AN INVESTIGATION INTO THE INTERACTIONS OF GOLD NANOPARTICLES WITH AQUATIC VASCULAR MACROPHYTES

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AN INVESTIGATION INTO THE INTERACTIONS OF GOLD NANOPARTICLES WITH AQUATIC VASCULAR MACROPHYTES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

By
James Bradley Glenn
December 2013

Accepted By
Dr. Stephen J. Klaine, Committee Chair
Dr. Sarah A. White
Dr. Cindy M. Lee
Dr. JoAn S. Hudson
Dr. Douglas G. Bielenberg
ABSTRACT

Advancements in technology are the driving force behind the science of nanomaterials. Nanomaterials (NMs) have promised lighter, stronger, smaller and more efficient products in areas such as electronics, medicines and even environmental sectors. Because of the increased production and use of nanomaterial containing compounds, unintentional release into the environment will occur. The consequences of nanomaterial entrance into the aquatic environment through use, disposal, spillage and runoff are unknown. This investigation started at the base of the food chain and characterized NMs interactions with aquatic plants. Citrate capped gold nanoparticles were used as a model nanoparticle to track fate and gain insight on factors that influence gold nanoparticle (AuNP) bioavailability and absorption. Four species of aquatic macrophytes were investigated. *Azolla caroliniana*, *Myriophyllum simulans*, *Egeria densa* and *Myriophyllum aquaticum* were selected due to growth habitat, leaf morphology and root structure. Because aquatic plants absorb the majority of their nutrients from the water column, it is logical to hypothesize that they may absorb nanomaterials in suspension, potentially facilitating trophic transfer.

*Azolla caroliniana*, *E. densa* and *M. simulans* were exposed to 4 nm and 18 nm AuNPs at a nominal concentration of 250 µg Au/L for 24 h. Macrophytes were harvested at six different time points (1, 3, 6, 12, 18 and 24 h), dried and then analyzed for gold concentration via inductively coupled plasma mass spectrometry. Concentrations were normalized to whole plant dry tissue mass.
Electron microscopy revealed that 4 nm and 18 nm AuNPs adsorbed to the roots of each species. Further, it was observed that 4 nm and 18 nm AuNPs were absorbed by *A. caroliniana*, however, only 4 nm AuNPs were absorbed by *M. simulans*; *E. densa* did not absorb AuNPs of either size.

To further identify factors that influence the bioavailability of gold nanoparticles to aquatic macrophytes, *A. caroliniana, E. densa* and *M. simulans* were exposed to 4, 18, and 30 nm gold nanoparticles. Results indicated that particle uptake was influenced by plant species, presence or absence of plant roots, particle size and dissolved organic carbon and their interactions; this suggests that nanoparticle bioavailability is influenced by multiple parameters. Absorption of AuNP was species specific and dependent upon the presence of roots and nanoparticle size. In the presence of dissolved organic carbon, the suspension of 4 and 18 nm gold nanoparticles formed a nanoparticle/organic matter association that resulted in 1) minimized particle aggregation and 2) a decrease of nanoparticle absorption by the aquatic plants. The same effect was not observed with the 30 nm nanoparticle treatment. Multiple factors, both biotic and abiotic, must be taken into account when predicting bioavailability of nanomaterials to aquatic plants.

