Relative Importance of Ions and Particles in Silver Nanoparticle Uptake and Accumulation in Keystone Estuarine Organisms; Prorocentrum minimum and Crassostrea virginica

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Relative importance of ions and particles in silver nanoparticle uptake and accumulation in keystone estuarine organisms; *Prorocentrum minimum* and *Crassostrea virginica*

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ABSTRACT

In marine systems, silver is regarded as being one of the most toxic and readily accumulated metals, and is known to be taken up and accumulate within marine invertebrates, fish, phytoplankton and seaweeds. Reported open ocean concentrations of silver range from 0.1 – 2 ng L\(^{-1}\) while silver concentrations in coastal waters are typically 5 to 10 times higher than open ocean values (0.1 - 50 ng L\(^{-1}\)). The determination of silver in marine and coastal waters presents several challenges including low concentrations and matrix complexity of seawater, which creates interferences and other challenges for many instrumental techniques creating a requirement for additional separation steps. On-line matrix removal of the salt matrix prior to inductively coupled plasma mass spectrometry (ICP-MS) analysis has been developed to overcome these problems. A solid phase extraction method for the quantification of dissolved silver in seawater was developed using Dowex 1-x8 anion exchange resin, 0.1 M thiourea/0.05% nitric acid solution, and quantification by ICP-MS. Recoveries of 102.8 ± 4.1% were obtained for 25 µg L\(^{-1}\) silver spiked natural seawater samples. Using a 50 mL sample, a preliminary method detection limit of 66 ng L\(^{-1}\) (n=14, DI water samples) was achieved.

Silver nanoparticles (AgNPs) have become a central component in a wide range of commercial and consumer products including electronic, biomedical, and pharmaceutical
drug delivery applications. Due to their unique properties, such as electronic constraint and high specific surface area, AgNPs exhibit novel behaviors that may affect biological processes such as uptake and accumulation, which might be significantly different to that of the bulk material or free silver (Ag) ion. Due to their partial solubility, studies show little agreement as to whether the effects and behavior of AgNPs are controlled by ions from dissolution of particles, or the particles themselves.

Bi-metallic nanoparticles (NPs), specifically core-shell NPs, offer a higher degree of functionality compared to single element NPs; Au and Ag have been used in this study in a core-shell conformation to allow better understanding of the impact of Ag solubility on bioavailability and bioaccumulation. The inner (Au or Ag107) core is protected from interacting with the media due to the Ag or Au/Ag109 outer shell. Therefore, Au or Ag107 can be used as a standard measurement of particle behavior and ratios of Ag and Au or isotopic ratios of Ag can be used to help explain the mechanisms influencing Ag and AgNP uptake. In this study we use Au@Ag NPs for exposure studies with an estuarine dinoflagellate, *Prorocentrum minimum*, and 107Ag@Au@109Ag NPs for exposure studies with an estuarine bivalve, *Crassostrea virginica*. This allowed the quantification of the contribution of ion and NP Ag bioavailability, *ie.*, to understand the relative roles of ion and NP in AgNP uptake and accumulation.

Results in all cases showed that 60-85% of added Ag remained in aqueous suspension, while 10-30% of added Ag was detected in the dissolved phase. After exposure to Au@Ag *P. minimum* accumulated less Ag mass than seen in the AgNO₃ exposures after 72 hours
with 0.17 µg-Ag 100,000 cells⁻¹ and 0.5 µg-Ag 100,000 cells⁻¹, respectively. Cell densities decreased in a dose-dependent manner after exposure to Au@Ag and we speculate that algae are able to recover from initial exposure to Au@Ag after 24 hours and continue to increase in cell density. Although algae were able to recover, densities remained 10-20% below the control growth densities at the same time point. Conversely, there was continual decrease in cell densities after exposure to AgNO₃ with a 94.9% decrease in the 50 µg L⁻¹ exposure. Thus, we speculate that the potential mechanisms of AgNP uptake and accumulation are likely to be associated with NP dissolution and the interaction of dissolved Ag in the form of silver-chloride complexes, ripened AgNPs, or smaller NPs formed from the nucleation of dissolved Ag. In addition, there is a nano-specific result in accumulation of Ag as shown by the Ag:Au ratios associated with the algal pellet and dissolution results (3 kDa, ultrafiltered fraction) which confirms Au did not dissolve and remained as a NP.

After exposure to 10⁷Ag@Au@10⁹Ag, the average Ag uptake by C. virginica was a small fraction (8.6 – 9.0 ± 3.4 %) of the total available Ag. Compared to the isotopic ratios measured for the original core-shell AgNPs, we found increased accumulation of 10⁷Ag in the 1 µg L⁻¹ exposures and the opposite trend (increased accumulation of 10⁹Ag) in the 50 µg L⁻¹ exposures. There was variability among individual oysters both within a size class and across size classes. The hepatopancreas accumulated the most Ag in the 1 µg L⁻¹. The same trend was not seen in the 50 µg L⁻¹ exposures. The F2 size class (reproductively mature females) accumulated the most Ag in the 1 and 50 µg L⁻¹ exposures, as compared to the other size classes. We interpreted results as preferential uptake of NPs with a reduced
Ag109 outer layer at 1 µg L⁻¹ 107Ag@Au@109Ag exposures. Furthermore, the increased accumulation in the hepatopancreas suggests that oysters are selecting AgNPs for ingestion. We explain higher 109Ag accumulation in 50 µg L⁻¹ exposures as preferential uptake of nucleated AgNPs (containing only 109Ag) formed from dissolution of the 109Ag outer layer. Individual variability may be due to differences in filtration rate (1-1.5 L h⁻¹ g⁻¹ tissue weight⁻¹), where larger oysters filter a larger volume of water which may results in greater amounts of Ag accumulated in tissues. Differences could also be caused by differences in energetic demands between oyster size classes or sex. For example, reproductively active individuals (M2 and F2) may require more nutrients to replenish energy spent on creating gametes as compared to not reproductively active individuals (M1 and F1).

The main reason for this study was to identify the mechanisms that facilitate AgNP uptake and accumulation by measuring ionic and NP Ag in estuarine organisms after exposure to AgNPs. We have shown that Ag is taken up and accumulated (or strongly bound) within both organisms after exposure to AgNPs with differences in uptake between the particles and ions observed. In both algae and oyster experiments, at low exposure concentrations (1 µg L⁻¹) there was either less Ag than Au accumulation or less 109Ag than 107Ag suggesting that Ag is take up in the NP form. In exposure concentrations of 50 µg L⁻¹ there was more Ag than Au uptake or more 109Ag than 107Ag for algae and oysters, respectively. This suggests that at higher concentrations, ionic Ag is preferentially taken up, most likely in a complexed or nucleated form. Consistent with previous research, our results confirm that Ag is taken up and accumulated by algae and oysters in a dose and
time dependent manner when exposed to AgNO₃ and has a greater accumulation and inhibition than seen in AgNP exposures. From these data and at current environmental levels of AgNPs, however, no immediate risk of AgNPs to *P. minimum* or *C. virginica* is indicated. Further testing and mechanistic understanding of AgNP interactions with marine algae and bivalves should be continued in order to fully understand the mechanisms of AgNP uptake and accumulation and the subsequent effect on estuarine ecosystems.
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LIST OF ABBREVIATIONS

Ag.................................................................Silver
AgENM(s).................................................. Silver engineered nanomaterial(s)
AgNM(s)....................................................... Silver nanomaterial(s)
AgNO₃........................................................ Silver nitrate
AgNP(s)....................................................... Silver nanoparticle(s)
Ag₂S ........................................................ Silver sulfide
Au@Ag ...................................................... Gold Core-Silver Shell Nanoparticles
Cl.................................................................Chloride
DI ..............................................................De-ionized Analytical Reagent-grade Water
DLS .............................................................Dynamic light scattering
ENP(s)........................................................ Engineered nanoparticle(s)
FI-AAS ......................................................Flow injection atomic absorption spectrometry
FI-ICP-MS ...............................................Flow injection inductively coupled plasma mass spectrometry
GF-AAS ....................................................Graphite furnace atomic absorption spectrometry
HCl .............................................................Hydrochloric acid
HNO₃ ..........................................................Nitric acid
HP ..............................................................Hepatopancreas
ICP-MS ......................................................Inductively coupled plasma mass spectrometry
ICP-OES ....................................................Inductively coupled plasma optical emission spectrometry
ID-ICP-MS.................. Isotope dilution inductively coupled plasma mass spectrometry
LOD .................................................................Limit of detection
NM(s).................................................................Nanomaterial(s)
NP(s).................................................................Nanoparticle(s)
pdi .................................................................Polydispersity index
PEC .................................................................Predicted environmental concentrations
PVP .................................................................Polyvinylpyrrolidone
S .................................................................Sulfur
SEM .................................................................Scanning electron microscopy
UV-vis...............................................................Ultraviolet visible spectrometry
VM .................................................................Visceral mass
INTRODUCTION

Engineered nanoparticles (ENPs), in the simplest definition, are particles that have at least one dimension that is less than 100 nm in size which exhibit novel size dependent properties (K Elaine et al. 2012). Since particles are three dimensional multiple agencies have defined descriptions of ENPs that vary slightly from one another. However, the American Society for Testing and Materials defines, a nanoparticle (NP) as a particle that has two or three dimensions between 1 – 100 nm (ASTM E2456). In October 2011 the European Union defined nanomaterials as a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or agglomerate; where 50% or more of the particles exhibited, one or more external dimensions in the size range 1–100 nm (Rauscher et al. 2019). Thus, for this research we define ENPs as 1 – 100 nm in two dimensions.

NPs often exhibit unique physio-chemical properties and reactivities due to their small size, homogenous structure, and surface characteristics that are not present at the larger scale such as electron spatial constraints, large specific surface area and high surface energy. Due to their small size, NPs have a high surface to volume ratio, thus the proportion of atoms on the surface versus the interior of the NP is much larger, with uncoordinated dangling bonds, than bulk sizes of the same material which aid in surface reactivity (Jefferson 2000, Poole and Owens 2003, Madden et al. 2006, Oberdörster et al. 2007,
Handy et al. 2008, Ju-Nam and Lead 2008). These properties give rise to unique environmental behaviors, such as toxicity increasing with surface area and considerable changes in crystalline structure that enhances surface reactivity, compared to their macroscale counterparts (Barlow et al. 2005, Auffan et al. 2009, Croteau et al. 2011). For example, research has shown that zinc oxide, aluminum oxide, and silicon oxide NPs are more toxic than their bulk counterparts (Wang et al. 2009, Jiang et al. 2009) and that cerium oxide NPs were more toxic than bulk cerium (Kuang et al. 2011, Arnold et al. 2013). In recent studies investigating the differences in toxicity between ions and NPs it has been reported that copper oxide and nickel oxide NPs are more toxic than copper and nickel ions (Baek and An 2011, Thit et al. 2017). It has also been shown that iron oxides can be thermodynamically stabilized at the nanoscale and that hematite NPs catalyze the oxidation of aqueous Mn$^{2+}$ which increases iron solubility in natural waters (Navrotsky et al. 2008, Hochella et al. 2008). However, for some ENPs such as zinc oxide and silver toxicity is still disputed (Wang et al. 2016, McGillicuddy et al. 2017). As a result of potential use, the U.S. National Nanotechnology Initiative (NNI) reported an estimated $1.4 billion in the President’s 2019 Budget allocated for investment and support of fundamental nanoscale, engineering technology research and development (National Science and Technology Council, 2018).

ENPs are currently used in a variety of applications due to novel behavior including, electronics, pharmaceutical drug design, cosmetics and clothing manufacturing, (Nowack and Bucheli 2007, Piccinino et al. 2012). For example, gold NPs are commonly used to coat sampling for scanning electron microscopy (SEM) to enhance the electron stream (Khan
et al. 2017). Carbon nanotubes are currently used as gas adsorbents for environmental remediation (Ngoy et al. 2014), energy storage and sensors (Baughman et al. 2002) while TiO$_2$ NPs are used in photocatalysis (Mital and Manoj 2011) and in commercial applications in coatings, plastics, papers, inks, and medicines (Shi et al. 2013). The unique physio-chemical properties of silver nanoparticles (AgNPs) make this type of NP of high interest and use are their high electrical and thermal conductivity (Capek 2004, Frattini et al. 2005) as well as their antimicrobial and bactericidal properties (Kim et al. 2007, Morones et al. 2005). Electrical and thermal conductivity is of special interest in microelectronic and medical imaging technologies while the antimicrobial properties are employed in consumer products including cosmetics (Vance et al. 2015), medical supplies (Chaloupka et al. 2010, Prabhu and Poulouse 2012) fabrics (Benn and Westerhoff 2008, Kulthong et al. 2010), children’s toys (Quadros et al. 2013) and industrial products such as building facades (Kaegi et al. 2010). As of 2016, The Project on Emerging Nanotechnologies has identified over 1800 products that contain ENPs and of that, 443 containing AgNPs (24%), thus, making AgNPs the most commonly used ENP in consumer products (Nanotechnologies 2017) 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, 2016, Wijnhoven et al. 2009, Weir et al. 2008, Yoon et al. 2008, Tulve et al. 2015).

As a consequence of their growing consumer use, NPs are introduced into the aquatic environment mainly through wastewater (Kakavandi et al. 2014, Adam et al. 2018) and
biosolids from consumer used products such as wearing and washing textiles (Benn and Westerhoff, 2008, Kulthong et al. 2010, Lorenz et al. 2012), cosmetics (Nowack and Bucheli 2007) and silver embedded washing machines (Farkas et al. 2011). When introduced to waste water treatment plants, AgNPs interact with sulfur (S) and chloride (Cl\textsuperscript{−}) and undergo transformations such as sulfidation or complexation with Cl\textsuperscript{−} (Adam et al.2018). Environmental concentration modeling showed that only 16-22% of AgNPs that enter the aquatic environment are in “pristine form” (here meaning in the same shape, size, and form as originally synthesized for commercial purposes) and that the majority (34-58%) of AgNPs present in surface waters were transformed with 90-95% of AgNPs being in the sulfidized form (Adam et al. 2018). Recent research further supports environmental modeling showing that the majority of Ag present in biosolids after wastewater treatment is in the form of silver sulfide (Ag\textsubscript{2}S) (Ma et al. 2014, Kaegi et al. 2015) and only a small fraction of AgNPs are introduced into aquatic surface waters (Kaegi et al. 2013). Following material flow through the AgNP lifecycle, Meier et al. (2016) found that after incineration, Ag\textsubscript{2}S was not detected in ash, rather, Ag was detected as metallic Ag and therefore was transformed again but was shown to remain at the nanoscale. During washing of textiles AgNPs can also complex with Cl\textsuperscript{−} or S to form either AgCl or Ag\textsubscript{2}C, respectively (Impellitteri et al. 2009, Lorenz et al. 2012, Mitrano et al. 2014) although AgCl seemed to be the dominate form. It has been hypothesized that in the sulfidized form, Ag become less available and less toxic to aquatic organisms (Leblanc et al. 1984, Hirsch 2009) but when ozone treatment is applied during wastewater treatment, toxicity increases to levels comparable to Ag\textsuperscript{+} treatments (Thalmann et al. 2015). In addition, interactions with S and Cl\textsuperscript{−} have been shown to decrease the dissolution of Ag ions from AgNPs in aquatic media,
most likely due to the formation of AgCl complexes, after Ag ion release from Ag$_2$S, which become less bioavailable (Li et al. 2015, Levard et al. 2013).

