looking at individual variability include the degree of sexual maturity. Oysters exemplify protandrous hermaphroditism where they start life as males and then switch to females as a result of sexual maturation (Aranda et al. 2014; Mann et al. 2014). Oysters were selected within the 6-10 cm shell size range to ensure the same gender of all test subjects.

3.3.3.2 LIPID PEROXIDATION

Results revealed the gill tissues experienced more LPx than the HP tissues. When looking at AgNP effect as a function of capping agent it can be determined that although

both AgNP types induced LPx, it was more prominent in the cit-AgNPs, i.e. caused more damage. Statistically significant LPx increases were observed in gill tissues in cit-AgNP treatments at concentrations of 15 and 50 ppb ($p<0.05$), $AgNO_3$ treatments at of 15 and 50 ppb ($p<0.05$), and there was no significant increase in LPx in the pvp-AgNP treatments ($p=0.674$) (Figure 3.5 A-C). Significant increases in LPx were observed in HP tissues in cit-AgNP treatments at concentrations of 15 ppb, in $AgNO_3$ treatments at 15 and 50 ppb ($p<0.05$), and there was no significant increase in LPx in the pvp-AgNP treatments ($p=0.497$) (Figure 3.6 A-C).

Cit-AgNPs caused oxidative stress in similar patterns in both the gills and HP. When comparing Figure 3.5 A and Figure 3.6 A, a relatable accumulation pattern can be seen in both tissues with LPx seen in the 15-50 ppb exposures and no toxic effect seen in 1-10 ppb. A nearly parallel pattern can also be seen in the $AgNO_3$ treatments. These results indicate that cit-AgNPs and $AgNO_3$ are toxic to *C. virginica* at high concentrations (15-50 ppb) causing MDA levels to increase. Findings also support the claim that cit-AgNPs are dissolving in solution and the oyster is being exposed to and accumulating Ag as free Ag ions or AgCl. Another possibility is that the cit-AgNPs are being taken up into the oyster and then dissolving inside the HP. Once inside the HP Ag ions are released directly to the HP tissues when digested causing a result similar to that of the $AgNO_3$ treatments. Results also signify that pvp-AgNPs are not toxic to *C. virginica* and do not cause oxidative stress, as seen by the observed MDA concentrations below the accepted control values for LPx at all exposure concentrations.

The background MDA concentration is produced by aerobic breathing of the organisms which causes some level of oxidative stress. Control values should be roughly

200 nM MDA for HP tissues and 150 nM for gill tissues. In pvp-AgNP treatments all exposure concentrations resulted in MDA levels that were less than the control. It is thought that the presence of pvp-AgNP stimulates a response in antioxidant production and therefore increases the amount of antioxidants such as catalase and superoxide dismutase (SOD) therefore decreasing the amount of MDA (McCarthy et al. 2013). Similar studies have shown that when exposed to pvp alone there is a depressed amount of MDA in oyster gill and HP tissue (McCarthy, unpublished). This indicates that pvp stimulates an antioxidant production and not oxidative stress (toxic response) which is the opposite of what is expected. Cit-AgNPs show the same response in the lower exposure concentrations (1-10 ppb) in both the gills and HP indicating that the same increase in antioxidant species is occurring here as it did in the pvp-AgNP exposures. However, increased levels of MDA were seen in the higher exposure concentrations (15-50 ppb) in both tissues, indicating that higher concentrations of cit-AgNPs induce a toxic response. The same pattern can be seen in the $AgNO_3$ treatments where 15 and 50 ppb cause a toxic effect but 1 and 10 ppb result in MDA levels below the control (Figure 3.5 A-C and Figure 3.6 A-C). The similarity in LPx between $AgNO_3$ and cit-AgNPs can again be explained by dissolution of cit-AgNPs and therefore exposure o Ag ions which are known to be toxic to estuarine organisms. The observed values of LPx for both cit-AgNP and pvp-AgNP treatments are not enough to conclude there is a toxic effect seen after exposure. However, as seen in the depression of MDA concentrations there is definitely some kind of response occurring within the organism.