Electron microscopy was used to further investigate the influence of AuNP size on species dependent uptake. Root micrographs of *E. densa, M. simulans* and *A. caroliniana* indicated that absorption of gold nanoparticles from suspension correlated with root microfibril density. The microfibril network
defines the porous structure of the root cell wall. The cell wall porosity of A. caroliniana was 4.5 – 5.0 nm, as measured by solute exclusion. The effect of evapotranspiration on AuNP uptake was measured over 16 days for emergent species A. caroliniana and M. aquaticum using 4 nm AuNPs. Disrupting boundary layers and varying humidity around the emergent plant achieved changes in the evapotranspiration rate. Placing plant units under a fan, in a sealed system or open (control) conditions correlated with increased, decreased or control measured evapotranspiration rates. Plant root and shoot samples were separated and analyzed for gold content. Increased evapotranspiration rates correlated with an increase in AuNP root loading (mg Au/kg dry tissue/24 h). An average of 18.83 ± 3.3 mg Au/kg dry tissue/24 h was observed in the fan treatment for A. caroliniana and in M. aquaticum, an increase of 1.07 ± 0.18 mg Au/kg dry tissue/24 h was observed in the fan treatment when compared to the control treatments. While an increase in evapotranspiration rate increased root loading, shoot concentrations of Au did not correlate with evapotranspiration rate. This suggested that shoot translocation was a diffusive process, not dependent on water movement into the root tissue. Shoot tissue concentrations in A. caroliniana increased from 14.58 ± 3.29 mg Au/kg dry tissue to 140.15 ± 6.73 mg Au/kg dry tissue over the course of 16 days. This corresponded with an average AuNP translocation rate of 6.79 ± 3.26 mg Au/kg dry tissue/24 h in A. caroliniana. At the same point, day 16, root tissue concentration for A. caroliniana is the highest observed, 1265.34 ± 139.3 mg Au/kg dry tissue.
Overall, my dissertation results indicate that absorption of gold nanoparticles by aquatic macrophytes from suspension is a complex interaction of plant species, nanomaterial size, levels of dissolved carbon in water, root structure, and evapotranspiration rate. Because no visual toxicity or deleterious effects were observed with the exposure of AuNPs to aquatic plants, there is potential for the use of AuNPs in tracking and fate studies within these macrophytes. Further, the results herein identify parameters that should be included in fate models of nanomaterials in aquatic systems, as well as environmental fate models of nanomaterials for future regulations and decision-making.
DEDICATION

I dedicate this work to my parents, William Kenneth Glenn Jr. and Donna Brackett Glenn. Without their constant support, love and encouragement, all of this work would not have been possible.
ACKNOWLEDGEMENTS

I would like to acknowledge my Ph.D. advisor, Dr. Stephen Klaine for providing me with the opportunity to work in his laboratory and pursue a degree under his mentorship. By his support, I was able to obtain much knowledge and understanding in the field of environmental toxicology and gain invaluable experiences that I could not have achieved without his guidance. I would also like to acknowledge Dr. JoAn Hudson for her guidance and expertise in training and refining my knowledge, skills and abilities with electron microscopy. Also, I must thank Dr. Sarah White for the countless office visits, guidance and questions I asked throughout my graduate tenure. I could not have completed this program without the support of Dr. Cindy Lee and Dr. Douglas Bielenberg, both of whom helped tremendously throughout the entire graduate school experience by serving on my committee as well as being an integral part of my coursework and dissertation research. I must also acknowledge Norman Ellis. Without his analytical knowledge and expertise, I would not have had my data returned in such a timely manner. Further, Donald Mulwee was a vital connection in my pursuit of electron microscopy. Without his expertise, selfless help and patience in teaching, the EM work would have been nearly impossible.

My research would not have been possible without the rest of the faculty and staff of both the Environmental Toxicology program as well as the department of Biological Sciences at Clemson University. A special thank you for all of their assistance, teaching of coursework and help with administrative
A big thank you to all of my colleagues and Klaine lab mates who made this adventure one to remember. Special acknowledgements go to my past lab mates, Dr. Joseph Bisesi and Dr. Aaron Edgington for all of their support and advice. Also, Austin Wray for helping with nanomaterials synthesis, along with Blair Paulik who not only helped several semesters with nanomaterials synthesis, but also through giving many long hours of her time to help with the harvesting of macrophytes and running bioassays.

Without my families support this experience would have not been possible. My mother, Donna Glenn and father Ken Glenn have been critical throughout this entire experience. Their loving support made this experience so much easier. Also, my brother Bill and sister-in-law Mary Ann with their family, Annabelle and Emmett helped to support me throughout this entire experience. And most importantly, I must thank my incredible wife Brooke P. Glenn, for her constant love, encouragement, support and aid. S.D.G
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CHAPTER ONE: LITERATURE REVIEW