AgNPs can also be discharged into the environment through diffuse sources such as runoff and stormwaters, which transport NP effluent from building facades (Kaegi et al. 2010), city roads and highways (Nowack and Bucheli 2007, Lowry and Casman 2009). It is estimated that about 80% of the total amount of Ag contained in the paints and building facades is released into the environment (Kaegi et al. 2010) and are readily absorbed to available surfaces such as organic material close to the emission source. AgNPs bound in organic matter facilitates further transport from the emission site and increases the difficulty to predict environmental transformations (McCarthy and Zachara 1989, Kretzschmar et al. 1999, Lecoanet et al. 2004, Kaegi et al. 2010). The speciation of AgNPs is a key parameter affecting bioavailability, uptake and accumulation in the environment (Ratte, 1999, Stasinakis and Thomaidis 2010). Current research suggests that the dominant form of Ag in sewage is Ag$_2$S (Lytle 1984, Herrin et al. 2001, Kim et al. 2010) and it has been shown that the potential transformations with S could reduce the antimicrobial ability of AgNPs (Ratte, 1999, Kaegi et al. 2010). These studies add to the importance to investigate and understand the transformations of AgNPs in complex environmental matrices to better understand the mechanisms that affect bioavailability, uptake and accumulation of Ag.

Figure 0.1 show a general structure of material flow of ENPs throughout the entire lifecycle (production, release, transport, and fate). Although there is no direct analytical
data of AgNP concentrations and form in the environment, currently there are attempts
made to model and predict the future environmental concentrations of some of the most
frequently used nanoparticles, such as AgNPs (Figure 0.2). Current predicted
environmental concentrations (PEC) modeled from substance flow analysis quantified
from NP product released into the environment from waste incineration plants, landfills,
and/or sewage treatment plants for AgNPs in the aquatic environment currently at 0.03 ±
0.0015 μg L\(^{-1}\) (Tiede et al. 2009, Mueller and Nowack, 2008). This model accounts for
what the author calls a “high exposure scenario”. These values would be indicative of the
worst-case scenario when determining the concentration of AgNPs in the environment and
reflect concentrations that may be present in highly urbanized or industrialized areas. Once
AgNPs are introduced to the aquatic environment a plethora of chemical and physical
interactions, such as sulfidization, scavenging, wave action, and currents, aid in diluting
and dispersing the initial introduction of AgNPs from the emission source. Therefore,
although it is beneficial to model high exposure scenarios, it is unlikely that these
concentrations of AgNPs are present in the natural aquatic environment. However, the
frequent and increasing use of AgNPs in commercial and industrial products increases the
potential for these particles to enter the environment and pose an environmental concern.

Release of AgNPs into the aquatic environment is of special concern because AgNPs
are partially soluble in complex aquatic media and therefore release Ag ions through
dissolution (Bondarenko et al. 2013, Krystek et al. 2015). It is known that dissolved Ag
ions are highly toxic to fish, crayfish, daphnia (Davies et al. 1978; Wood et al. 1996;
Grosell et al. 2002; Bianchini and Wood 2002) and aquatic microorganisms (Spadaro et al.
1974, Slawson et al. 1992, Ratte, 1999, Zhao and Stevens 1998, Sondi and Salopek-Sondi 2004, Shahverdi et al., 2007). However, AgNP studies show little agreement as to whether the effects and behavior are controlled by Ag ions from resulting from NP dissolution (Chernousova and Epple 2013), the direct effects of AgNPs on organisms (Piccapietra et al. 2012, Kroll et al. 2014, Shah et al. 2014, Akter et al. 2018), or a combination of both with mainly ion causing the effect (Leclerc and Wilkinson 2014). It is suggested that free Ag ions are the most toxic of Ag species (Hogstrand and Wood 1998), however previous research suggests that complexation of Ag by Cl-, S, and dissolved organic carbon (DOC) reduce ionic Ag bioavailability and toxicity (Leblanc et al. 1984, Hogstrand and Wood 1998). The aquatic marine and estuarine environments contain ppm levels of chlorides, sulfides, and DOC (Menzel and Vaccaro 1964, Howarth and Teal 1980, Luther et al. 1986, Vetter et al. 1989, Millero et al. 2008), therefore it is suggested that Ag₂S dominates speciation under reducing conditions while other Ag species are more important in oxidizing waters (Hogstrand and Wood 1998). Therefore, it is reasonable to conclude that the roles of ions and particles in AgNP uptake and accumulation have not been fully elucidated.

Biouptake of NPs and ions released by their dissolution is often a prerequisite to subsequent human or environmental toxicity. Bioaccumulation has been measured in a number of studies (Leclerc and Wilkinson 2014, Croteau et al. 2013, Hou et al. 2013) and nano-specific differences in comparison to the dissolved and larger particle biouptake have been observed (Croteau et al. 2011, Croteau et al. 2014, Khan et al. 2012). In some cases, such as zinc oxide NPs, dissolution alone has often been sufficient to explain toxicity
In the case of poorly soluble NPs such as ceria, dissolution plays a lesser role (Collin et al. 2014, Merrifield et al. 2013). However, the methods for understanding the relative role of ion and particle in uptake and accumulation are not fully developed. Leclerc and Wilkinson (2014) suggests that, for AgNPs, accumulation can be largely explained by the Ag ion which dissolves from the AgNP, while others suggest that in addition to mainly ionic there is residual accumulation of the NP (Piccapietra et al. 2012, Kroll et al. 2014). To resolve this uncertainty further development and research using relevant biological models is needed.

Bi-metallic NPs have a higher degree of functionality by combining multiple NMs, each possessing unique and novel advantages and have the potential to provide more detail about bioaccumulation. These nanohybrids are multifunctional and can be used to help enhance NP characteristics or to understand NP behavior, which can be applied to simpler, mono-metallic NPs (Li et al. 2010, Khan et al. 2012, Nguyen 2013, Aich et al. 2014, Banin et al. 2014, Shi et al. 2015). For example, iron NPs hybrids have been used to enhance magnetic properties for drug targeting (Alexiou et al. 2000), heat dissipation for hyperthermia treatments (Jordan et al. 2009), and to investigate magnetic properties (Pankhurst et al. 2003, Colombo et al. 2012). Au- and AgNPs have been used in a wide spread range of applications including photothermal imagine (Leduc et al. 2011, 124), enhancement of antibacterial agents (Mahmoudi and Serpooshan 2012), high efficiency localized surface plasmon resonance and surface enhanced Raman scattering (Major et al. 2009) and to determine AgNP transformations and behavior in complex environmental media (Li et al. 2010, Mott et al. 2012, Merrifield et al. 2017). Recent studies have looked
at the use of Au-Ag NPs specifically to investigate the bioavailability AgNPs due to enhanced stability (Mott et al. 2012, Shi et al. 2015). These Au-AgNPs offer a new tool to understand the mechanism controlling bioavailability and uptake of AgNPs for the reason that the Au core is protected from interacting with the media due to the Ag outer shell, therefore Au can be used as an internal standard and ratios of Ag and Au can be used to help explain the complex transformations of Ag (Saleh et al. 2015, Merrifield et al. 2017). Isotopically labeled NPs add another level of certainty to bioaccumulation studies. In addition to the benefits of using bi-metallic NPs listed above, the isotopic labels enhance the signal to noise ratio of the NP from the background element by measuring the ratio of the enriched isotope against a non-enriched (background) isotope. This has allowed for a significant reduction in detection limits within organisms by up to two orders of magnitude (Croteau et al. 2014). The labeled core-shell NPs allow the direct calculation of ion and NP bioaccumulation, since the core always remains as a particle, while the shell partially dissolves.

Various organisms including algae, daphnia, fish, and bivalves have been used to examine the biological interactions of engineered AgNPs (Handy et al. 2008, Lapresta-Fernandez and Blasco 2012, Moreno-Garrido and Blasco 2015). Microalgae have been an organism of interest in recent AgNP uptake and toxicity studies (Ivask et al. 2014, Turner et al. 2012, Sendra et al. 2017). 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. Although previous studies have addressed the impact of NPs
on algae, research has focused mainly on either freshwater algae or marine diatom species (Lodeiro et al. 2017, Huang and Cheng 2016, Sendra et al. 2017) and there is little information regarding the actual mechanism of uptake and bioaccumulation (Ivask et al. 2014, Sorensen and Baun 2015, Ribeiro et al. 2014, Navarro et al. 2015). Dinoflagellates are one of the largest groups of marine eukaryotes (Gomez 2005), they are mixotrophic, meaning they can obtain energy through photosynthesis or phagocytosis and are possess flagella which assist in locomotion. Examining AgNP uptake and accumulation on a primary producer could highlight the possible implications for biomagnification and trophic transfer within an ecosystem. The dinoflagellate *Prorocentrum minimum* is a mixotrophic, bloom forming estuarine dinoflagellate with a pan-global distribution and is potentially harmful to other organisms and humans via shellfish poisoning (Heil et al. 2005) and harmful algae blooms such as red tides (Heil et al. 2005).

Bivalves have been historically used in many studies as an indicator species for marine pollution monitoring (Boening 1999, Sarkar et al. 2006, Anajjar et al. 2008, Fukunaga and Zuykov et al. 2009, Anderson 2011). More recently, bivalves have been used as a biological test organism in NP accumulation and toxicity studies (Zuykov et al. 2011, Buffet et al. 2014, Volker et al. 2015), including the estuarine bivalve *Crassostrea virginica* (Trevisan et al. 2014, Johnson et al. 2015, Doyle et al. 2016). *C. virginica* is an intertidal reef forming bivalve and a valuable keystone species in south-eastern USA and gulf coast estuaries where salinities range from 5 to 30 ppt (Buroker 1983, Dame and Prins 1998). *C. virginica* is an ideal bioindicator species due to the constant gaping of its valves and continuous beating of cilia along the shell rim, which allows for the pumping of water
through the mantle cavity across the gills as a continuous flow which increases exposure
to the surrounding water (Jorgensen 1975). Oysters indiscriminately filter the surrounding
water into their mantle cavity, over the gills, to their labial palps which are used to sort and
identify phytoplankton-sized particles within a 1-12 μm size range for consumption based
on size and chemical composition (Shumway et al. 1985, Rosa et al. 2013). On average, *C.
virginica* filters 1 – 1.5 L hour⁻¹ g⁻¹ tissue weight (50 gallons/day) (Jorgensen 1966, Beck
et al. 2011). *C. virginica* 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).

*C. virginica* is considered an ecosystem engineer in that their reefs provide multiple
benefits and ecosystem services (Jones et al. 1994, Newell and Koch 2004, Grabowski and
Peterson 2007, Grabowski et al. 2012, Hoellein and Zarnoch 2014). Oyster reefs provide
habitat, refuge and foraging for commercially and recreationally important fishes, benthic
invertebrates, and crustaceans (Meyer et al.1993, Rutledge et al. 2018) as well as improve
water quality through filtration. Reefs also reduce erosion of other estuarine habitats such
as salt marshes and tidal creeks by stabilizing sediments and serving as a living breakwater
that absorbs and dissipates wave energy (Meyer et al. 1997). Filtration also benefits
submerged aquatic vegetation (Meyer et al. 1997, Grabowski et al. 2012) which is also a
critical habitat for fishes. Through biodeposition of feces and pseudo-feces benthic plants
are fertilized and filtration of suspended matter in the water column allows for more light
penetration (Newell 1988, Everett et al. 1995, Newell and Koch 2004, Carroll et al. 2008,
Wall et al. 2008). *In situ* water measurements have demonstrated that oysters actively
reduce the quantity of suspended solids and phytoplankton through filtration (Nelson et al. 2004, Grizzle et al. 2006, Pomeroy et al. 2006) and therefore improve water quality. Natural oyster reefs also facilitate bacterial denitrification in estuaries of anthropogenic nitrogen loading through the deposition of feces and pseudofeces into sediment (Newell and Langdon 1996, Newell et al. 2002, Piehler and Smyth 2011, Kellogg et al. 2016), with estimates of 51.3 to 502.5 kg N km$^{-2}$ removal through biodeposits (Pollack et al. 2013). Thus, playing a vital role in facilitating the nitrogen cycle and maintaining water quality (DePiper et al. 2016). Oysters can also sequester nitrogen, phosphorus, and other pollutants in their shell and tissues in high concentrations (Newell and Mann 2012). *C. virginica* is of special interest in biological studies due to numerous ecosystem services and their importance as a commercial and recreational fishery in the southeastern and Gulf coastlines of the US.

Although there has been research conducted on AgNPs and associated adverse effects to algae and bivalves, these studies have not been able to definitely conclude if uptake and accumulation is caused by the AgNP or Ag ion released through dissolution. In this study we used bi-metallic Au@Ag NPs and isotopically labeled NPs, 107Ag@Au@109Ag NPs and algae in this study to better understand Ag accumulation. We use the isotopically labeled NPs, 107Ag@Au@109Ag, synthesized in house from Merrifield and Lead (2016), as a tool for the assessment of relative particle and ion driven uptake and accumulation. The idea is that the 107Ag core remains intact, protected by the gold barrier layer, while the outer 109Ag layer partially dissolves producing silver ions. Stable elemental and
isotopic ratios in suspension and in the organism will provide quantification of the relative importance of ions and NPs in bioaccumulation.

The overall aim of this study was to understand the relative roles of ion and NP uptake and accumulation. We use Au@Ag NPs and 107Ag@Au@109Ag NPs to quantify ionic and NP Ag contributions. The specific objectives were:

(1) Develop an analytical method to measure ionic silver in seawater. We hypothesize that silver is detectable in seawater after matrix removal. To investigate this hypothesis, we tested the effect of various chelating and anion exchange resins, and method parameters on the recovery of silver from seawater.

(2) Quantify the uptake and bioaccumulation of nanoparticulate and ionic Ag in algae. We hypothesize that algae take up ionic Ag dissolved from the AgNPs. To test this hypothesis, we exposed algae to Au@Ag NPs and measured Au and Ag associated with (strongly bound or internalized Ag) cells over time.

(3) Quantify the uptake and bioaccumulation of nanoparticulate and ionic Ag in oysters. We hypothesize that oysters take up ionic Ag dissolved from the AgNPs. To test this hypothesis, we allowed oysters to filter natural media with 107Ag@Au@109Ag and measured 107Ag (NP) and 109Ag (ionic) concentrations in tissues over time.

In order to fully understand the impact nanotechnology has on our environment as well as the associated hazards and risks, the relative role of ion and NP in uptake and
accumulation need to be determined. The ultimate goal of this research is to provide insights into NP characteristics that lead to low hazard and low risk nanotechnologies for the benefit of human and environment health.
Figure 0.1: General Structure of Nanomaterial-flow Model. The model’s principle is to track engineered NM flows throughout the entire life cycle: engineered NM production; incorporation into products; engineered NM release from products during use; transport and fate of engineered NM between and within sewage treatment plants, waste incineration plants, landfill, and recycling processes (technosphere); transfer from technosphere to air, soil, water, and sediments (ecosphere); and transport within environmental compartments. The amounts of engineered NM in the compartments provide the basis for calculating the overall environmental concentrations of engineered NMs. (Adapted from Lead et al. 2018).
Figure 0.2. Predicted Future Environmental AgNM Concentrations. Modeled and predicted future environmental concentrations of nano-Ag flows from the products to the different environmental compartments, WIP, STP, and landfill. Depicted is a high exposure scenario, that relied on estimations that would lead to higher concentrations in the environment. Arrows indicate flow values in tons/year. The thickness of the arrows is proportional to the amount of silver flowing between the compartments. Dashed arrows represent the lowest volume. (Adapted from Mueller and Nowack 2008).
CHAPTER 1

DETERMINATION OF DISSOLVED SILVER IN SEAWATER USING OFFLINE PRECONCENTRATION AND QUANTIFICATION BY ICP-MS

1.1 ABSTRACT

A solid phase extraction method for the quantification of dissolved silver in seawater is described. A polypropylene column packed with ca. 0.50 g of Dowex 1-x8 anion exchange resin was used to separate and preconcentrate silver from seawater prior to elution and quantitation by inductively coupled plasma mass spectrometry (ICP-MS). A volume of 50 mL (acidified to 0.1% nitric acid v/v) was loaded onto the column at 4 mL min\(^{-1}\) and eluted at 1 mL min\(^{-1}\) with 25 mL of 0.1 M thiourea/0.05% nitric acid solution. Recoveries of 102.8 ± 4.1% were obtained for 25 µg L\(^{-1}\) silver spiked natural seawater samples. Using a 50 mL sample, a preliminary method detection limit (3 times the standard deviation of the blank) of 66 ng L\(^{-1}\) (n=14 DI water samples) was obtained. Far better detection limits should be achieved when using fully optimized ICP-MS operating conditions. The total processing time for a 50 mL samples was 45 minutes including cleaning and reconditioning of the resin column between samples. If samples are processed in parallel as many as 10 samples can easily be processed offline in working day. The solid phase extraction method was also applied to the analysis of coastal seawaters in NSW Australia. Dissolved Ag concentrations range from 34 to 89 ng L\(^{-1}\).