At first, the results seen in this study appear to be related to the phenomenon of hormesis. Currently, there is a great deal of confusion and controversy surrounding the

concept and definition of this phenomenon. Pickrell and Oehme (2005) describe hormesis as the stimulation of a biological process at low concentrations of a toxin, followed by inhibition as doses increases (Pickrell and Oehme 2005). Mattson and Calabrese (2009) believe it to be a beneficial response to low levels of toxicants, while Kendig et al. (2010) describe hormesis as a reversal of the responses between low and high doses based on the traditional dose response curve and does not create a beneficial response. It cannot be determined if there was a beneficial response in the oysters during exposure to low concentrations of AgNPs, therefore the results of this study best fit the definition provide by Pickrell and Oehme (2005). In a review of pre-existing literature Pickrell and Oehme (2005) determined that while hormesis provides a more complete understanding of information than the traditional dose response it cannot be used to generally describe all toxicant interactions and responses. In the case of certain contaminants and effects (pulmonary fibrosis, colorectal cancer, and malodorants) hormesis does not provide sufficient information for solving the problem of reversed results between low and high exposures. Much of the confusion over generalizing hormesis comes from the lack of research and approaches to specifically quantify its characteristics. Most of the research supporting and rejecting hormesis come from literature searches and reviews instead of studies directed specifically at hormesis. It has been argued that hormesis cannot be used to describe generalities in data and cannot be used as a default or dose-response model for contamination and regulation until 100% hormesis frequency is reached (Crump 2001; Shrader-Frechette 2010; Mushak 2013).

Due to the uncertainty and controversy of hormesis, we cannot for certain say our data is demonstrating this biological response, therefore we propose the idea of bio-

reactivity. The presence of low concentrations of AgNPs is enough of a signal to stimulate a protective response, not necessarily toxicity. In an unexposed oyster a response to a contaminant or unknown foreign particle would cause a response that creates a stress itself (Moore 2009). This stress induces the up regulation of antioxidants that balances out the oxidative stress, therefore MDA and antioxidant levels would neither raise nor fall, keeping the balance between oxidative damage and antioxidant production.

Due to short exposure time and low concentrations, the absence of a significant toxic effect is not unexpected. The majority of studies have investigated NP uptake and toxicity under longer exposure times that range from 48 hours to week long studies at higher concentrations than we tested (20-500ppb) (Ringwood et al. 2009; Kadar et al. 2010, Ringwood et al. 2010). Although we did see a negative impact at higher concentrations, the lower concentrations showed LPx values that were lower than the control. Bio-reactivity of oysters can explain the low levels of MDA within the tissues and is logical due to the short 4 hour exposure time and low concentration (1 and 10 ppb). Nonetheless, in order to claim that it is a bio-reactive response or hormesis that is occurring in our samples more trials need to be conducted where we specifically look for each of these phenomena properly.

3.1 CONCLUSION

This study showed that AgNPs are taken up by *Crassostrea virginica* and accumulated in the gills and HP. This provides evidence that *C. virginica* can be used as a sentinel species for monitoring and aquatic contamination programs. It was determined

that when exposed to two different types of AgNPs there is a larger amount of Ag accumulated in the HP than in the gills, resulting in a tissue specific response. However, we were not able to identify a concrete effect due to capping agent of the AgNP. The high exposure concentrations (15 and 50 ppb) of both particles accumulated more Ag than the low concentrations (1 and 10 ppb). After exposure to these AgNPs we did see a significant difference from the controls in LPx in the 15 and 50 ppb exposures in cit-AgNP and $AgNO_3$ treatments but no difference in pvp-AgNPs. Overall there was more LPx seen in the gills than in the HP, and exposure to cit-AgNPs resulted in a larger amount of LPx than pvp-AgNPs which showed to have relatively no toxic effect at an exposure concentration. Although a tissue specific and AgNP specific affect were seen in this study and MDA concentrations were abnormally low as compared to controls, there is not enough evidence to claim if the response is hormesis, bio-reactivity, or toxicity. It is thought that the presence of AgNPs stimulate a response in antioxidant production and therefore increase the amount of antioxidants such as catalase and SOD resulting in a suppressed amount of MDA. The results of this study provide valuable information on the potential risks that can occur from exposure to AgNPs. Estuarine bivalves are taking up and ingesting AgNPs, there may not be an identifiable source of toxicity but the accumulation of AgNPs creates issues by itself. Oysters couple benthic and pelagic components of ecosystems through filtration and excretion. If the NPs are present in the surrounding water column the oysters will filter the NPs into its mantle cavity. We have shown that AgNPs do accumulate in oysters, however we did not test the amount of Ag released as feces or pseudo feces. These are two key components that future studies should examine because multiple organisms that inhabit oyster reefs or feed off of both

these excretions. If AgNPs are incorporated into these excretions as they are in oyster tissues then the associated organisms are also at risk as these AgNPs are reintroduced to the environment through aggregation, dissolution, flocculation, sedimentation, or resuspension.