Introduction to nanomaterials

In 1959, Richard Feynman addressed the American Physical Society with an invitation to enter a new field of physics in his address entitled “There’s Plenty of Room at the Bottom” [1]. Twenty years later with the advancements of high-resolution electron microscopy, visualization of nano-sized structures became a reality. In 1986 Eric Drexler published the book “Engines of Creation: The Coming Era of Nanotechnology” coining the term nanotechnology. The foundation of Drexler’s book is that atoms are the principle building blocks of all things; manipulation of these atoms can change soil, air and water into ripe strawberries [2]. Although Drexler’s ideas on nanotechnology are not feasible, nanotechnology and the manipulation of matter at the atomic and molecular scale does exist. Fifty-four years since Feynman’s address, nanotechnology is estimated to be worth over 1 trillion dollars by the year 2015 [3]. The British Standards Institution and the American Society for Testing and Materials define nanomaterial (NM) as having at least one dimension that is between 1 and 100 nanometers (nm), while a nanoparticle (NP) is defined as materials with at least two dimensions between 1 and 100 nm [4,5].

By engineering matter at the atomic level, nanomaterials possess unique properties due to the surface area to volume ratio. Properties that emerge when a material is reduced to this level include unique mechanical, catalytic, optical and electrical properties [6,7,8]. These properties can then be fine-tuned with
slight changes in synthesis or the addition of surface chemistries, offering a myriad of applications. Engineered nanomaterials are being used to enhance everything from personal care products to car tires [9]. Engineered nanomaterials are not the only nanostructures that can be found in nature; natural nanoparticles have been present since the creation of the earth. Iron (hematite) nanoparticles and silicate nanoparticles have been discovered in the sediments at the Cretaceous-Tertiary (K-T) boundary layer in Gubbio, Italy [10]. Also, carbon based nanomaterials such as carbon nanotubes and fullerenes as well as silicon dioxide nanoparticles have been discovered in ice cores dating over 10,000 years old [11]. Natural nanomaterials can form through a variety of geochemical processes including volcanic heat and eruption. For example, bismuth oxide nanoparticles and crystalline silica are present in volcanic ash and dust [12,13]. Even freshwater contains colloids that fit into the definition of nanomaterials, including mineral particles and various organic macromolecules [14]. However, a large difference between naturally occurring and engineered nanomaterials is that engineered nanomaterials (NMs) are created with specific shapes, sizes, surface chemistries and core compositions that may not be found in naturally derived nanomaterials. Due to the mass production of engineered NMs and NM-containing compounds, there is concern about the eventual entry of NM into the environment. Colvin [9] was one of the first scientists to discuss the potential ecological impact of these NMs. Nanomaterials are of the appropriate size to interact with biochemical molecules such as DNA [15]. Further, they are
often designed for enhanced bioavailability, stability, and biological activity increasing their potential to interact with biological systems in unintended ways. From a historical prospective, there are few, if any examples of anthropogenic chemicals and compounds that have not been released into the environment. This release may be intentional, through improper waste management, or through the use and aging of materials. Classic examples of anthropogenic chemicals found to have adverse effects long after use and release include industrial chemicals such as polychlorinated biphenyls (PCBs) and insecticides such as dichlorodiphenyltrichloroethane (DDT). Currently, uses of NMs in consumer products are regulated under the toxic substances control act and require little data submission in order to begin use in a substance that is not a food, drug or pesticide. However, given the focus on NM many US government agencies are beginning to require more data about NMs currently in use [16]. Because NMs may interact with the environment differently than natural particulates, understanding NP fate and bioavailability is a vital first step in proactive research as a foundation for characterization of exposure in risk assessment and possibly implementing regulations for protection.

**Synthesis of Nanomaterials**

Nanomaterials can be synthesized in two basic ways, often referred to as a top down (mechanical grinding), or bottom up (chemical synthesis) approach. Top down approaches are based on patterning bulk materials and reducing large-scale dimensions to the nanoscale. The top down methods can refer to
grinding or milling bulk materials into a nanoscale size range, typically to form dry powders. In this approach, controlling size and particle shape is difficult as physical processes such as grinding are often associated with high variability. Leela and Vivekanandan [17] indicate that a bottom up synthesis approach has more homogenous synthesis with less chance of defects, because it occurs in a chamber driven by the reduction of Gibb’s free energy.