1.2 INTRODUCTION

In marine systems, silver is regarded as being one of the most toxic, readily accumulated trace metals, second only to mercury (Luoma et al. 1995, Ratte 1999, Reinfelder and Chang 1999). It is strongly bioaccumulated by marine invertebrates, fish, phytoplankton and seaweeds (Luoma et al. 1995, Ratte 1999).
Reported open ocean concentrations of silver (Table 1.1) range from ≤ 0.7 to 20 pM (0.08 – 2.2 ng L^{-1}) in eastern Atlantic and North Pacific Oceans (Flegal et al. 1995). Silver follows a nutrient type distribution similar to that of silicate where it is has a depleted surface water concentration with direct surface input mainly from aeolian transport associated with Saharan dust, and a systematic enrichment of deep-water concentrations along advective flow lines from the North Atlantic to the North Pacific (Barriada et al. 2007). Silver concentrations in coastal waters are typically 5 to 10 times higher than open ocean values (Bruland et al. 1979, Gallon and Flegal 2015) with a rough average estimate concentration of 9 ng L^{-1}, lower range value of 0.1 ng L^{-1} and upper range value of 50 ng L^{-1} (Beck and Sanudo-Wilhelmy 2007, Squire et al. 2002). The higher concentrations of silver in coastal zones and estuaries are largely due to runoff from land and anthropogenic sources of silver such as municipal and industrial discharges (Flegal and Sanudo-Wilhelmy 1993). Important sources include mining, electronics, nano-silver embedded consumer products, photographic and metal-processing industries (Sanudo-Wilhelmy and Flegal 1992, Benoit et al. 1994, Benn et al. 2010). Decadal data from the San Francisco and San Diego Bays show that anthropogenic sources have historically added to the concentration of silver in marine waters fluctuating from 0.1 to 27 ng L^{-1} and 7 to 34 ng L^{-1}, respectively (Sanudo-Wilhelmy and Flegal 1992, Squire et al. 2002, Barriada et al. 2007).

The determination of silver in marine and coastal waters presents several challenges including low concentrations and matrix complexity of seawater, which creates interference problems for many instrumental techniques creating a requirement for additional separation steps. Dissolved silver analysis typically utilizes techniques for
matrix isolation to remove the high salt background, some form of preconcentration to increase sensitivity followed by quantification by optical or mass spectrometry (Barriada et al. 2007, Zhang et al. 2001).

Typical approaches to dissolved silver analysis in marine waters are summarized in Table 1.2. Preconcentration by solvent extraction/complexation with dithiocarbamate ligands followed by quantitation by atomic spectrometry has been a popular approach (Bruland and Coale 1985, Bruland et al. 1979), however this process is time consuming, requires large sample volumes, multiple extraction steps and reported limits of detection of (LOD) ranging from 0.1 – 700 ng L\(^{-1}\) (Bruland and Coale 1985, Adams and Kramer 1999, Bloom and Crecelius 1984). Voltammetric techniques require low sample volume and no matrix removal, however these techniques are typically non-robust and a pretreatment normally required to achieve a stable electrode response which can be complex and time consuming. In addition, voltammetric techniques can suffer from interferences arising from other metals present in solution (Svancara et al. 1996).

Direct introduction of undiluted seawater samples to ICP-MS is not feasible as the salt matrix causes interferences, and deposition of salt on the nebulizer, cones and lenses resulting in severe signal suppression. In order to overcome these problems, samples are typically diluted 10–100 times with high purity water which significantly reduces detection limits (Field et al. 1999). Isotope dilution has been paired with ICP-MS and yields good LODs (0.001 ng L\(^{-1}\)) but is time consuming with procedures stating 48 hours to reach isotopic equilibrium (Kato et al. 1990) and complicated with multiple steps of added
isotopes and reagents (Yang and Sturgeon 2002). Online matrix removal of the salt matrix prior to ICP-MS analysis has been developed to evade these problems (Dressler et al. 2001, Barriada et al. 2003). This approach offers short analysis times and low detection limits (Table 1.2).

Ndung’u et al. (2006) published a paper describing the analysis of silver in estuarine waters. They developed an automated flow system using a 1 cm mini column packed with a strong anion exchange resin (Dowex 1-x8), and a flow-injection system to separate and preconcentrate silver from the seawater samples before acid elution of the silver and online analysis by ICP-MS. This method yielded a LOD of 0.076 ng L⁻¹, however, a number of potential interferences such as dissolved organic matter were highlighted which required sample pretreatment. In this paper we re-examine their operational conditions and describe an improved procedure which was tailored for use in experimental studies on the fate of silver nanomaterials in marine systems. This new procedure has the added advantage of offline preconcentration prior to quantification by ICP-MS which allows parallel sample processing of multiple samples and reduces instrument time.

1.3 EXPERIMENTAL

1.3.1 GENERAL INFORMATION

All glassware and plastic containers were acid soaked (10% v/v) for 24 hours, rinsed with copious amounts of deionized water, and allowed to dry in a laminar flow hood. Developmental work was conducted in a general use laboratory while all low-level analysis was conducted in a purpose-built trace metals clean room. Silver concentrations were
measured by ICP-MS (Agilent 8800) using matrix matched standards and standard operating conditions recommended by the manufacturer.

1.3.2 REAGENTS AND RESINS

All solutions were prepared with ultra-pure water (18 Ωcm) obtained from a Milli-Q analytical reagent-grade water purification system (denoted at DI), and all acids were of Optimal grade (Sigma Aldrich). A 0.1M stock standard of thiourea (Sigma Aldrich) was prepared daily. All resins trialed in this study were first washed three times using DI water. The supernatant washings were separated by centrifugation. Natural seawater used in the method development studies was collected from Cronulla Beach, NSW, Australia (35° 4’ 12” S, 151°9’36” E) and filtered through a 0.45 µm membrane before use.

The resins tested for their ability to quantitatively remove silver from seawater were: a chelating ion exchange resin, Chelex 100 (200-400 mesh size, BioRad, Analytical Grade), and the following anion exchange resins: Dowex 1-x4 (100-200 mesh size Sigma Aldrich), Dowex 1-x8 (20-50 mesh size, ACROS Organics) and Dowex 1-x8 (200-400 mesh size, Sigma Aldrich).

1.3.3 COLUMN PREPARATION

Approximately 0.50 g of resin slurry was packed into a polypropylene column (Supelco, Sigma-Aldrich, USA) between two polyethylene frits with a pore size of 20 µm. The column had an internal volume of approximately 1 mL. The column was connected to a 60 mL Luer slip plastic syringe which was placed into a vertical syringe pump (New Era,
NE-1000 Programmable Single Syringe Pump). The pump was programmed according to manufacturer’s guidelines for the internal diameter of the 60 mL volume syringe. Effluent was collected in 50 mL polystyrene vials.

1.3.4 METHOD OPTIMIZATION

Univariate optimization was performed on the following parameters: eluent concentration, sample loading flow rate, eluent flow rate and eluent volume. The influence of eluent composition, light and DOM on method performance were also investigated. Unless otherwise stated, optimization experiments were conducted using acidified, filtered natural seawater solution spiked with 25 µg/L silver. For the resin selection studies, a known volume of solution was passed through the column and the effluent collected and analyzed for dissolved silver.

1.3.5 OPTIMIZED PROCEDURE

The column was first conditioned with 50 mL of 1% acidified seawater at a flow rate of 4 mL min⁻¹. Using the same flow rate, 50 mL of acidified seawater sample (0.2% nitric acid) was loaded onto the column then rinsed with DI water for 5 minutes at a flow rate of 1 mL min⁻¹. The sample was then eluted with 0.1 M thiourea /0.05% nitric acid solution for 20 minutes at 1 mL min⁻¹ into an acid washed 50 mL polystyrene conical vial. The eluent was stabilized to a total nitric acid concentration of 2% and stored in dark at 4°C. The column was then rinsed for 5 minutes at a flow rate of 4 mL min⁻¹ with DI water then with 1% acidified seawater for 5 minutes at 1 mL min⁻¹ to recondition the resin prior to the analysis of the next sample. Extracts were analyzed by ICP-MS (Agilent 8800) using
matrix matched standards and standard operating conditions recommended by the manufacturer. Higher concentrations of Ag used in the early method optimization experiments were measured by ICP-AES (Varian 730 ES).

1.4 RESULTS AND DISCUSSION

1.4.1 RESIN SELECTION

A number of resins were screened for their ability to quantitatively take up Ag from acidified seawater (0.2% nitric acid). Uptake onto Chelex 100 was < 35%, Dowex 1-x4 (100-200) was < 60%, and Dowex 1-x8 (20-50) was < 70%. The best resin was the anion exchanger Dowex 1-x8 (200-400) which afforded quantitative uptake of Ag (>95%). This resin was selected for further investigation. As noted by previous workers (N’dungu et al. 1996), the resin has a high affinity for negatively charged silver-chloro complexes which are present in chloride (Cl\(^{-}\)) containing media such as a seawater.

1.4.2 METHOD OPTIMIZATION

The effects of varying loading flow rate, elution flow rate, eluent composition parameters, and elution volume are shown in Figure 1.1 and Figure 1.2. The main trends are summarised below.

1.4.3 ELUENT COMPOSITION

Using the eluent described by N’dungu et al. (2006) (5 mL of 10% HNO\(_3\), flow rate 1 ml min\(^{-1}\)) resulted in incomplete elution of silver from the Dowex 1-x8 loaded column (77.08 ± 15.6%). In their study, N’dungu et al. (2006) quantified uptake of silver onto their
mini-column but did not actually measure recovery during elution. They consequently assumed that there was an interference in natural seawater samples which prevented silver from binding to the resin. Based on recent literature (Wen et al. 2002, Ndung’u et al. 2006, Yin et al. 2012) there have been suggestions that a reaction between DOM, light, and elemental Ag on the column could be the cause of reduced recovery. However, our more detailed investigations indicate that the issue is not with the binding of silver to the column but its quantitative recovery during the elution phase.

With the goal to fully elute bound silver from the resin column, we trialled a range of eluents including nitric and hydrochloric acid solutions. The effects of elution composition on recovery are summarised in Table 1.3. Nitric acid solutions yielded better percent recoveries (average ± 1 standard deviation, 77.1 ± 15.6) than hydrochloric acid solutions (19.5 ± 13.3). This can be attributed to the delicate relationship between silver and chloride due to silver solubility and the formation of silver precipitates in high chloride solutions. Nitric acid does not introduce an additional source of chloride to the seawater sample, thus reducing the chance of silver precipitating and formation of insoluble chlorides. As nitric acid concentration in the eluent increased, recovery decreased (Figure 1.1-A). To improve recoveries, thiourea was added to the eluant. Thiourea was selected as it is known to form strong complexes with silver and other metals under acidic conditions (Henrichs et al. 1977, Loo 1982, Jing-Ying et al. 2012). Recovery of silver increased with a higher thiourea concentration in the eluent solution (Figure 1.1-B). The optimum concentration was in the range 0.1 M thiourea. When low concentrations of nitric acid were combined with thiourea
recoveries improved resulting in quantitative recovery. The best combination was 0.1 M thiourea with 0.05% HNO₃ resulting in recovery of (102.8 ± 4.1%, n=14).

The effects of varying loading flow rate, elution flow rate, elution composition parameters, and elution volume are shown in Figure 1.1.

1.4.4 LOADING FLOW RATE AND VOLUME

The optimal loading flow rate was 4 mL min⁻¹. As the loading flow rate increased beyond this value, percent recovery slowly decreased due to reduced contact time between silver ions and binding sites on the resin (Figure 1.1-C). Increasing sample volume from 50 to 100 mL resulted in a 90% recovery of silver as compared to 100% recovery, respectively. Further work is required to elucidate why recovery is slightly lower at higher sample volumes.

1.4.5 ELUTION FLOW RATE

Elution flow rate was tested from 1 mL min⁻¹ to 4 mL min⁻¹ but did not significantly reduce or improve the recovery of silver (Figure 1-D). As a safeguard a flow rate of 1 mL min⁻¹ was selected to ensure enough time for the eluent to interact with silver on the resin for full recovery.

In an attempt to reduce the amount of eluent (and thereby increase the preconcentration factor) we investigated the effluent volume needed to recover 100% of silver loaded onto the column. Figure 1-E clearly shows 100% recovery of silver from the column at 20-25
mL of elution. A volume of 25 mL of eluent (1 mL min\(^{-1}\) flow rate) is required for comprehensive silver recovery. A greater eluent volume does not increase or decrease recovery, but less than 20 mL resulted in less than 100% recovery (Figure 1-E).

The kinetics of Ag elution were investigated using stop-flow elution experiments. The column loading flow was stopped for 5-10 minutes to allow equilibration of the eluent with the column prior to the flow being restarted. However, results indicated a decrease in recovery with increased stoppage time (Figure 1.2). The reasons for this counterintuitive trend are not fully understood.

1.4.6 EFFECT OF LIGHT AND DOC

To shield experiments from light, samples were prepared in a darkened room. The sample and column were also covered in aluminum foil to block light penetration during all steps of the loading and elution procedure. Samples that were not light shielded were prepared on the lab bench and not covered in aluminum foil. For these tests, 10% nitric acid eluent was used. The effect of light on elution efficiency showed no significant difference between light shielded (78.5 ± 12.5%, n=4) and not light shielded samples (79.5 ± 6.5%, n=4) (p > 0.05).

1.4.7 PERFORMANCE CHARACTERISTICS

Based on these results presented above, the optimum operating conditions were selected (see recommended protocol for details). The recovery tested at 25 µg L\(^{-1}\) was 102.8 ± 4.1% (n=14). The preliminary method detection limit (3 times the standard deviation of
the blank) obtained under sub-optimal ICP-MS operating conditions was 66 ng L\(^{-1}\) (n=10 DI water samples). Further tests are required to rigorously define the actual method detection limit.

The total processing time for a 50 mL samples was 45 minutes including cleaning and reconditioning of the resin column between samples. If continuous samples are processed over 10 samples can easily be processed offline in an 8-hour working day.

Dissolved Ag concentrations measured in various coastal waters sampled from the east coast of New South Wales, Australia are presented in Table 1.4. Dissolved Ag concentrations in surface waters are significantly higher than those reported previously for other locations (Table 1.1), and it appears that the variability of Ag between locations is greater than an order of magnitude. This may reflect anthropogenic sources of silver or natural inputs along this part of the NSW coastline.

1.5 CONCLUSION

This study resulted in a successful, time efficient, and low-cost method for the determination of silver in seawater. We argue recent results from literature where an interference of dissolved organic material causes low recovery rates due to loading of silver onto the resin. Instead, we suggest that there is no issue with the separation and preconcentration of silver using anion exchange resins, but with the removal of silver from the resin and elution efficiency. A thiourea and nitric acid mixture was shown to be an effective means of removing silver from the anion exchange resin Dowex 1-x8, enhancing
the elution efficiency of silver from acidified estuarine samples. The enhancement is suggested to be due the addition of thiourea to the eluent solution and its affinity to complex with silver and other metals under acidic conditions.
**Table 1.1. Dissolved Silver in Seawater Summary.** Summary of dissolved Silver concentrations in seawater from various locations.