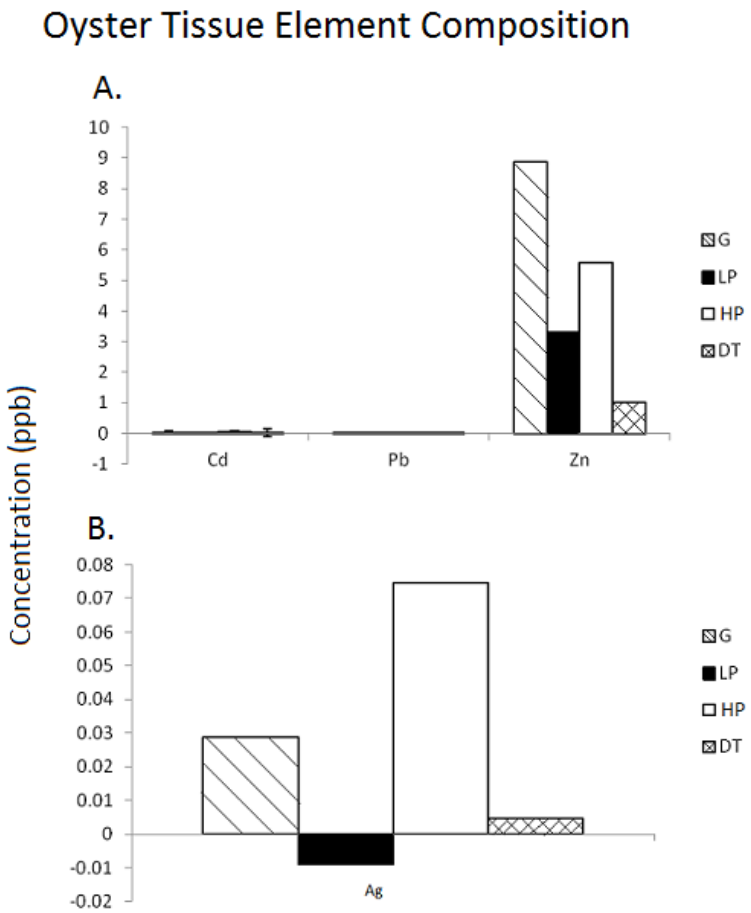

Figure 3.1 A-B: Elemental composition analysis of tissues from an unexposed oyster via ICP-OES. Tissues sampled include the gills (G), labial palps (LP) digestive glad (DG), and the digestive tract (DT). This was replicated twice with two different oysters. A) Includes the elements cadmium (Cd), Lead (Pb), and zinc (Zn). B) Includes only silver (Ag) to better visualize the minimal amount of silver present in tissues before exposures.