The top down approach can also refer to the construction of nanostructures using machining techniques. Mijatovic et al. [18] define three categories of top down nanostructure production including bulk-/film-machining, surface machining, and mold machining. These fabrication methods are also used to make nanochannels, which are defined as a channel with at least one dimension in the 1-100 nm range. Nanochannels have been used to study molecular behavior at the nanoscale. These channels have lead to the creation of a new class of electronics referred to as nanoelectromechanical systems.

The bottom up approach involves the use of controlled chemical reactions to self assemble atoms into nanostructures. This approach typically yields the smallest nanostructures with dimensions between 2-10 nanometers and below as technology advances [18]. Typically, two methods of bottom up approaches have been utilized. The first are gas phase methods, such as the case with building carbon nanotubes (CNTs) where tubes are synthesized under defined conditions to control growth, size and diameter. Carbon nanotubes can be synthesized from graphite using arc discharge, laser ablation, or from deposition
from carbon-containing gas utilizing chemical vapor deposition [6,19]. Gas phase methods can also be used to synthesize metallic particles through the evaporation of a metal by a high-energy source. Then, the metal vapor in controlled conditions including pressure, various inert gases and temperature is allowed to condense, forming the nanoparticles. However, these methods do present drawbacks as the lack of surface functional groups or capping agents results in particle aggregation and oxidation [20].

Wet chemical synthesis is the second method to synthesize nanomaterial using the bottom up approach and is commonly used in the synthesis of metallic nanoparticles. Wet chemical synthesis occurs via a reduction reaction where ionic metallic salts are reduced to zero-valent state in controlled conditions. The reducing agent can be from electrochemical reactions, photo-reduction, or thermal treatment. This reduction causes the zero-valent metal ions to interact forming crystal packages that are the building blocks of the nanoparticle. Through varying temperature, or the addition of surface chemistries of specific concentrations, size and shape of the nanostructure can be fine-tuned. Capping agents in wet synthesis methods are easily added and can be used to cease growth of nanomaterials, stabilize particles, change solubility, shape and many other particle properties. The use of capping agents can also increase monodispersity of the particles being synthesized [20]. A good example of wet synthesis methods is the synthesis of gold nanoparticles. Gold nanoparticles are commonly synthesized by the chemical reduction of tetrachloroaurate (III) in the
presence of citrate as a capping agent. Depending on the ratio of gold to citrate, different size ranges of monodispersed gold nanoparticles can be synthesized [21-23].

**Types of Nanomaterials**

The exponential growth of engineered nanomaterials can be explained by unique mechanical, electrical and optical properties that can be achieved by manipulating matter at the atomic scale. Nanoparticles can remain bare, or functionalization groups or chemistry moieties can by attached to further expand properties and stability of each nanomaterial type, producing literally endless combinations. Below, each major class is discussed with emphasis on properties, special characteristics and current global uses.

**Carbon nanomaterials**

A new allotropic form of carbon was first recognized in 1985 with the discovery of the 60-carbon atom hollow sphere named the Buckminsterfullerene. Also known as C_{60}, fullerene or buckyball, this carbon sphere is composed of highly structured carbon atoms that are positioned in an icosahedron like structure [24,25]. C_{60} is synthesized in the laboratory by evaporating graphite electrodes in ~100 Torr of helium gas and then purifying the resulting soot in a series of organic solvent washes [26]. Generally this soot contains approximately 15% fullerenes [25]. The C_{60} structures have high thermal and electrical conductivity giving this nanomaterial a large number of applications in optics, electronics and biomedicines [27,28].
In the early 1990s, a derivative of C$_{60}$ was produced known as the carbon nanotube (CNT). Carbon nanotubes are seamless cylinders of one or more layers of graphene rolled into a tube shape. Graphene is a single layer sheet of carbon atoms forming a hexagonal lattice, resembling a honeycomb pattern. Carbon nanotubes can exist with a single carbon wall or with multiple carbon walls, both of which can be made in varying lengths and aspect ratios depending on synthesis methods and catalysts present. Multi-walled carbon nanotubes (MWCNTs) are 5 to 40 nm in diameter, and single-walled carbon nanotubes (SWNTs) are 0.8 to 2 nm in diameter. The CNT may have open, or closed ends [28]. The length of the carbon nanotubes is variable and can be from less than 100 nm up to several centimeters. Single-walled CNTs have strength to weight ratios exceeding 460 times that of steel [6]. Individual SWNTs can exceed the thermal conductivity of diamonds, showing thermal conductivity measurements of 3500 W m$^{-1}$ K$^{-1}$ at 25 ºC [7]. Multi-walled CNTs possess special properties such as high tensile strength, which has been measured ten-fold higher than any industrial fiber produced. Also, individual MWCNTs can show metallic properties and carry currents of up to $10^9$ Amp/cm$^2$ [30]. These MWCNTs can act as semiconductors depending on the orientation of the graphene lattice with respect to the tube axis [29]. These unique properties of CNTs have led to increased production for use in industry and electronic applications. In 2003, the first fullerene plant owned by Mitsubishi, opened in Japan to produce fullerene for
applications from bowling balls to fuel cells [9,31,32]. Since 2006, worldwide CNT production has increased ten-fold [29].