<table>
<thead>
<tr>
<th>Location</th>
<th>Dissolved Ag Concentration (ng L(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE Pacific Ocean (surface)</td>
<td>0.033</td>
<td>Martin et al. (1983)</td>
</tr>
<tr>
<td>NE Pacific Ocean (deep waters)</td>
<td>2.54</td>
<td>Martin et al. (1983)</td>
</tr>
<tr>
<td>N Pacific Ocean (Japan) (surface)</td>
<td>0.46 – 0.88</td>
<td>Zhang et al. (2001)</td>
</tr>
<tr>
<td>N Pacific (surface)</td>
<td>0.12 – 1.27</td>
<td>Ranville et al. (2005)</td>
</tr>
<tr>
<td>N Atlantic Ocean (surface)</td>
<td>0.074 – 0.78</td>
<td>Rivera-Duarte et al. (1999)</td>
</tr>
<tr>
<td>NE Atlantic Ocean (surface)</td>
<td>≤ 0.076</td>
<td>Flegal at al. (1995)</td>
</tr>
<tr>
<td>NE Atlantic Ocean (deep waters)</td>
<td>0.76</td>
<td>Flegal at al. (1995)</td>
</tr>
<tr>
<td>Adriatic Sea (surface)</td>
<td>0.73 – 2.29</td>
<td>Tappin et al. (2010)</td>
</tr>
<tr>
<td>Southern Ocean (surface)</td>
<td>0.98 – 2.47</td>
<td>Sañudo-Wilhelmy et al. (2002)</td>
</tr>
<tr>
<td>Bering Sea</td>
<td>0.3 – 11.5</td>
<td>Zhang et al. 2004</td>
</tr>
<tr>
<td>San Francisco Bay</td>
<td>0.65 – 26.2</td>
<td>Smith &amp; Flegal (1993)</td>
</tr>
<tr>
<td>San Francisco Bay</td>
<td>0.11 – 27.0</td>
<td>Squire et al. (2002)</td>
</tr>
<tr>
<td>San Diego Bay</td>
<td>7.3 – 33.9</td>
<td>Sañudo-Wilhelmy and Flegal (1992)</td>
</tr>
<tr>
<td>Long Island Sound</td>
<td>0.33 – 39.0</td>
<td>Buck et al. (2005)</td>
</tr>
<tr>
<td>Jamaica Bay</td>
<td>0.22 – 49.9</td>
<td>Beck and Sañudo-Wilhelmy 2007</td>
</tr>
<tr>
<td>NE Atlantic Ocean (deep waters)</td>
<td>0.76</td>
<td>Flegal at al. (1995)</td>
</tr>
<tr>
<td>Tamar Estuary, UK (surface)</td>
<td>1.04 – 0.67</td>
<td>Tappin et al. (2010)</td>
</tr>
<tr>
<td>Seine Estuary</td>
<td>6.6 – 5.85</td>
<td>Cozic et al. (2008)</td>
</tr>
<tr>
<td>Fal Estuary</td>
<td>1.55 – 9.76</td>
<td>Tappin et al. (2010)</td>
</tr>
<tr>
<td>Tokyo Bay</td>
<td>0.55 – 1.66</td>
<td>Zhang et al. (2008)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection Limit (ng L⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent extraction/GF-AAS</td>
<td>0.10</td>
<td>Bloom and Crecelius 1984</td>
</tr>
<tr>
<td>Solvent extraction/GF-AAS</td>
<td>0.04</td>
<td>Rivera-Duarte and Flegal 1997</td>
</tr>
<tr>
<td>FI-AAS using column preconcentration</td>
<td>700</td>
<td>Dadfarnia et al. 2004</td>
</tr>
<tr>
<td>FI-ICP-MS using column preconcentration</td>
<td>0.89</td>
<td>Dressler et al. 2001</td>
</tr>
<tr>
<td>FI-ICP-MS using column preconcentration</td>
<td>0.054</td>
<td>Barriada et al. 2003</td>
</tr>
<tr>
<td>FI-ICP-MS using column preconcentration</td>
<td>0.07</td>
<td>Ndung’u et al. 2006</td>
</tr>
<tr>
<td>ID-ICP-MS</td>
<td>0.0001</td>
<td>Yang and Sturgeon 2002</td>
</tr>
</tbody>
</table>
Table 1.3: Summary of Tested Eluent Compositions. Summary of eluent compositions tested for effects on recovery. Results presented as the average percent recovery ± 1 standard deviation from the mean.

**Sample:** Natural Seawater (0.2% HNO3).

<table>
<thead>
<tr>
<th>Elution Composition</th>
<th>% Recovery</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% HNO3</td>
<td>77.1 ± 15.6</td>
<td>7</td>
</tr>
<tr>
<td>20% HNO3</td>
<td>59.2 ± 10.7</td>
<td>3</td>
</tr>
<tr>
<td>10% HCl</td>
<td>19.5 ± 13.3</td>
<td>2</td>
</tr>
<tr>
<td>0.2M thiourea + 1% HCl</td>
<td>95.3 ± 2.8</td>
<td>2</td>
</tr>
<tr>
<td>0.1M thiourea + 1% HCl</td>
<td>94.9 ± 6.8</td>
<td>5</td>
</tr>
<tr>
<td>0.05M thiourea + 1% HCl</td>
<td>47.2 ± 6.7</td>
<td>2</td>
</tr>
<tr>
<td>0.1M thiourea + 1% HNO3</td>
<td>76.9 ± 2.7</td>
<td>2</td>
</tr>
<tr>
<td>0.1M thiourea + 0.1% HNO3</td>
<td>99.8 ± 2.4</td>
<td>2</td>
</tr>
<tr>
<td>0.1M thiourea + 0.05% HNO3</td>
<td>102.8 ± 4.1</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 1.4. **Dissolved Silver in Natural Waters.** Dissolved Ag concentrations measured in various coastal waters sampled from the east coast of New South Wales, Australia. Results presented as the average percent recovery ± 1 standard deviation from the mean.

<table>
<thead>
<tr>
<th>Location</th>
<th>Dissolved Ag Concentration (ng L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulli Beach</td>
<td>34 ± 10</td>
</tr>
<tr>
<td>Cronulla Beach</td>
<td>89 ± 46</td>
</tr>
<tr>
<td>Sydney Harbour</td>
<td>85 ± 81</td>
</tr>
</tbody>
</table>
Figure 1.1: Univariate Optimisation. Effect of (A) loading flow rate, (B) elution flow rate, (C) thiourea concentration, (D) nitric acid concentration and (E) elution volume on recovery of silver from 50 mL of spiked seawater (25 µg L\(^{-1}\)).
**Figure 1.2: Residence Time.** Effect of stop-flow experiments on elution efficiency and recovery of silver from 50 mL of spiked seawater (25 µg L\(^{-1}\)) using 0.1 M thiourea/0.05% HNO\(_3\). Error bars represent standard deviation of 5.17 for 0 minutes, 0.672 for 5 minutes, and 0.997 for 15 minutes.
CHAPTER 2

RELATIVE ROLES OF IONS AND PARTICLES IN SILVER NANOPARTICLE UPTAKE AND ACCUMULATION USING AN ESTUARINE DINOFLAGELLATE

2.1. ABSTRACT

Silver nanoparticles (AgNPs) are widely used and have an inherently high toxicity to a range of organisms. Biouptake of AgNPs and Ag ions released by AgNP dissolution is often a prerequisite to subsequent human or environmental toxicity. However, the methods for understanding the relative role of ion and particle in uptake and accumulation are not fully developed. Bi-metallic nanoparticles (NPs) offer a higher degree of functionality by combining multiple NMs, each possessing unique and novel advantages, and have recently been used to assess bioavailability to organisms with the aim to provide more detail about AgNP bioaccumulation. In this study we use the estuarine dinoflagellate, *Prorocentrum minimum* to determine the relative roles of ions and particles in AgNP uptake and accumulation. We show that dissolution is dependent on concentration where there is more dissolution at lower concentrations and more aggregation at higher concentrations. In addition, several transformations may be occurring simultaneously. Due to complex transformations in seawater media algae are exposed to original Au@Ag with a reduced Ag outer layer, ripened AgNPs, or silver-chloro complexes during the exposure. After exposure to Au@Ag, although there was not a significant relationship between Ag detected in the pellet and cell inhibition, cell densities decrease as exposure concentration increases with 10-15% decrease in cell density compared to the control. After 48 hours of exposure, we speculate that algae are able to recover from the initial exposure to Au@Ag and continue to increase in cell density. Consistent with previous research, our results confirm that dissolved Ag is more toxic than AgNPs and algae are not able to recover after exposure to AgNO$_3$ exposure concentrations above 10 µg L$^{-1}$. 
2.2. INTRODUCTION

Nanoparticles (NPs) have become a central component in a wide range of commercial and consumer products having electronic, biomedical, and pharmaceutical drug delivery applications, as well as, cosmetics and personal care products (Piccinno et al. 2012, Nowack and Bucheli 2007). AgNPs are used frequently in consumer products (Nanotechnologies Consumer Products Inventory) because of their strong antimicrobial properties (Sondi and Salopek-Sondi 2004). Since AgNPs are known to be toxic and to be present in the environment (and also potentially to have novel properties), they have a potential environmental risk, which might be significantly different to that of the bulk material or free silver ion. Bi-metallic NPs have recently been used to assess bioavailability to organisms (Khan et al. 2017, Marcato et al. 2013, Shi et al. 2015). Bi-metallic NPs have a higher degree of functionality by combining multiple NMs, each possessing unique and novel advantages and have the potential to provide more detail about bioaccumulation. The Au core is protected from interacting with the media due to the Ag outer shell, therefore Au can be used as an internal standard and ratios of Ag and Au can be used to help explain the complex transformations of Ag (Merrifield et al. 2017, Saleh et al. 2015).

Microalgae have been an organism of interest in NP exposure studies and potential mechanisms of AgNP toxicity are likely to be largely associated with NP dissolution and the interaction of dissolved Ag with the cells (Ivask et al. 2014, Zhang et al. 2016, Turner et al. 2012). However, in several studies, dissolution was not the only process contributing to algal toxicity and therefore the role of NP-cell interactions cannot be discounted (Zhang et al. 2016, Turner et al. 2012, Leclerc and Wilkinson 2014, Sorensen and Baun 2015).
Dinoflagellates are one the largest groups of marine eukaryotes (Gomez 2005), they are mixotrophic, meaning they can obtain energy through photosynthesis or phagocytosis, and are able to cause harmful algae blooms such as red tides (Heil et al. 2005). The dinoflagellate *Prorocentrum minimum* is a mixotrophic, bloom forming estuarine species that is widely distributed geographically and is potentially harmful to humans via shellfish poisoning (Heil et al. 2005). Examining AgNP toxicity on the primary production level is important to ensure the base of estuarine food webs are not contaminated or compromised by toxicants that may drastically alter phytoplankton community cell density, function, or structure. The objective of this study was to determine the relative roles of ions and particles in silver nanoparticle uptake and accumulation using estuarine dinoflagellate.

2.3. METHODS

2.3.1. SILVER NANOPARTICLES

Bi-metallic Au@Ag NPs were purchased from NanoComposix Inc. (San Diego, CA) as aqueous 60 nm silver shelled gold nanospheres capped with sodium citrate, at a concentration of 1 mg mL$^{-1}$.

2.3.2. NP CHARACTERIZATION

Stock solutions were analyzed for total Ag and Au concentrations via inductively coupled plasma optical mass spectrometry (ICP-MS) (Perkin Elmer NexION 350D). Size, polydispersity index (pdi), zeta potential (Malvern Nanosizer Nano-Zs) and absorption spectra over a wavelength of 200-800 nm using a UV-visible spectrophotometer (Shimadzu UV2600) of stock NPs were measured of purchased Au@Ag NPs as a stock solution. The
mean of at least three measurements were reported (Tejamaya et al. 2012). The same measurements were made of stock NPs in media after 0, 24, 48, and 72 hours of exposure. Dissolution was measured at 0, 24, 48 and 72 hours. An aliquot was taken from each exposure concentration and ultrafiltered (EMD Millipore Amicon™ Ultra-4 Centrifugal Filter Unit, 3kDa membrane) via centrifugation (400 RPM, 20°C, 30 minutes) then preconcentrated using an anion exchange resin to remove the seawater matrix (Butz et al. 2019 in prep) and measured by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer NexION 350D) for dissolved Ag. The 0-hour sample aliquot was taken immediately after Au@Ag NP addition, however due to procedure and sample processing time this sample was measured 25-30 minutes after initial addition.

2.3.3. BIOLOGICAL MEDIA AND TEST ORGANISM

A standard culturing media, denoted L1 -Si was prepared according to the National Center for Marine Algae and Microbiota (NCMA) instructions. The complete recipe can be found in the supporting information (Tables 2.1-2.3). This media was used for all NP exposures. The estuarine dinoflagellate *Prorocentrum minimum* (CCMP# 695) was obtained from the NCMA, Bigelow, Maine, USA and grown in L1 –Si enriched seawater media (Guillard and Ryther 1962, Guillard 1975) at 23 ± 2°C, on a 12 hour light and dark cycle, with irradiance range of 90-100 μmol quanta m⁻² s⁻¹ to mimic natural conditions. The dinoflagellate culture was allowed to grow for four days prior to transfer to individual exposure bottles to ensure cells were in exponential growth (supported by cell count and fluorescence measurements).
2.3.4. EXPOSURES

Exposures were performed for 72 hours in triplicate at concentrations ranging from 0 to 50 μg-Ag L\(^{-1}\) at 23 ± 2°C on a 12-hour light/dark cycle with an irradiance range of 90-100 μmol quanta m\(^{-2}\) s\(^{-1}\) to mimic incubation and natural conditions. This length of time was chosen for exposures based on standard protocol for proper bio-assessment of phytoplankton growth (Munawar and Munawar 1987, Schafer et al. 1994). For each exposure, 200 mL of media and 25 mL of cultured dinoflagellates (cell density greater than 100,000 cells mL\(^{-1}\)) were added to individual glass bottles along with either an aliquot of Au@Ag NPs or AgNO\(_3\) (dissolved Ag control). To ensure consistent light exposure, an ultraviolet spherical quantum probe (Biospherical Instruments Inc., model QSI2101) was used along with the Logger 2100 program to measure the exposure irradiances.

2.3.5. GROWTH INHIBITION

Four separate inoculum cultures were measured every 24 hours for 4 days for chlorophyll a (Chl. A) in raw fluorescence units (RFUs) (Turner Trilogy Fluorometer with Chl \(a\) in vivo module) and cell density enumerated using inverted light microscopy (Olympus IMT-2 microscope, 10 μL Burker counting chamber). A linear regression analysis was performed to relate the parameters of fluorescence (RFU) and cell density (cells mL\(^{-1}\)) (adj. \(R^2\) = 0.781, p<0.05) (Butz et al. 2019 submitted). Once this relationship was determined only fluorescence was measured and used to calculate cell density during exposures.
After 72 hours, an aliquot was taken from each bottle and centrifuged (2000 rpm, 4°C, 10 min) to precipitate suspended cells. The supernatant (10 mL) was removed and acidified (1% nitric acid). The pellet of cells was washed with 10 mL of fresh media by centrifugation under same conditions. This process was repeated twice. The washes were removed, combined, and acidified (1% nitric acid). The remaining algal pellet was digested with concentrated nitric acid. All acidified samples were diluted to 1% acid and measured by ICP-MS. We assumed the Ag remaining with the precipitate was either internalized or strongly bound to the cell wall and Ag removed during the washing process was weakly bound to the cell wall. Both binding types were purely operational.