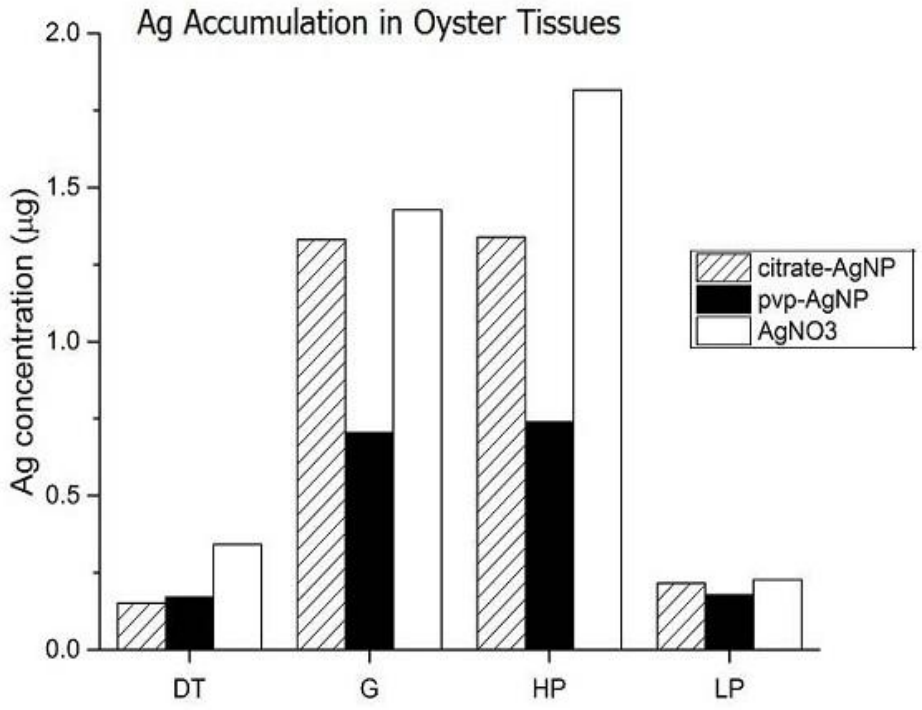

Figure 3.2: Accumulation of silver within oyster tissues after a 30 minute exposure time via ICP-OES. Tissues sampled include the gills (G), labial palps (LP) digestive glad (DG), and the digestive tract (DT). This exposure was duplicated.