Applications of carbon nanotubes are broad, and have a wide market range. Due to their semiconducting ability and tensile strength, MWCNTs have found a niche as an additive to strengthen plastics. These MWCNTs can also be used as electrically conductive fillers. The addition of MWCNTs allows for the creation of conductive plastics that assist auto manufactures in electrostatic painting and dissipation of electrostatic charges, reducing the risk of accidental fuel ignition in fuel lines and fuel filters [29]. Carbon nanotube powders mixed with plastics and resins can increase the stiffness, strength and toughness of load-bearing parts as well. Gojny et al. [33] and Chou et al. [34] observed that the addition of approximately 1 wt.% MWCNTs to epoxy resins enhanced the stiffness and fracture toughness by 6 and 23%, respectively, without the part function compromised. However, CNTs are not limited to use in plastics. Many consumer products, such as sporting goods, often contain CNTs. Carbon nanotubes can be found in bicycle frames, tennis racquets and baseball bats, just to name a few. Carbon nanotubes have been used to strengthen and lighten wind turbine blades and also boat hulls. Due to their semiconducting abilities, CNT powders are mixed in polymers that are used in constructing transistors, lithium ion batteries, biosensors and other electronics such as transparent conducting films to possibly replace or alleviate the need for indium tin oxide used in touch screen devices [29,35-37]. Interestingly, Beigbeder et al. [38]
found that the addition of MWCNTs as low as 0.05 wt.% significantly reduced the adhesion strength of adult barnacles to a silicon elastomer fouling release paint. All of the diverse applications utilizing CNTs have increased production of these nanomaterials exponentially.

Metal Oxides

Metal oxides are chemical compounds that contain a metal atom combined with at least one oxygen molecule. The presence of the oxygen molecule makes these compounds chemically different from their parent metal. A common example of a metal oxide is iron(III)oxide, commonly known as rust. Metal oxides are naturally occurring minerals that can be found in the earth’s crust. Refinement of these minerals into fine powders has been used in many applications, such as pigments in pottery dating back thousands of years [39]. More modern uses include food grade additives, cosmetics, and even electronic devices and sensors in the 21st century. Metal oxides are used in electronics due to their photocatalytic activity and semiconducting nature. Nano-sized zinc oxide and titanium dioxide have become common ingredients in cosmetics, especially sunscreens due to their ability to absorb electromagnetic radiation in the UVA spectrum [40,41,42]. Although bulk zinc oxide and titanium dioxide have been used in sunscreen agents for several decades, the nanoscale versions tend to be more aesthetically pleasing as they no longer scatter visible light, avoiding the traditional heavy white paste appearance. It has been estimated that 30% of the sunscreen market is comprised of nano-based products [http://www.ewg.org/].
Due to the photolytic and semi-conductive properties of nano-sized TiO\textsubscript{2} and ZnO, commercial applications range from use in electronic devices to self-cleaning glass, water purification systems and solar cells components [43].