2.4. RESULTS

2.4.1. NP CHARACTERIZATION

Characterization of stock bi-metallic Au@Ag NPs in water resulted in an absorption spectrum indicative of Au@Ag NPs (Figure 2.1). Due to the interchange of light with electrons on the exterior of the NP, at a specific wavelength (about 400 nm for AgNPs and about 540 nm for AuNPs) plasmon resonance occurs, meaning that the frequency of the incident photon is resonant with the collective electrons resonance occurs when oscillations on the surface of the nanoparticle (Link et al. 1999, Shafiqu et al. 2018). When both Ag and Au are present the wavelength corresponding to the maximum extinction, absorption as well as scattering, redshifts, as compared to a mono-layer AgNP, due to the additional of gold. An average hydrodynamic diameter of 70.1 ± 2.5 nm and a polydispersity index (pdi) of 0.2 ± 0.03 (Figure 2.1) were also measured by DLS. Based on total metal concentrations measured by ICP-MS an Ag:Au mass ratio of 2.96 was determined.
At 1, 10, 20, and 50 µg L⁻¹ exposures Au@Ag NPs were undetectable by UV-vis in media due to their low concentration and a signal was not detected. An additional experiment was performed at 50 and 200 µg L⁻¹ dispersed in MQ water to determine the LOD. Results showed a signature peak between 400 and 500 nm indicating Au@Ag NPs in the 200 µg L⁻¹ exposure. In the 50 µg L⁻¹ there was no peak detected and therefore was considered to be below the LOD of the UV-vis using the standard short path-length cuvette (Figure 2.2). Results from the LOD, 20 and 50 µg L⁻¹ experiments can be found in Figure 2. DLS results were inconclusive because NP signals were not distinguishable from the background of seawater media. Results from 0, 20 and 50 µg L⁻¹ exposures are included in Figures 2.3 and 2.4, respectively.

2.4.2. METAL ACCUMULATION

2.4.2.1. GENERAL OBSERVATIONS

In all Au@Ag NP treatments, Ag and Au were detected in the algal pellet, here referred to as internalized and strongly bound to the dinoflagellate cell wall. For all exposures the mass of Ag accumulated increased with increasing exposure concentration and accumulation shows a decline over time (Figures 2.5) and aside from the 50 µg L⁻¹ Au@Ag NP exposures, as exposure concentration increased percent accumulated decreased (Figure 2.6). There was a concentration and time dependent accumulation of Ag in the AgNO₃ exposures (Figure 2.7) and decreasing percent accumulation with increasing exposure concentration (Figure 2.8). AgNO₃ exposures resulted in the highest amounts of Ag mass accumulation in the algal pellet after 72 hours with 0.6 µg-Ag 100,000 cells⁻¹ in the 50 µg
L$^{-1}$ exposure, compared to 0.13 µg-Ag 100,000 cells$^{-1}$ at 72 hours accumulated in the Au@Ag 50 µg L$^{-1}$ exposure.

2.4.2.2. METAL ACCUMULATION IN THE WASH SOLUTION

No silver or Au was detected in the wash (weakly bound) proportion in any of the Au@Ag exposures or in the 0, 1 and 10 µg L$^{-1}$ AgNO$_3$ exposures. In the 20 and 50 µg L$^{-1}$ AgNO$_3$ exposures sub nanogram levels of Ag mass detected, however this value was below the LOD of 1.4 ug L$^{-1}$ (mean ± 3SD) and therefore not reported in this paper.

2.4.2.3. METAL ACCUMULATION IN THE ALGAL PELLET

In the 1 µg L$^{-1}$ Au@Ag exposures silver and gold accumulation in the pellet was less than 0.005 µg-Ag and -Au 100,000 cells$^{-1}$ (Figure 2.5) and less than 2% uptake of total Ag- Au- added. These values were below the LOD (1.4 ug L$^{-1}$) and are therefore not discussed in this paper. Results from AgNO$_3$ exposures at 1 µg L$^{-1}$ were not below the LOD with 0.04 µg-Ag 100,000 cells$^{-1}$ (Figure 2.7) and about 6% uptake of initially added Ag (Figure 2.8). In the 10 and 20 µg L$^{-1}$ Au@Ag exposures, silver accumulation was not significantly different with an average of 0.02 ± 0.005 µg-Ag 100,000 cells$^{-1}$. Concentrations of Au in the pellet were measured at less than 0.01 ug-Au 100,000 cells$^{-1}$ for both exposure concentrations (Figure 2.5). Figure 2.6 shows relatively constant percent uptake of initially added Ag and Au during the exposure for both 10 and 20 µg L$^{-1}$ Au@Ag exposures. However, there was twice as much percent uptake in the 10 µg L$^{-1}$ exposure (5.4%) than in the 20 µg L$^{-1}$ exposure (2.6%) (Figure 2.6). Figure 2.7 shows that Ag was accumulated nearly 2 – 4 times higher in the 10 and 20 µg L$^{-1}$ AgNO$_3$ exposures (0.04 and
0.08 µg-Ag 100,000 cells\(^{-1}\) respectively) compared to the 10 and 20 µg L\(^{-1}\) Au@Ag exposures. Figure 8 shows that 7% of initially added Ag was detected in the pellet in both the 10 and 20 µg L\(^{-1}\) AgNO\(_3\) exposures.

In the 50 µg L\(^{-1}\) Au@Ag NP exposures there was 0.16 ug-Ag 100,000 cells\(^{-1}\) at 0-24 hours and then a decrease in silver accumulation over time to 0.12 µg-Ag 100,000 cells\(^{-1}\) at 72-hours. This was the highest accumulation in the Au@Ag exposures. Au accumulation resulted in 0.01 µg-Au 100,000 cells\(^{-1}\) over time (Figure 2.5). Figure 2.6 shows relatively constant uptake over time of Ag and Au with 7% and 0.9% uptake of initially added Ag and Au, respectively. Figure 7 shows time and concentration dependent accumulation of Ag in the 50 µg L\(^{-1}\) AgNO\(_3\) exposures, with 0.60 µg-Ag 100,000 cells\(^{-1}\) at 72 hours. Figure 2.8 shows an increase over time in the percent uptake of initially added Ag measured at 0.1 – 3.4% over the 72-hour AgNO\(_3\) exposures.

2.4.2.4. ISOTOPIC RATIOS

Aside from the 72-hour time point in the 20 µg L\(^{-1}\) Au@Ag exposure, ratios of Ag:Au in Au@Ag NP 10 and 20 µg L\(^{-1}\) exposures were relatively equal to the original NP ratio with values ranging from 3.5 to 3.8 (Figure 2.9). The Ag:Au ratio in the 20 µg L\(^{-1}\) Au@Ag 72-hour time point was greater than the original Au@Ag NP ratio at a value of 4.0. Figure 2.9 shows that Ag:Au ratios in the 50 µg L\(^{-1}\) decreased over time and at 0, 24, and 48-hour measurements Ag:Au ratios were greater than the original NP ratio, while in the final measurement at 72 hours the ratio (2.6) was less than the original NP ratio (2.96).
2.4.2.5. ALGAL GROWTH

Figure 2.10 shows inhibition in cell densities over time after initial exposure to all concentrations of Au@Ag exposures (1, 10, 20, 50 µg L\(^{-1}\)) over time. All concentrations showed a 10-15% decrease in cell densities in 24 hours but increased after 48 hours of exposure to all concentrations of Au@Ag. However, cultures were not able to recovery to densities equal to that of the control and remained at an average of 14.4 ± 6.9 % less than the control at 72 hours of exposure. There was greater inhibition measured in the AgNO\(_3\) exposures than in the Au@Ag exposures with the lowest and highest concentration (1 and 50 µg L\(^{-1}\)) resulting in 61.9 and 94.9% growth inhibition by 72 hours. In the 1 and 10 µg L\(^{-1}\) exposures of AgNO\(_3\) cell density increased after 48 hours of exposure to 8.2 and 32.4% less than the control. Conversely, there was continual decrease in cell densities in the 20 and 50 µg L\(^{-1}\) exposures over the full 72 hours exposure resulting in 86.7 and 94.9% decrease at 72 hours.

2.4.2.6. DISTRIBUTION OF SILVER

Based on the initial masses added to the exposure bottles, Figure 2.11 shows that for all Au@Ag exposure concentrations the majority of was detected in the “in suspension or lost” fraction which was calculated based on the difference between initially added Ag and measured Ag masses in the wash, pellet, and dissolved phases. Percentages ranged from 90-95% of Ag accumulated in this fraction. The second highest accumulating fraction was the pellet (internal or strongly bound fraction) at 1-7.3% accumulation (Figure 2.11). Au was also determined to be mainly in the “in suspension or lost” phase at 98-100% accumulation of initially added Au followed by the pellet with 1-2% accumulation (Figure
Figure 2.13 shows that in the AgNO₃ exposures the majority of Ag was in the “in suspension or lost” fraction with 60-85% of initially added Ag detected. This fraction was followed by the “dissolved” fraction with 10-36% and the “internal or strongly bound” fraction with 3.4-7% of initially added Ag accounted for.

2.5. DISCUSSION

2.5.1. CONCENTRATION EFFECT ON ACCUMULATION

In the 10 and 20 µg L⁻¹ Au@Ag exposures silver and gold were taken up at similar levels and accumulated ratios of Ag:Au that were not different from the original Au@Ag ratio of 2.96. This suggests that there is the same amount of Au and Ag uptake. Based on dissolution results (Figures 2.11-2.13) Au remains in the NP form throughout the exposure and therefore the increased Ag uptake compared to Au suggests that Au@Ag NPs are transformations such as dissolution and aggregation are occurring but that aggregation is the dominant process at these concentrations. These dynamic changes and aggregation dominant regime were also seen in recent studies (Merrifield et al. 2017, Butz et al. 2019 submitted). The Au@Ag NPs are partially dissolving releasing Ag ions into solution which combined with the high chloride concentration of seawater, for silver-chloro complexes and become unavailable to algae for uptake. Algae then take up the original Au@Ag NPs with reduced Ag on the outer shell due to dissolution. The Ag:Au ratios measured in the algal pellet being nearly equal to that of the original Ag:Au ratio (2.96) suggests that the Au@Ag NP undergoes little dissolution. Another explanation could be that the original Au@Ag NP is dissolving and the additional Ag is taken up in the form of nucleated AgNPs and/or silver-chloro complexes to account for the additional Ag. At the 10 and 20 µg L⁻¹
Au@Ag exposures there is decreased accumulation in μg-Ag 100,000 cells$^{-1}$ over time at relatively constant uptake. For both concentrations there is also consistent accumulation of Ag and Au in ratios less than or equal to that of the original NP ratio over time, indicating more Ag uptake than Au or similar uptake of both metals. We explain this as dissolution and aggregation of Au@Ag NPs and an overall ripening of the original Au@Ag NPs. Therefore, uptake in 10 and 20 μg L$^{-1}$ Au@Ag exposures is preferential to the ripened Au@Ag NPs over the Ag ions or silver-chloro complexes in solution. Current research conducted on similar concentrations further supports our theory that at 10 and 20 μg L$^{-1}$, both aggregation and dissolution are important in NP behavior (Merrifield et al. 2017). It has been shown dinoflagellates are mixotrophic and can switch from autotrophy to heterotrophy (Jacobson and Anderson 1996, Li et al. 1996, Johnson 2014, Jeong et al. 2018). It is possible that in the 10 and 20 μg L$^{-1}$ exposures dinoflagellates may be taking up AgNPs by phagocytosis thus causing an increase in Ag accumulation. However, it was shown that P. minimum specifically may switch to heterotrophy in the dark under nutrient poor conditions to potentially restore the anabolic demand for nitrogen and phosphorous after a natural 12-hour light cycle (Heil et al. 2005). Nutrients were not limited in this study; thus, phagocytosis is unlikely.

At the 50 μg L$^{-1}$ Au@Ag exposures there was a time dependent accumulation of Ag and Au due to a mix of processes that occur during the exposure time. Over time there is more dissolution of Au@Ag NPs and starts to resemble lower concentration exposures as seen in previous research (Merrifield and Lead 2016) toward end of the exposure (48-72 hours) where Ag is unavailable to dinoflagellates. Although there is no direct evidence that
AgNPs are unavailable there is no direct evidence that they are available. If available, then the original NPs are more bioavailable as supported by Ag:Au ratios (discussed in detail in section 2.5.2) depleted in Ag, which means that if the chloro-complexes are available, then the Au@Ag bioavailability must be even higher to account for ratio. We explain this as ripening of Au@Ag NPs at the 0-hour time point followed by increasing dissolution toward the end of the exposure. Ag ions released from dissolution form silver-chloro complexes in suspension and potentially new smaller AgNPs, as seen in Merrifield et al. 2016, which become unavailable to dinoflagellates. Nucleation of secondary AgNPs may also be occurring in solution which may be taken up in preference to the original Au@Ag NPs that, due to dissolution, have less Ag on the outside. An aggregation dominant system is also supported by the Ag:Au ratios in the 50 µg L⁻¹ Au@Ag exposures. Ratios in the pellet were greater than the original NP ratio were detected aside from the final measurement at 72 hours where the ratio is less than the original NP ratio indicating more Ag than Au uptake (Figure 2.9). We postulate both aggregation and dissolution are occurring, although aggregation is dominant, and that free ions complex with chloride in solution or form smaller AgNPs in solution. We also expect the ripening of Au@Ag NPs where Ag ions from dissolution re-precipitate onto the original NP forming a larger NP, thus becoming less bioavailable to algae than the smaller AgNPs form from nucleation of free Ag ions and chloride. This theory is further supported by research conducted on Au@Ag NPs by Merrifield et al. (2017) where the highest exposure concentrations of 50 and 100 µg L⁻¹ showed a significant increase in the Ag particle diameter and a decrease in particle number indicating that NPs are primarily aggregating in moderately hard water. The media used in this study was more complex and had a higher ionic strength than moderately hard water.
and therefore we suspect that there would be more and faster transformations compared to moderately hard water.

### 2.5.2. ISOTOPIC RATIOS

Isotopic ratios further support our suggestion of dissolution being dependent on exposure concentration. There was relatively more uptake of Au than Ag at lower concentrations than higher concentrations. Dissolution data shows that the Au core remains protected and does not dissolve during the exposure, therefore uptake of Au is in the NP form. Thus, uptake of Au at lower concentrations indicated the uptake of the Au@Ag NP with a reduced Ag outer layer. More uptake of Ag at the higher concentrations indicates more aggregation of Au@Ag NPs that makes the original NP unavailable to algae for uptake. The increased uptake of Ag is in the form of silver chloride-complexes or ripened AgNPs. This is further supported by results in Figure 2.13 where Ag in the AgNO$_3$ exposures was mainly detected in the “in suspension or lost” fraction. This suggests that free Ag ions released form dissolution are either forming silver-chloride complexes in solution or aggregating to form new AgNPs.