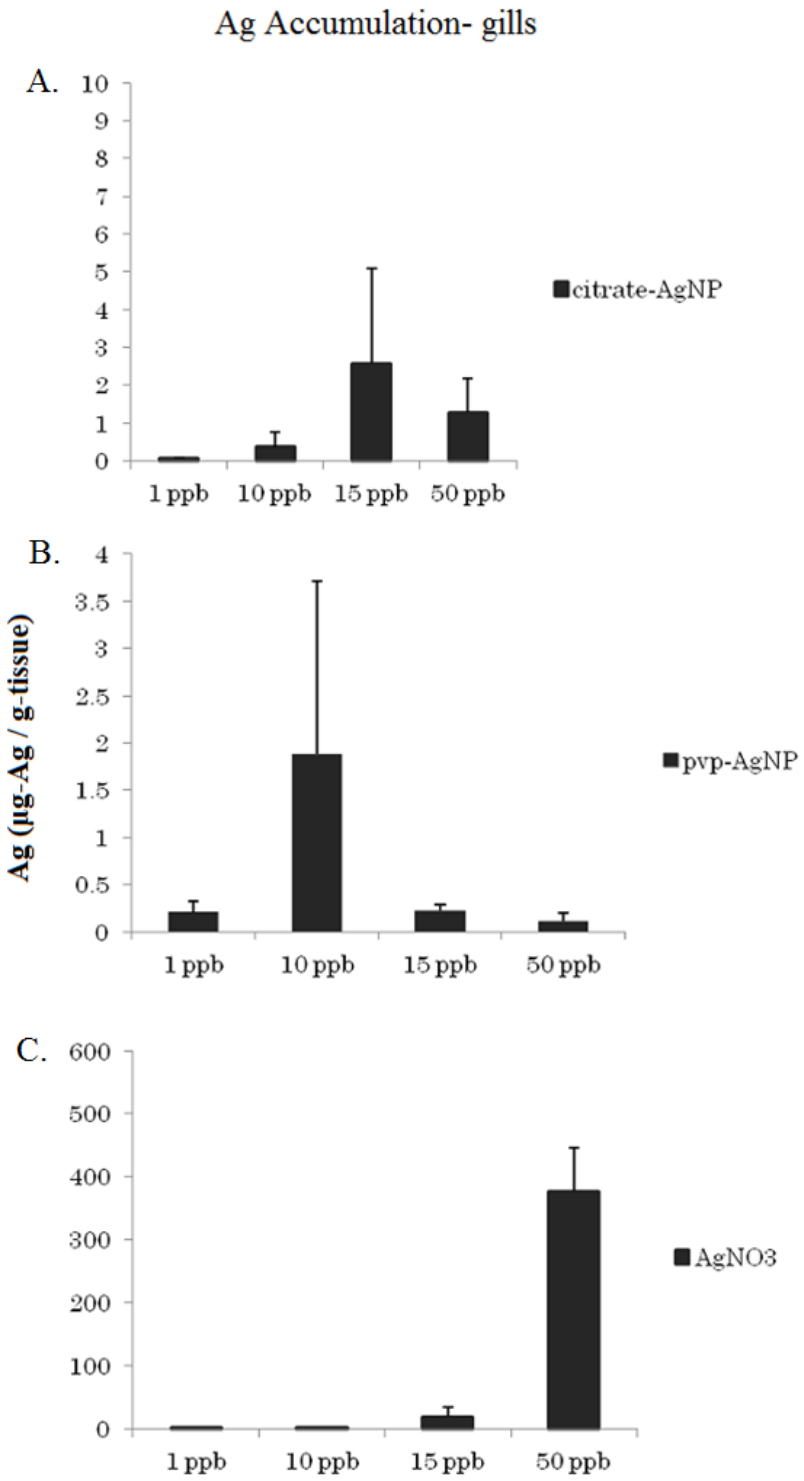

Figure 3.3 A-C: Accumulation of Ag detected after 4 hour exposures in oyster gill tissue. Each panel represents a different treatment A) cit-AgNP B) pvp-AgNP C) $AgNO_3$. Data are means and standard deviations, based on wet weight. Asterisks (*) indicate a statistically significant difference from the control.