### 2.5.3. GROWTH INHIBITION

For Au@Ag NP exposures, as concentration increased cell density decreased (Figure 2.10). After 24 hours of exposure cell densities were able to recover but densities remained less than the control. When examining our data of cell density compared to accumulated Ag in the pellet, there seems to be an inverse relationship between the amount of Ag accumulated and cell density; as accumulated Ag increased, cell density decreased.
However, there was no significant relationship between the mass associated with the pellet and cell density (p = 0.21, regression analysis). However, current research has suggested that intracellular and cell-associated Ag is the main determinant of AgNP toxicity (Ivask 2016, Malysheva et al. 2016, Collins et al. 2017). However, with EC\textsubscript{50} values of 38.3 ng-Ag mL\textsuperscript{-1} for 30 nm citrate coated AgNPs and 118.1 ng-Ag mL\textsuperscript{-1} for 70 nm citrate coated AgNPs, which relate to the size of the Au@Ag NPs used in this study (70 nm total size, 30 nm core size) (Malysheva et al. 2016), we speculate that due to our low exposure concentrations (µg L\textsuperscript{-1}) as compared to other recent studies using higher exposure concentrations (µg mL\textsuperscript{-1}) is the reason that our results to do not reflect this trend. Meaning that higher exposure concentrations are needed in order to see a significant relationship between internalized Ag and cell inhibition. In the AgNO\textsubscript{3} exposures a concentration and time dependent trend can be seen in Figure 10 where cell densities decrease with increasing exposure concentration. However, aside from the 1 µg L\textsuperscript{-1} exposure cultures were not able to recover over time as seen in the Au@Ag exposures. There was a significant relationship determined between Ag accumulation and cell density in the AgNO\textsubscript{3} exposures (p << 0.05, regression analysis). This further explains the lack of inhibition see in Au@Ag exposures where less Ag was accumulated as compared to masses detected in the AgNO\textsubscript{3} exposures. The similarities and differences between Au@Ag NP and AgNO\textsubscript{3} suggest a nano-specific effect but that AgNO\textsubscript{3} (ie. Ag ions as silver-chloride complexes) causes more cell inhibition than AgNPs. Based on these results combined with evidence from the NP characterization and Ag accumulation sections, we hypothesize that lower concentrations of Au@Ag NPs are dominated by dissolution during exposure and algae are exposed to silver-chloro
complexes in suspension. Thus, over time Ag becomes unavailable to algae and cell densities are able to recover from the initial exposure.

2.6. CONCLUSION

In this study we hypothesize that dissolution is dependent on concentration where there is more dissolution at lower concentrations and more aggregation at higher concentrations. In addition, several transformations may be occurring simultaneously. For instance, where aggregation is dominant, dissolution is likely to still happen due to decreased aggregation at lower concentrations there is more surface area of the NP exposed to the high chloride media which acts as a sink for free Ag ions and forces more dissolution. Due to these complex transformations in seawater media algae are exposed to original Au@Ag with a reduced Ag outer layer, ripened AgNPs, or silver-chloro complexes during the exposure. We have shown that Ag is associated with *P. minimum* cells, as shown by Ag measured in the algal pellet, with differences between the particles and ions observed. After exposure to Au@Ag cell densities decrease in a dose-dependent manner and we speculate that algae are able to recover from the initial exposure to exposure concentrations of 1, 10, 20, and 50 µg L\(^{-1}\) of Au@Ag after 24 hours and continue to increase in cell density. Consistent with previous research, our results confirm that Ag is taken up and accumulated by algae when exposed to AgNO\(_3\) and that AgNO\(_3\) is detrimental to algae growth. From these data and at current environmental levels of AgNPs, however, no immediate risk of AgNPs to *P. minimum* is indicated. Further testing and mechanistic understanding of AgNP interactions with marine algae should be continued.
**Table 2.1. NCMA L1 –Si Medium.** Enriched seawater medium (Guillard and Ryther 1962; Guillard and Harraves 1993) -To prepare, begin with 950 mL of filtered natural seawater. Add the quantity of each component as indicated below, and then bring the final volume to 1 liter using filtered natural seawater. The trace element solution and vitamin solutions are given below. Autoclave. Final pH should be between 8.0 and 8.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Solution</th>
<th>Quantity</th>
<th>Molar Concentration in Final Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>75.00 g L⁻¹ dH₂O</td>
<td>1 mL</td>
<td>8.82 x 10⁻⁴ M</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>5.00 g L⁻¹ dH₂O</td>
<td>1 mL</td>
<td>3.62 x 10⁻⁵ M</td>
</tr>
<tr>
<td>Trace element solution</td>
<td>(see recipe below)</td>
<td>1 mL</td>
<td>---</td>
</tr>
<tr>
<td>Vitamin solution</td>
<td>(see recipe below)</td>
<td>0.5 mL</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 2.2. NCMA L1 Medium Trace Element Solution. To 950 mL dH$_2$O add the following components and adjust to a final volume to 1 liter with dH$_2$O. Autoclave.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Solution</th>
<th>Quantity</th>
<th>Molar Concentration in Final Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$EDTA · 2H$_2$O</td>
<td>---</td>
<td>4.36 g</td>
<td>1.17 x 10$^{-5}$ M</td>
</tr>
<tr>
<td>FeCl$_3$ · 6H$_2$O</td>
<td>---</td>
<td>3.15 g</td>
<td>1.17 x 10$^{-5}$ M</td>
</tr>
<tr>
<td>MnCl$_2$ · 4H$_2$O</td>
<td>178.10 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>9.09 x 10$^{-7}$ M</td>
</tr>
<tr>
<td>ZnSO$_4$ · 7H$_2$O</td>
<td>23.00 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>8.00 x 10$^{-8}$ M</td>
</tr>
<tr>
<td>CoCl$_2$ · 6H$_2$O</td>
<td>11.90 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>5.00 x 10$^{-8}$ M</td>
</tr>
<tr>
<td>CuSO$_4$ · 5H$_2$O</td>
<td>2.50 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>1.00 x 10$^{-8}$ M</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$ · 2H$_2$O</td>
<td>19.9 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>8.22 x 10$^{-8}$ M</td>
</tr>
<tr>
<td>H$_2$SeO$_3$</td>
<td>1.29 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>1.00 x 10$^{-8}$ M</td>
</tr>
<tr>
<td>NiSO$_4$ · 6H$_2$O</td>
<td>2.63 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>1.00 x 10$^{-8}$ M</td>
</tr>
<tr>
<td>Na$_3$VO$_4$</td>
<td>1.84 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>1.00 x 10$^{-8}$ M</td>
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<tr>
<td>K$_2$CrO$_4$</td>
<td>1.94 g L$^{-1}$dH$_2$O</td>
<td>1 mL</td>
<td>1.00 x 10$^{-8}$ M</td>
</tr>
</tbody>
</table>
Table 2.3. NCMA L1 Medium Vitamin Solution. First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH₂O, dissolve the thiamine, add the amounts of the primary stocks as indicated in the quantity column below, and bring final volume to 1 liter with dH₂O. Store in refrigerator or freezer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Primary Stock Solution</th>
<th>Quantity</th>
<th>Molar Concentration in Final Medium</th>
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</thead>
<tbody>
<tr>
<td>Thiamine · HCl (vit. B₁)</td>
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<td>200 mg</td>
<td>2.96 x 10⁻⁷ M</td>
</tr>
<tr>
<td>Biotin (vit. H)</td>
<td>0.1 g L⁻¹ dH₂O</td>
<td>10 mL</td>
<td>2.05 x 10⁻⁹ M</td>
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<tr>
<td>Cyanovobalamin (vit. B₁₂)</td>
<td>1.0 g L⁻¹ dH₂O</td>
<td>1 mL</td>
<td>3.69 x 10⁻¹⁰ M</td>
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</tbody>
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Figure 2.1. **Stock Nanoparticle Characteristics.** Each condition was conducted using 100 µg L⁻¹ Au@Ag NPs. (A) Dynamic Light Scattering hydrodynamic size of stock Au@Ag NPs dispersed in ultra high purity water. (B) Ultraviolet visible spectrometry absorbance spectra of stock Au@Ag NPs dispersed in ultra high purity water.
Figure 2.2. UV-vis Results; Au@Ag in Media. Absorption spectra by ultraviolet visible spectrometry; Au@Ag NP. UV-vis analysis of Au@Ag NPs dispersed in exposure media with algae at 0-hour time point (A) 20 µg L\(^{-1}\) Au@Ag NP (B) 50 µg L\(^{-1}\) Au@Ag NP (C) 200 and 50 µg L\(^{-1}\) Au@Ag NPs dispersed in MQ water.
Figure 2.3. DLS Size Distribution in Media; 20 µg L⁻¹ Au@Ag. Size distribution as measured by dynamic light scattering; 20 µg L⁻¹ Au@Ag NP. Dynamic light scattering hydrodynamic size of stock Au@Ag NPs dispersed in exposure media with algae at (A) seawater blank (B) 0-hour time point (C) 24-hour time point (D) 48-hour time point (E) 72-hour time point. Each line represents an individual measurement of the same dispersed solution.
Figure 2.4. DLS Size Distribution in Media; 50 µg L⁻¹ Au@Ag. Size distribution as measured by dynamic light scattering; 50 µg L⁻¹ Au@Ag NP. Dynamic light scattering hydrodynamic size of stock Au@Ag NPs dispersed in exposure media with algae at (A) seawater blank (B) 0-hour time point (C) 24-hour time point (D) 48-hour time point (E) 72-hour time point. Each line represents an individual measurement of the same dispersed solution.
Figure 2.5. Silver and Gold Mass Accumulation in Cells Over Time; Au@Ag. Mass of Ag in µg per 100,000 cells. Values were normalized to the control (0 µg L⁻¹) exposures. Results from the 1 µg L⁻¹ Au@Ag exposures were below the instrumentation detection limit (0.5 µg L⁻¹) and not reported in this figure. Internalization analysis was performed at each time point for (A) 10 µg L⁻¹ (B) 20 µg L⁻¹ (C) 50 µg L⁻¹ Au@Ag exposures. Note that all y-axis units are the same but y-axis scale varies between figures.
Figure 2.6. Silver and Gold Percent Accumulation in Cells Over Time; Au@Ag. Percentage of Ag accumulated by cells over 72-hour exposure time. Internalization analysis was performed at each time point for (A) 0 µg L⁻¹ (B) 1 µg L⁻¹ (C) 10 µg L⁻¹ (D) 20 µg L⁻¹ and (E) 50 µg L⁻¹ Au@Ag exposures.
Figure 2.7. Silver and Gold Mass Accumulation in Cells Over Time; AgNO₃. Mass of Ag in µg per 100,000 cells. Internalization analysis was performed at each time point for (A) 0 µg L⁻¹ (B) 1 µg L⁻¹ (C) 10 µg L⁻¹ (D) 20 µg L⁻¹ and (E) 50 µg L⁻¹ AgNO₃ exposures. Note that all y-axis units are the same but y-axis scale varies between figures.
Figure 2.8. Silver Percent Accumulation in Cells Over Time; AgNO₃. Percentage of Ag accumulated by cells over 72-hour exposure time. Internalization analysis was performed at each time point for (A) 0 µg L⁻¹ (B) 1 µg L⁻¹ (C) 10 µg L⁻¹ (D) 20 µg L⁻¹ and (E) 50 µg L⁻¹ AgNO₃ exposures.
Figure 2.9. Silver to Gold Ratios in Algae Over Time; Au@Ag. Results from the 1 µg L\(^{-1}\) Au@Ag exposures were below the instrumentation detection limit (0.5 µg L\(^{-1}\)) and not reported in this figure. (A) 10 µg L\(^{-1}\) (B) 20 µg L\(^{-1}\) (C) 50 µg L\(^{-1}\) Au@Ag exposures. The original Au@Ag NP Ag:Au ratio is indicated as a straight horizontal line at a value of 2.96. Each bar represents the average of three replicates. Error bars represent standard deviation.
Figure 2.10. *P. minimum* Growth Inhibition Tests. Cell densities of *P. minimum* cells over time (72-hours) after exposure to 0, 1, 10, 20 and 50 µg L$^{-1}$ of (A) Au@Ag exposures (B) AgNO$_3$ exposures. Results were normalized to the control culture.
Figure 2.11. Silver Distribution; Au@Ag. Distribution of silver at the end of the exposure time (72-hours) for 0, 1, 10, 20 and 50 µg L$^{-1}$ Au@Ag exposures. (A) Distribution of silver in mass (µg-Ag) (B) Distribution of silver in percent of initially added silver (%). Initially added Ag masses correspond to 0.23, 2.25, 4.50, and 11.25 µg-Ag.
Figure 2.12. Gold Distribution; Au@Ag. Distribution of gold at the end of the exposure time (72-hours) for 0, 1, 10, 20 and 50 µg L⁻¹ Au@Ag exposures. (A) Distribution of silver in mass (µg-Au) (B) Distribution of gold in percent of initially added silver (%). Initially added Au masses correspond to 0.08, 0.76, 1.52 and 3.80 µg-Au.
Figure 2.13. Silver Distribution; AgNO₃. Distribution of silver at the end of the exposure time (72-hours) for 0, 1, 10, 20 and 50 µg L⁻¹ AgNO₃ exposures. (A) Distribution of silver in mass (µg-Ag) (B) Distribution of silver in percent of initially added silver (%). Initially added Ag masses correspond to 0.225, 2.25, 4.50, and 11.25 µg-Ag.
CHAPTER 3

UPTAKE AND ACCUMULATION OF ISOTOPICALLY LABELED SILVER-GOLD-SILVER NANOPARTICLES BY *CRASSOSTREA VIRGINICA*\(^1\)

3.1 ABSTRACT

Silver nanoparticles (AgNPs) are widely used as an anti-bacterial agent in numerous consumer products and therefore discharged to the environment. AgNPs are partially soluble in complex aquatic media and studies show little agreement as to whether effects and behavior are controlled by ions from dissolution or particles. At this point in time, the roles of ions and particles in AgNP uptake and accumulation have not been fully elucidated. In this study, we exposed the Eastern oyster, *Crassostrea virginica*, to 0, 1 and 50 µg L\(^{-1}\) of novel isotopically labeled, core-shell NPs (107Ag@Au@109Ag; 109Ag is the outer layer, coated in PVP) to determine the mechanism of uptake and quantify AgNP accumulation. Oysters (n=4) were exposed to AgNPs in natural estuarine water at ambient salinity, water temperature, and pH for a time period of two hours which was sufficient for them to filter all of the available media. At both test conditions, the average total Ag uptake was a small fraction (8.6 – 9.0 ± 3.4 %) of the total available Ag. Compared to the isotopic ratios measured for the core-shell AgNPs, we found increased accumulation of 107Ag in the 1 µg L\(^{-1}\) exposures and the opposite trend (increased accumulation of 109Ag) in the 50 µg L\(^{-1}\) exposures. Previous work has shown that as AgNPs dissolve, polyvinylpyrrolidone (PVP) prevents aggregation at these concentrations and silver ions likely reprecipitate as AgNPs or silver chloride complexes. We interpreted uptake as follows. At the 1 µg L\(^{-1}\) exposures, the higher 107Ag accumulation implies that the oysters are preferentially taking up the partially solubilized NPs. There is no evidence for the direct uptake of dissolved ions. It is also possible that some of the dissolved ions form secondary silver and silver chloride complexes and again there is no evidence that these are taken up based on ratios and total uptake, although this is possible. We explain higher 109Ag accumulation in 50
µg L⁻¹ exposures as follows. Dissolution still occurs but at the higher NP concentrations is less important as a percentage of the total Ag. At the higher concentrations, we hypothesize that ions form new Ag NPs (containing only 109Ag) and that these are taken up preferentially, but along with some of the core-shell NPs. Other processes likely occur (dissolution, formation of silver chloride precipitates and reprecipitation of ions on existing core-shell NPs) but do not explain uptake ratios.

3.2 INTRODUCTION

Engineered nanoparticles (ENPs) have been increasingly incorporated into consumer products, although research is still ongoing on their potential effects to the environment and human health. ENPs have numerous industrial applications including fuel cell technology (Stark et al. 2015), pharmaceutical drug-delivery (Parveen et al. 2012) and are frequently incorporated into food matrices (Tiede et al. 2008, Weir et al. 2012, Yang et al. 2013). Silver nanoparticles (AgNPs) are specific interest because of their antimicrobial and bactericidal properties (Morones et al. 2005) and wide use leading to extensive environmental exposure (Gottschalk et al. 2013, Sun et al. 2016). AgNPs are partially soluble, releasing ions and NPs in media but the relative importance of these two Ag sources in bioaccumulation and toxicity has not been determined (Lead et al. 2018). The current paradigm is that ions are the cause of toxicity (Leclerc and Wilkinson 2014), but there is likely a role for NPs (Piccapietra et al. 2012, Kroll et al. 2014, Shah et al. 2014, Shah et al. 2014). The relative importance of ion and ENPs is likely dependent on organism/cell type, ENP properties and media properties. Some recent work has suggested
a significant role for ENPs in bioaccumulation and that this is partially concentration dependent (Croteau et al. 2011, Croteau et al. 2014, Khan et al. 2012).