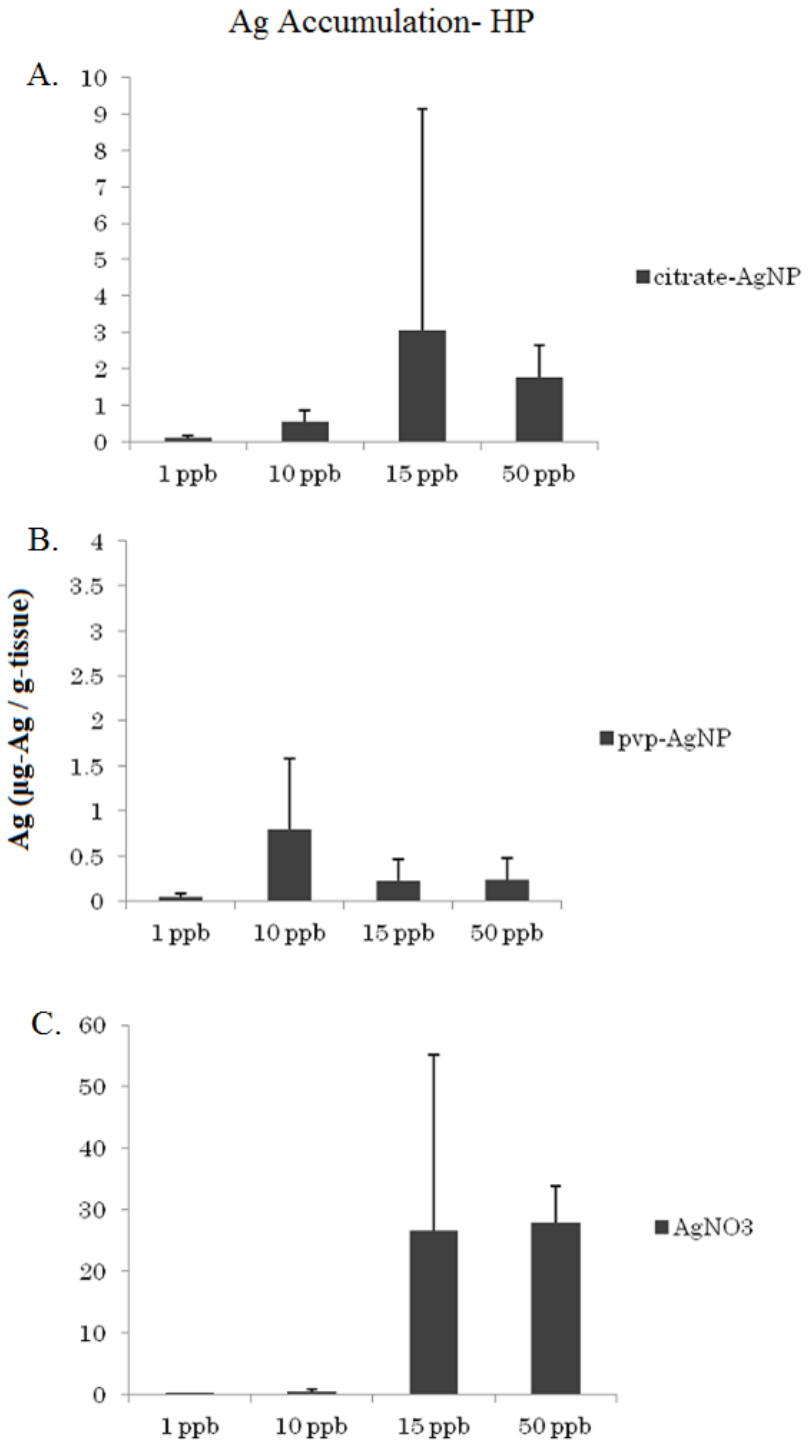

Figure 3.4 A-C: Accumulation of Ag detected after 4 hour exposures in oyster hepatopancreas (HP) tissue. Each panel represents a different treatment A) cit-AgNP B) pvp-AgNP C) $AgNO_3$. Data are means and standard deviations, based on wet weight. Asterisks (*) indicate a statistically significant difference from the control.

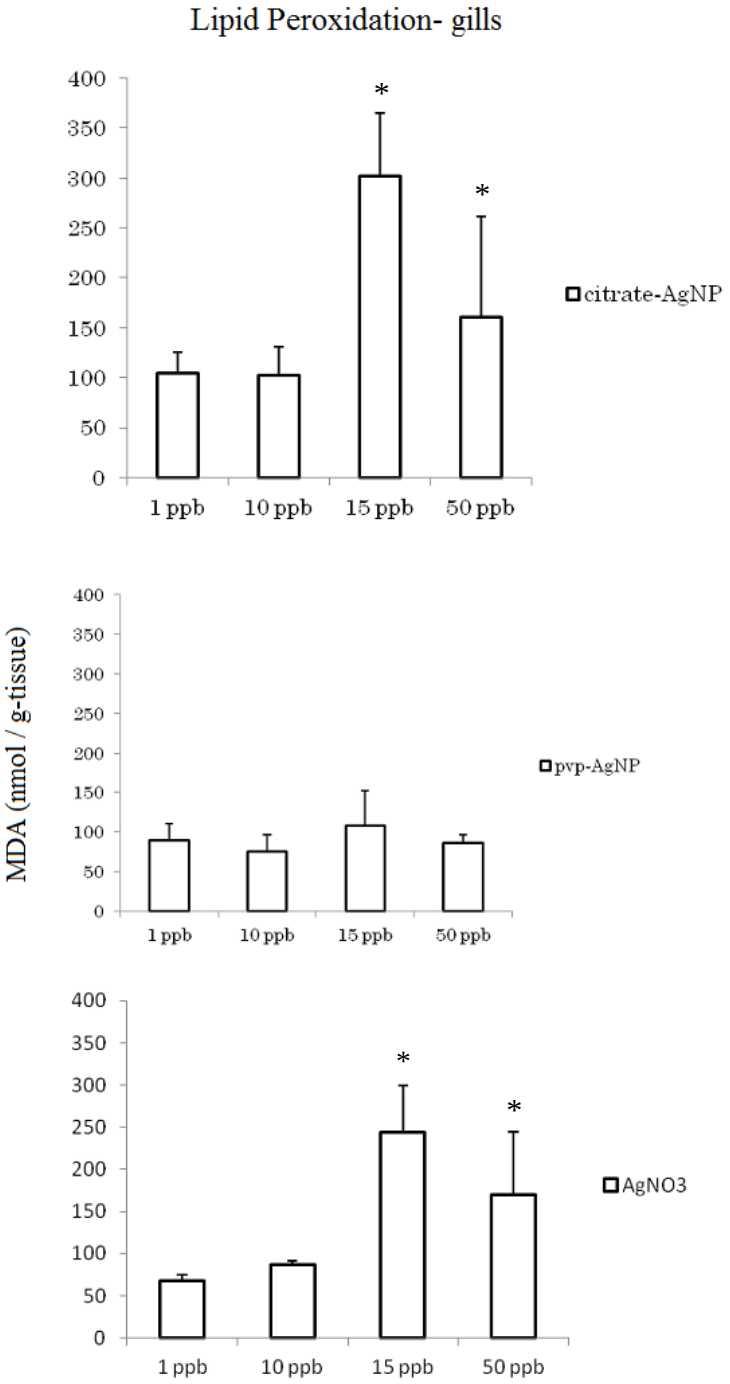

Figure 3.5 A-C: Effects of AgNP treatment on lipid peroxidation of oyster gill tissue after 4 hour exposures A) cit-AgNP, B) pvp-AgNP, C) $AgNO_3$. Data are means and standard deviations, based on wet weight. Asterisks (*) indicate a statistically significant difference from the control.

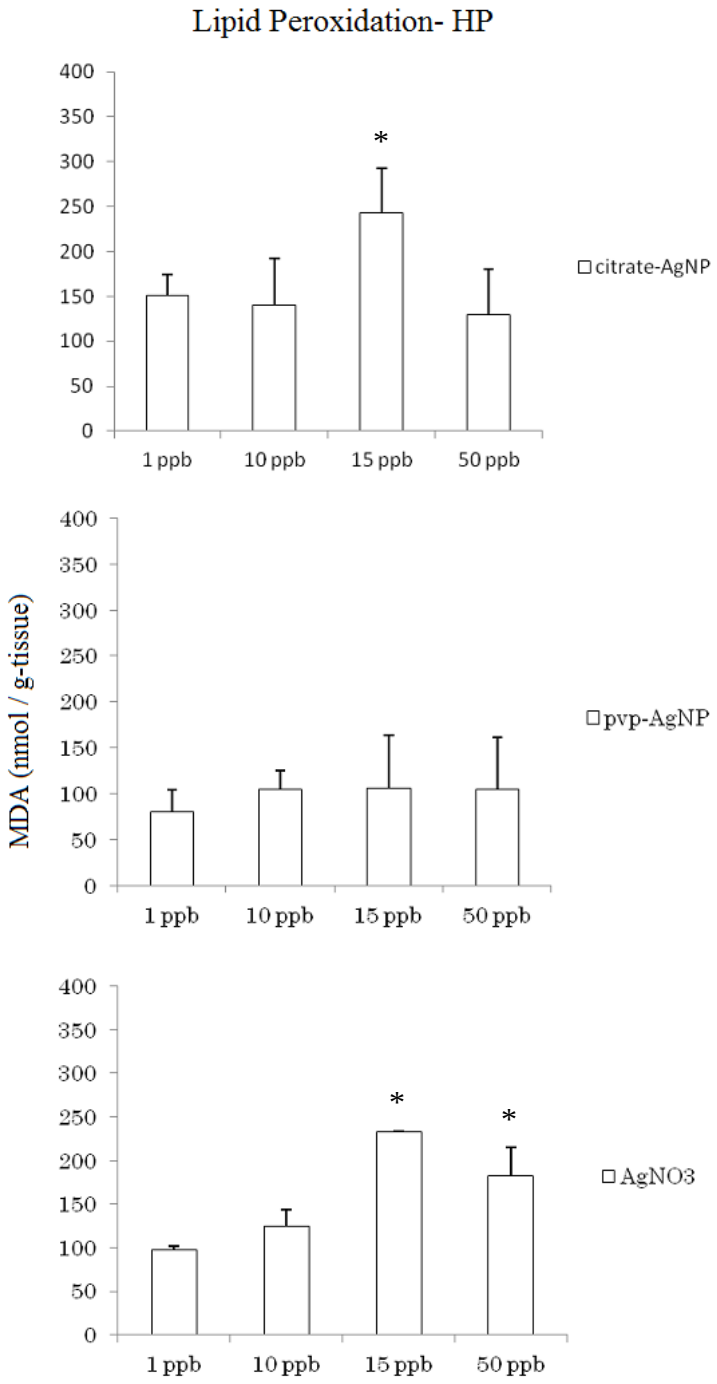

Figure 3.6 A-C: Effects of AgNP treatment on lipid peroxidation of oyster hepatopancreas tissue after 4 hour exposures A) cit-AgNP, B) pvp-AgNP, C) $AgNO_3$. Data are means and standard deviations, based on wet weight. Asterisks (*) indicate a statistically significant difference from the control.

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