Historically, bivalves have been used as an indicator species for marine pollution monitoring (Boening et al. 1999, 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. This can lead to toxicity and, as many bivalves are widely consumed to food chain transfer and potential risk to human health. Bivalves have the ability to integrate and indicate physiochemical and biological variations in their surrounding environment (Bryan and Langston 1992, Chapman et al. 2001, Zuykov et al. 2013). More recently, bivalves have also been used as a biological test organism in NP accumulation and toxicity studies (Zuykov et al. 2011, Buffet et al. 2014, Volker et al. 2015), including the estuarine bivalve Crassostrea virginica (Trevisan et al. 2014, Johnson et al. 2015, Doyle et al. 2016). C. virginica is an intertidal reef forming bivalve and a valuable keystone species in southeast-ern estuaries where salinities range from 5 to 30 ppt (Buroker 1983, Dame and Prins 1998). 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). C. virginica is considered an ecosystem engineer in that their reefs provide multiple benefits for the ecosystem including water filtration, structure, habitat, and increased biodiversity (Newell and Koch 2004; Grabowski and Peterson 2007; Hoellein and Zarnoch 2014).
Bioaccumulation is an important property for the regulation and understanding fundamental mechanisms of ENP behavior and toxicity (Pettitt and Lead 2013). Recently, there has been a focus on nanohybrids (two or more nanomaterials to achieve increased multifunctionality (Aich et al. 2014, Saleh et al. 2015), and the use of specific nanohybrid types can facilitate our understanding of biological processes. In previous work, isotopically labeled 3-layer core-shell AgNPs (107Ag@Au@109Ag, with different capping agents) have been synthesized and physio-chemical behavior tested (Merrifield and Lead 2016). The formulation of the ENP is such that the outer layer of 109Ag is allowed to partially dissolve while still retaining a layer of Ag to ensure that organisms are exposed to Ag and not the Au middle layer. The middle Au layer is insoluble and prevents the Ag107 from dissolving so that it always remains as a particle. The isotopes of Ag are expected to behave identically but can be analytically distinguished. The objective of this study was to quantify particle and ion biouptake and accumulation based on the isotopic ratios of Ag taken up, using the test species *C. virginica*.

3.3 METHOD

3.3.1 SILVER NANOPARTICLES

Three layered isotopically labeled Ag107@Au@Ag109 NPs were previously synthesized (Merrifield and Lead 2016), and the nanohybrid dissolution and transformations were quantified in USEPA moderately hard water showing a 10-25% dissolution rate for the outer layer of 109Ag over 24 hours (Merrifield and Lead 2016), dependent on the capping agent. The data provides strong support that the outer (Ag109)
layer partially dissolves, and that the Au layer protects the Ag107 inner core, allowing for no dissolution of Ag107. All data on the ENP synthesis and behavior can be found in the Merrifield and Lead (2016). Core-shell AgNP were used 1-2 weeks after synthesis and were stored at 4°C in the dark between synthesis and exposures.

3.3.2 OYSTER AND WATER COLLECTION

Oysters between 2.60 and 9.20 cm in shell length were collected from Oyster Landing, North Inlet, Georgetown, South Carolina, at the Belle W. Baruch Institute for Marine and Coastal Sciences (National Estuarine Reserve Station ID: 8662245, 33° 21.1’ N, 79° 11.2’ W). Oysters were kept in an acclimation tank containing water from the collection site (which had previously been sand filtered on site) at ambient salinity and pH (34-35 ppt and a pH value of 7.6 – 8.0) for 4 days. Tides were established in the acclimation tank using tubing, water pumps and a reserve tank, on a 6-hour schedule, including 2 low and 2 high tides to mimic natural tidal variation. The sex of the oysters was determined using established relationships between size and gender (Coe 1934, Powell et al. 2012, Harding et al. 2013). Based on size and sex information the oysters were split into four groups (Table 3.1).

3.3.3 EXPOSURE

Exposures were performed in 2-liter glass beakers for 2 hours at concentrations of 0, 1 and 50 µg-Ag L⁻¹ treatment. Each beaker contained a single oyster and 2 L of natural seawater (BFSW, sand filtered) kept at ambient salinity and pH (34-35 ppt, 7.6 – 8.0 pH). Oysters were removed from the acclimation tank at the beginning of high tide and placed
into the beakers described above for exposures. This was done to induce filtration from the
oysters. Oysters were not fed during the 2-hour exposures. On average oysters filter 1 L
hour\(^{-1}\) g-tissue weight \(^{-1}\) (50 gallons day\(^{-1}\)) (Jorgensen 1966, Casas et al. 2018). This
filtration rate was taken into consideration when setting the exposure volume (2 L) and
exposure time (2 hours) to ensure that the total volume and mass of AgNPs would be
filtered by the oyster, on average. Filtration rate was also taken into account when
calculating Ag mass balances for each experiment. Each concentration of
Ag\(^{107}\)Au@Ag\(^{109}\) NPs was repeated in replicates of four, with an individual oyster in each
beaker.

3.3.4 ANALYSIS

Once the exposure time (2 hours) elapsed, the oyster was sacrificed and dissected
carefully separating the gills (G), hepatopancreas (HP), and visceral mass (VM). Tissues
were blotted with tissue to remove excess water and particles not incorporated into oyster
tissues and weighed. Tissues were digested in 15 ml centrifuge tubes with concentrated
trace metal grade nitric acid and placed in an incubator shaker (New Brunswick Scientific
Innova 44 Incubator Shaker Series) at 300 rpm and 20\(^{\circ}\)C, for 24 hours. Digested tissues
were made to a total acid concentration of 1%, filtered with a 0.45 \(\mu\)m filter (sterile PTFE
membrane) and analyzed for Ag\(^{107}\) and Ag\(^{109}\) via ICP-MS (Perkin Elmer NexION
350D). Both isotopes were quantified separately and added together to calculate total Ag.
3.3.5 SILVER SPECIATION MODELING

Silver speciation modeling was conducted using Visual Minteq 3.1 using a provided database of thermodynamic constants and using the Davies activity correction. Ag concentrations considered were 1 and 50 µg L\(^{-1}\). The following parameters were kept constant for all concentrations (mimicking the test media) pH 8.2, ionic strength 0.7, temperature 25°C. Calculations assumed thermodynamic equilibrium and therefore do not take into account reaction kinetics and are more accurate for inorganic speciation and have not been experimentally tested against Ag ENPs, to our knowledge. The data is assumed to be indicative and semi-quantitative for these reasons.

3.4 RESULTS

3.4.1 GENERAL OBSERVATIONS

Control exposures of 0 µg L\(^{-1}\) Au@AgNPs resulted in less than 0.5% uptake in all tissues indicating a background of 0.001 µg-Ag for all tissues (Figure 3.1). Figure 3.1 shows that at the 1 µg L\(^{-1}\) exposure concentration between 10 and 50% of the total Ag accumulated within tissues at the 1 µg L\(^{-1}\) exposure, which corresponded to a mass of 0.1 to 1 µg-Ag (wet tissue). There was 50-100% of the total Ag accumulated in tissues at the 50 µg L\(^{-1}\) exposure, which corresponds to a mass of 50-100 µg-Ag. However, if the largest size class of oysters (F2) was excluded accumulation decreases to 2-3% (2-3 µg-Ag) of the total Ag accumulated in the 50 µg L\(^{-1}\) exposures. This results in a lower percentage of initially added Ag accumulated than in the 1 µg L\(^{-1}\) exposures, but a greater mass of Ag accumulated. This resulted in a higher percentage of initially added Ag accumulated than in the 50 µg L\(^{-1}\) exposures, but less mass of Ag accumulated.
3.4.2 EFFECT OF AGNP CONCENTRATION ON TUSSE ACCUMULATION

While all three tissue sections accumulated Ag after exposure at 1 µg L\(^{-1}\), the main target organ for silver accumulation for all size classes was the hepatopancreas with an average of 10.6 ± 4.7% uptake of initially added Ag and an average of 1.32 ± 0.54 µg-Ag g tissue mass\(^{-1}\) in this tissue section (Figure 3.2, Tables 3.2 and 3.3). Accumulation of Ag was also detected in all tissues after exposure to 50 µg L\(^{-1}\) with the hepatopancreas accumulating the most Ag with an average of 7.1 ± 3.6% uptake of initially added Ag and an average of 16.31 ± 3.0 µg-Ag g tissue weight\(^{-1}\) in this tissue section (Figure 3.3, Tables 3.4 and 3.5). At 50 µg L\(^{-1}\) exposures the size class F2 (7.0 – 9.2 cm shell length) accumulated more Ag by mass than the other three size classes across all tissue sections. The other three size classes (M1, M2, F1) showed relatively similar Ag mass uptake in the gills (2-7 µg-Ag) and visceral mass (1-6 µg-Ag). There was a larger range in Ag mass accumulation detected in the hepatopancreas (2-11 µg-Ag) (Figure 3.3).

3.4.3 EFFECT OF SIZE AND SEX ON ACCUMULATION

Overall, larger oysters (shell length) accumulated more Ag than smaller oysters with relatively more 107Ag uptake at lower concentrations and more 109Ag at higher concentrations. More accumulation was detected in the 50 µg L\(^{-1}\) than the 1 µg L\(^{-1}\) exposures across all size classes and tissues. In the 1 µg L\(^{-1}\) exposures the size class F2, rep 1 was shown to be significantly different (ANOVA) and treated as an outlier. In 1 µg L\(^{-1}\) exposures male and female size classes accumulated between 0.1 – 2 µg-Ag g-tissue weight\(^{-1}\), with relatively high variability between individuals (Figure 3.2, Table 3.3). In the
50 µg L⁻¹ exposures F2 accumulated the highest amounts of Ag but in variable amounts amongst individuals within this group. It was shown that F2 as a group accumulated significantly more Ag than M1, M2, and F1 size classes. However, within the size class of F2, rep 2 was shown to be significantly different than the other reps in visceral mass and hepatopancreas but not in gill accumulation.

3.4.4 ISOTOPIC RATIOS

In 1 µg L⁻¹ exposures isotopic ratios of ¹⁰⁹Ag:¹⁰⁷Ag in oyster tissues (1.22 ± 0.26) showed depleted ¹⁰⁹Ag accumulation in organisms compared to the original NP ratio (2) (Figure 3.2). Aside from M1 rep 1 gill tissue and F2 rep 4 gill tissue, all ratios were consistently lower than the original NP ratio. M1 rep 1 and F2 rep 4 were determined to be significantly different than other ratios within the respective size class and treated as outliers. Conversely, exposures of 50 µg L⁻¹ Au@AgNPs resulted in increased isotopic ratios of ¹⁰⁹Ag:¹⁰⁷Ag (4.70 ± 2.77) and increased accumulation of ¹⁰⁹Ag in organisms compared to the original AgNP ratio (2) (Figure 3.3). This is consistent across tissues except for M1 rep 1 and F2 reps 2 and 3. These individuals were found to be significantly different from other ratios in the gill and visceral mass tissues, however M1 rep 1 and F2 reps 2 and 3 were not found to be significantly different from other individuals in their respective size classes with respect to the hepatopancreas tissues and could not be discounted.
3.4.5 SILVER SPECIATION IN SEAWATER

The two major species of Ag in seawater are AgCl$^{2-}$ and AgCl$^{3-}$. At 1 µg L$^{-1}$ Ag in seawater speciation is 60.6% AgCl$^{2-}$ and 37.7% AgCl$^{3-}$, with less than 0.01% in the ionic (Ag$^{+}$) form. At 50 µg L$^{-1}$ Ag in seawater speciation is 60.6% AgCl$^{2-}$ and 37.7% AgCl$^{3-}$, with less than 0.01% in the ionic (Ag$^{+}$) form. At 100 µg L$^{-1}$ Ag in seawater speciation is 60.7% AgCl$^{2-}$ and 37.7% AgCl$^{3-}$, with less than 0.01% in the ionic (Ag$^{+}$) form (Table 3.6). Calculations suggest that a dissolved Ag concentration of greater than 100 µg L$^{-1}$ Ag is required to precipitate Ag in exposure media.

3.1 DISCUSSION

3.1.1 GENERAL OBSERVATIONS

In all treatments, Ag was accumulated within oyster tissues and in all exposures, the mass of Ag accumulated increased with increasing exposure concentration; as exposure concentration increased the percentage of total silver accumulated decreased (Figure 3.1). Accumulation shows a concentration dependent trend where higher exposure concentrations accumulated more Ag mass. About 10 and 50% of the total Ag accumulated within tissues at the 1 µg L$^{-1}$ exposure, which corresponded to a mass of 0.1-1 µg-Ag (wet tissue). This resulted in a higher percentage of initially added Ag accumulated than in the 50 µg L$^{-1}$ exposures, but less mass of Ag accumulated. There was 50-100% of the total Ag accumulated in tissues at the 50 µg L$^{-1}$ exposure, which corresponds to a mass of 50-100 µg-Ag. However, if the largest size class of oysters (F2) is excluded, accumulation decreases to 2-3% (2-3 µg-Ag) of the total Ag accumulated in the 50 µg L$^{-1}$ exposures. This
results in a lower percentage of initially added Ag accumulated than in the 1 µg L\textsuperscript{-1} exposures, but a greater mass of Ag accumulated.

### 3.1.2 ACCUMULATION AND ISOTOPIC RATIOS

There was an increased accumulation of 107Ag in oyster tissues in the 1 µg L\textsuperscript{-1} exposures as compared to 109Ag accumulation (Figure 3.1). We interpret this as partial dissolution of the 109Ag outer shell partially dissolves while the 107Ag core remains contained by the Au layer and the original core-shell NPs are taken up most as shown by enhanced 107Ag uptake. These results are further supported by Merrifield and Lead (2016) where 10-25% dissolution of the outer 109Ag shell was detected. Representing free ions and/or AgCl complexes, 109Ag uptake is depleted further supporting that oysters are taking up core-shell NPs depleted in 109Ag, most likely due to partial dissolution of the outer shell. Partial dissolution and increased percent dissolution at low concentrations was also previously reported in EPA moderately hard water (Merrifield and Lead 2016). Ag uptake ratios of 109:107 show more 107Ag uptake than 109Ag. We explain this as preferential uptake of original core-shell-shell NPs over Ag ions, present as Ag chloride complexes. We speculate that Ag ion uptake is through the gills which results in a slow uptake with potential dissolution of the AgNP while in contact with the gill tissue. Whereas, NP uptake would be through the digestive system and the Ag uptake would be of the whole AgNP.

In 50 µg L\textsuperscript{-1} exposures there is a depletion in 107Ag uptake and an increase in 109Ag uptake (Figure 3.3). We interpret this as dissolution and uptake of the 109Ag outer shell. This would suggest that Ag ions are taken up readily which contradicts results in 1 µg L\textsuperscript{-1}
exposures. However, based on percent uptake data (Figure 3.1) we assume that because there is a larger concentration in 50 than 1 µg L\(^{-1}\) exposures, secondary 109AgNPs may be more likely to form from dissolution of the outermost shell. These secondary 109AgNPs are more bioavailable than the primary core-shell-shell NPs with a reduced 109 shell, and free ions (in form of silver chloride complexes). Thus, oysters may be taking up secondary particles of 109Ag. Ag uptake ratios of 109:107 show more 109 uptake than 107 further supporting the idea of preferential uptake of secondary 109AgNPs over primary core-shell-shell AgNPs and ions which are present as AgCl complexes.

3.1.1 ACCUMULATION AND SILVER SPECIATION

Silver speciation in seawater indicates that Ag speciation is constant and due to the amount of Cl\(^{-}\) in seawater it would take massive amounts of Ag in seawater to change speciation. At 100 µg L\(^{-1}\) Ag addition, 25°C, from 0 mg L\(^{-1}\) to 2.1 mg L\(^{-1}\) addition of Ag\(^{+}\) to seawater dissolution occurs releasing free ions into solution which increases the total amount of Ag in solution (Figure 3.1). After this point a plateau is reached in which the total amount of Ag in solution does not increase although Ag\(^{+}\) is still being added. The excess amount of Ag is precipitated out of solution in the form of Cerargyrite. Based on speciation modeling the chance of free ions being taken up is low but uptake of AgCl complexes is likely due to the high Cl\(^{-}\) concentration in seawater. In addition, Ag concentration in 1 and 50 µg L\(^{-1}\) exposures is not large enough to surpass the Ag solubility point and form solid precipitates. Therefore, we speculate that due to the high ionic strength of seawater and the low amount of ionic Ag, Ag forms into silver chloride species and becomes unavailable to the oyster in the 1 µg L\(^{-1}\) exposures. However, in the 50 µg L\(^{-1}\)
exposures based on silver solubility data, we explain increased uptake of 109Ag as the complexation of dissolved 109Ag to silver-chloro complexes in suspension or nucleation of new NPs that may be more likely to form from dissolution of the outermost shell, which agrees with results from previous research at similar concentrations (Merrifield et al. 2017).

3.1.1 ACCUMULATION, SEX AND SIZE

Aside from treatment F2, the hepatopancreas was determined to accumulate the most Ag. Consistent accumulation among specific tissues was also detected by Abbe et al. (1994). There was variable uptake in individual oysters both between and within size classes, in both 1 µg L\(^{-1}\) and 50 µg L\(^{-1}\) exposures. However, in 50 µg L\(^{-1}\) exposures there was a significant increase in accumulation in the F2 size class (Figure 3.3). Variable and size-related differences in accumulation of Ag by oysters has also been reported in previous research (Cunningham and Tripp 1975, Fowler et al. 1978, Phelps et al. 1985, Abbe et al 1994). When determining uptake and storage of chemicals in oysters, it has been shown that in general, body burden increases with size of the organism (Abbe et al. 1994). The F2 size class consists of the largest shell lengths in this study and corresponds to reproductively mature females (Coe 1934, Powell et al. 2013, Harding et al. 2013). On average intertidal oysters in North Inlet allocate roughly 0 – 48.4% of total energy on reproduction depending on the season (Dame 1976). Other research shows that male and female oysters have different bioenergetic functioning and that energy demands for reproduction are higher in reproductively active individuals (Mathieu and Lubet 2011) and furthermore higher for reproductively active females than males (Chavez-Villalba 2013, Bayne and Newell 1983). We speculate that due to difference in energetic requirements in
males and females that these individuals would have an increased need to filter more
nutrients from the water column and consequently take up more Ag in the process of
filtering. This reasoning is why the F2 size class of reproductively active females
accumulated the largest amounts of Ag.

3.1 CONCLUSION

At lower concentrations of AgNPs there was enhanced uptake of $^{107}\text{Ag}$, and a higher
percent uptake of total Ag added, while at higher concentrations of AgNPs there is more
$^{109}\text{Ag}$ uptake than $^{107}\text{Ag}$ and a lower percent uptake of total Ag added. We assume that
there is more mass of Ag ions in solution but a lower percent dissolution at $50 \mu g \text{ L}^{-1}$, which
suggests ions could form secondary $^{109}\text{AgNPs}$. We know that aggregation dominates in
high concentrations, but dissolution still occurs. Therefore, we interpret this data as
dissolution and aggregation of $^{109}\text{Ag}$ and uptake of secondary $^{109}\text{AgNPs}$ in $50 \mu g \text{ L}^{-1}$
exposures. We know low concentrations of Ag are dominated by dissolution which releases
free ions. Coupled with speciation data we explain $1 \mu g \text{ L}^{-1}$ exposure results of enhanced
uptake of $^{107}\text{Ag}$ as dissolution of the $^{109}\text{Ag}$ outermost shell and preferential uptake of the
primary core-shell-shell with reduced $^{109}\text{Ag}$ or free Ag ions which form silver chloride
complexes and become unavailable to oysters.
Table 3.1. Oyster Characteristics. Oyster shell length, assigned sex, and labels for 107Ag@Au@109Ag NPs exposure experiments.

<table>
<thead>
<tr>
<th>Shell length (cm)</th>
<th>Assigned sex</th>
<th>Exposure group label</th>
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</thead>
<tbody>
<tr>
<td>2.6 – 3.5</td>
<td>Male</td>
<td>M1</td>
</tr>
<tr>
<td>3.6 – 5.0</td>
<td>Male</td>
<td>M2</td>
</tr>
<tr>
<td>5.1 – 6.7</td>
<td>Female</td>
<td>F1</td>
</tr>
<tr>
<td>7.0 – 9.2</td>
<td>Female</td>
<td>F2</td>
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</table>
**Table 3.2. Silver Speciation in Seawater.** Speciation of silver in seawater represented in percent of total concentration added. Generated by silver speciation modeling in Visual Minteq 3.1. parameters were kept constant for all concentrations, pH 8.2, ionic strength 0.7, temperature 25°C, and possible precipitating phases of bromyrite and cerargyrite.

<table>
<thead>
<tr>
<th>Ag concentration added (µg- Ag L⁻¹)</th>
<th>Silver speciation (%)</th>
</tr>
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<tr>
<td></td>
<td>AgCl (aq)</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>50</td>
<td>1.5</td>
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</tbody>
</table>
Table 3.3. Percentage of Silver Uptake in Specific Oyster Tissues; 1µg L⁻¹. Average percent uptake of total Ag in individual oysters in specific tissue sections after exposure to 1µg L⁻¹ 107Ag@Au@109Ag NPs. Values were determined based on silver accumulation in specific tissues and recorded weights of organisms, not adjusted to µg-Ag g tissue weight⁻¹.

<table>
<thead>
<tr>
<th>Size classes</th>
<th>Shell size (cm)</th>
<th>Silver accumulation in specific tissue (%)</th>
<th>109:107 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td>Visceral mass</td>
</tr>
<tr>
<td>M1</td>
<td>2.6 – 3.5</td>
<td>2.1 ± 0.9</td>
<td>6.3 ± 2.4</td>
</tr>
<tr>
<td>M2</td>
<td>3.6 – 5.0</td>
<td>6.0 ± 4.9</td>
<td>16.8 ± 0.8</td>
</tr>
<tr>
<td>F1</td>
<td>5.1 – 6.7</td>
<td>3.2 ± 1.9</td>
<td>10.7 ± 4.5</td>
</tr>
<tr>
<td>F2</td>
<td>7.0 – 9.2</td>
<td>6.9 ± 2.9</td>
<td>13.7 ± 4.7</td>
</tr>
</tbody>
</table>
Table 3.4. Percentage of Silver Uptake in Specific Oyster Tissues; 50 µg L$^{-1}$. Average percent uptake of total Ag in individual oysters in specific tissue sections after exposure to 50 µg L$^{-1}$ 107Ag@Au@109Ag NPs.

<table>
<thead>
<tr>
<th>Size classes</th>
<th>Shell size (cm)</th>
<th>Silver accumulation in specific tissue (%)</th>
<th>109:107 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td>Visceral mass</td>
</tr>
<tr>
<td>M1</td>
<td>2.6 – 3.5</td>
<td>0.37 ± 0.2</td>
<td>0.39 ± 0.2</td>
</tr>
<tr>
<td>M2</td>
<td>3.6 – 5.0</td>
<td>0.66 ± 0.4</td>
<td>0.69 ± 0.6</td>
</tr>
<tr>
<td>F1</td>
<td>5.1 – 6.7</td>
<td>0.33 ± 0.2</td>
<td>0.46 ± 0.1</td>
</tr>
<tr>
<td>F2</td>
<td>7.0 – 9.2</td>
<td>17.41 ± 6.94</td>
<td>60.27 ± 29.78</td>
</tr>
</tbody>
</table>
Table 3.5. Silver Mass Uptake in Specific Oyster Tissues; 1 µg L⁻¹. Uptake in mass (µg) of total Ag in individual oysters in specific tissue sections after exposure to 1 µg L⁻¹ 107Ag@Au@109Ag NPs.

<table>
<thead>
<tr>
<th>Size classes</th>
<th>Shell size (cm)</th>
<th>Silver accumulation in specific tissue µg-Ag g tissue weight⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gills</td>
</tr>
<tr>
<td>M1</td>
<td>2.6 – 3.5</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>M2</td>
<td>3.6 – 5.0</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>F1</td>
<td>5.1 – 6.7</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>F2</td>
<td>7.0 – 9.2</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>
Table 3.6. Silver Mass Uptake in Specific Oyster Tissues; 50 µg L\(^{-1}\). Uptake in mass (µg) of total Ag in individual oysters in specific tissue sections after exposure to 50 µg L\(^{-1}\) 107Ag@Au@109Ag NPs.

<table>
<thead>
<tr>
<th>Size classes</th>
<th>Shell size (cm)</th>
<th>Silver accumulation in specific tissue (µg-Ag g tissue weight(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Gills</strong></td>
</tr>
<tr>
<td>M1</td>
<td>2.6 – 3.5</td>
<td>7.5 ± 5.4</td>
</tr>
<tr>
<td>M2</td>
<td>3.6 – 5.0</td>
<td>5.9 ± 3.4</td>
</tr>
<tr>
<td>F1</td>
<td>5.1 – 6.7</td>
<td>2.1 ± 3.0</td>
</tr>
<tr>
<td>F2</td>
<td>7.0 – 9.2</td>
<td>25.8 ± 11.6</td>
</tr>
</tbody>
</table>
Figure 3.1. Percent Uptake of Silver in Oysters. Percentage of total silver uptake in individual oysters based on initial addition of 107Ag@Au@109Ag NP. (A) 1 µg L$^{-1}$ 107Ag@Au@109Ag NP (B) 50 µg L$^{-1}$ 107Ag@Au@109Ag NP (C) 50 µg L$^{-1}$ 107Ag@Au@109Ag NP, without F2 size group for scale. Each vertical bar represents an individual oyster.
Figure 3.2. Silver Mass and Ratio Accumulation in Oyster Tissues; 1 µg L\textsuperscript{-1}. Silver mass accumulation and isotopic ratios of 109Ag:107Ag in gills, visceral mass, and hepatopancreas tissues of individual oysters after a 2-hour exposure to 1 µg L\textsuperscript{-1} 107Ag@Au@109Ag NP. Size ranges were determined off of shell length and include M1 (2.6 – 3.5 cm), M2 (3.6 – 5.0 cm), F1 (5.1 – 6.7 cm), and F2 (7.0 – 9.2 cm). (A) gills Ag accumulation (B) gills 109:107 ratio (C) visceral mass Ag accumulation (D) visceral mass 109:107 ratio (E) hepatopancreas Ag accumulation (F) hepatopancreas 109:107 ratio. Each vertical bar represents an individual oyster. The 109:107 silver ratio of stock particles is indicated on the figure as a straight horizontal line at a value of 2. Significantly different results are indicated by a star (*).
Figure 3.3. Silver Mass and Ratio Accumulation in Oyster Tissues; 50 µg L\(^{-1}\). Silver mass accumulation and isotopic ratios of 109Ag:107Ag in gills, visceral mass, and hepatopancreas tissues of individual oysters after a 2-hour exposure to 50 µg L\(^{-1}\) 107Ag@Au@109Ag NP. Size ranges were determined off of shell length and include M1 (2.6 – 3.5 cm), M2 (3.6 – 5.0 cm), F1 (5.1 – 6.7 cm), and F2 (7.0 – 9.2 cm). (A) gills Ag accumulation (B) gills 109:107 ratio (C) visceral mass Ag accumulation (D) visceral mass 109:107 ratio (E) hepatopancreas Ag accumulation (F) hepatopancreas 109:107 ratio. Each vertical bar represents an individual oyster. The 109:107 silver ratio of stock particles is indicated on the figure as a straight horizontal line at a value of 2. Significantly different results are indicated by a star (*).
CONCLUSION

The methods for understanding the relative role of ion and particle in AgNP uptake and accumulation are not fully developed. Current research does not fully describe the mechanisms of bioaccumulation with reports of accumulation being largely explained by the Ag ion which dissolves from the AgNP, while others suggest that in addition to mainly ionic there is residual accumulation of the NP. To resolve this uncertainty further development and research using relevant biological models is needed. The overall aim of this study was to understand the relative roles of ion and NP uptake and accumulation using bi-metallic Au-AgNPs (Au@Ag and 107Ag@Au@109Ag) to quantify ionic and NP Ag contributions to these two mechanisms.

Results show that dissolution is the primary mechanism controlling bioavailability at lower concentrations such as 1 µg L⁻¹ AgNPs. At higher concentrations of 50 µg L⁻¹ AgNPs aggregation is dominant and, although dissolution is still occurring it is less important when explaining the bioavailability of Ag to estuarine organisms. At exposure concentrations of 1-50 µg L⁻¹ AgNPs, free Ag ions are not present in solution and are most likely present as silver-chloride complexes. Due to complex transformations of AgNPs in seawater, dissolved Ag may reabsorb onto the original AgNP which causes ripening, or Ag in solution may be forming new smaller AgNPs through nucleation.
This study showed that Ag is taken up or strongly bound to *P. minimum* cells and *C. virginica* tissues after exposure to AgNPs. Due to these complex transformations in seawater media algae are exposed to original Au@Ag with a reduced Ag outer layer, ripened AgNPs, or silver-chloro complexes during the exposure. We have shown that Ag is taken up and accumulated within *P. minimum* cells with differences between the particles and ions observed. After exposure to Au@Ag cell densities decrease in a dose-dependent manner and we speculate that algae are able to recover from the initial exposure to Au@Ag after 24 hours and continue to increase in cell density. Oyster accumulation results showed that at lower concentrations of AgNPs (1 µg L⁻¹) there was enhanced uptake of 107Ag, and a higher percent uptake of total Ag added, while at higher concentrations of AgNPs (50 µg L⁻¹) there is more 109Ag uptake than 107Ag and a lower percent uptake of total Ag added. We interpret this data as dissolution and aggregation of 109Ag and uptake of secondary 109AgNPs in 50 µg L⁻¹ exposures. In the 1 µg L⁻¹ we suggest enhanced uptake of 107Ag as dissolution of the 109Ag outermost shell and preferential uptake of the primary core-shell-shell with reduced 109Ag or free Ag ions which form silver chloride complexes and become unavailable to oysters.

From these data and at current environmental levels of AgNPs, no immediate risk of AgNPs to *P. minimum* or *C. virginica* is indicated. However, this research confirms that AgNPs are accumulated in both algae and oysters and are capable of causing a toxic effect on dinoflagellate population growth resulting in a decrease in cell densities. Although the Ag mass accumulated poses no immediate risk and the change in abundance of a single species of dinoflagellates is a small-scale impact, greater accumulation has the potential to
change population dynamics and community composition of primary producers and therefore estuarine ecosystem trophic dynamics and transfer efficiencies. By removing the primary producer and primary consumer 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. Furthermore, our results postulate that once Ag is accumulated within algae there is a potential for consumption by *C. virginica* and may have the ability to pass Ag via trophic transfer. Previous and current research shows that Ag can cause adverse effects in humans. Oysters are a recreational and commercial fishery organism and are frequently consumed by humans, therefore, accumulation of Ag in oysters and their food source can have consequential effects on human health.

Our research suggests a complex mix of transformations occurring and influencing AgNP behavior, uptake, and accumulation. Further testing and mechanistic understanding of AgNP interactions with marine species should be continued in tandem with AgNO₃ exposures for comparison. In order to fully understand the impact nanotechnology has on our environment as well as the associated hazards and risks, the relative role of ion and NP in uptake and accumulation need to be determined.
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