Upon decreasing the crystal structure into the nano-range, metal oxides offers unique properties that make them valuable for many applications. The properties that emerge when reduced to the nanoscale include increased surface area to volume ratios, reactivity, and unique quantum effects that are valuable in the advancements of electronics and optical technologies. Nano-zinc oxide is of particular interest due to its semiconductor properties and direct band gap energy of 3.36 eV, at room temperature [44]. The band gap energy is used to determine the electrical conductivity of a compound. The higher the band gap energy, the more insulating properties it possesses. The lower the band gap energy indicates the compounds conducting ability. With a band gap energy of 3.36 eV, nano-ZnO is considered a semiconductor, having both insulating and conductive properties. Nano-zinc oxide has a high dielectric constant and excitation binding energy of 60 meV, meaning this metal oxide has the ability to hold an electric charge. The high excitation energy has made nano-ZnO attractive for applications in photoluminescent devices, solar cells and light emitting devices. These properties of nano-ZnO have also aided in the development of semiconductors in the form of thin nanofilms, nanowires or nanoparticles [25,45].

Titania has three distinct crystalline forms, rutile, anatase and brookite. Of these, anatase and rutile are the predominant forms. Titania (TiO\textsubscript{2}) nanoparticles
stability and surface area are dependent on the crystal structure. However, it is very difficult to achieve pure crystalline forms, making phase-specific effects elusive [25]. Similarly to ZnO, titania nanoparticles are also semiconductors. Titania nanoparticles have a band gap energy of 3.2 eV, slightly lower than that of ZnO, making this material more conductive [25]. TiO$_2$ is also excellent at absorbing and blocking UV light. Because of ZnO and TiO$_2$ photoactivity, these nanomaterials have become commercially produced as the active ingredients in sunscreens and UV protective cosmetics [46]. Titania nanoparticles have been utilized to enhance the photodegradation processes that result in the breakdown and mineralization of potentially toxic organic compounds. Because TiO$_2$ is photoactive in the UV spectra, oxidative decomposition, the transfer of electrons in the form of free radicals, can be used to breakdown neighboring compounds. Instead of introducing free particles into the water environment, Nakata et al. [47] have found that structures such as the titania monolith, or thin porous sheets that are impregnated with TiO$_2$ prove to be effective at producing hydroxyl radicals and super oxide ions. From this study, both rutile and anatase predominant forms were studied. Predominant titania crystalline structure was achieved by calcination over a temperature range of 500-1500ºC. Using X-ray diffraction (XRD), crystal faces were measured and it was determined that at 500ºC, the predominant form was anatase, and heated to 700ºC, the crystal faces indicated predominantly rutile. It was observed that the rutile phase performed better at decolorizing methylene blue, an indicator of free radical production [47].
Because of the ability of TiO$_2$ to produce free radicals through photoactivation, there is concern of unintentional toxicity from titania coming in contact with microorganisms and biofilms.

Another common example of metal oxide nanomaterials includes iron oxide (Fe$_2$O$_3$). This compound has become commercially important due to its reactivity and photolytic properties [25]. Iron oxide nanoparticles have received attention due to their applications in biological systems, use as pigments and their magnetic properties. Iron oxide nanomaterials can be synthesized in stable, monodispersed batches that are easily scalable to produce both small and large volumes. Iron oxide nanoparticles have applications ranging from stimulating marine algal growth for reducing carbon dioxide levels in the atmosphere to contrast enhancements in magnetic imaging resonance (MRI) [48]. In marine systems, iron is often a limited essential micronutrient, acting as a fertilizer for the production of algal blooms that would consume CO$_2$ [25]. Synthesis of iron oxide nanoparticles can be achieved through numerous chemical methods. The classic synthesis technique involves the co-precipitation of iron oxide particles using iron salts. In this synthesis procedure, mixtures of ferrous and ferric salts are combined in an aqueous medium in a stoichiometric ration of 2:1 (Fe$^{3+}$/Fe$^{2+}$) in a non-oxidizing environment. With a pH between 8 and 14, complete precipitation of Fe$_3$O$_4$ should be expected [48]. Other methods include hydrothermal and high temperature reactions, sol-gel reactions, aerosol/vapor methods and decomposition of organometallic precursors [48]. Environmental
risk of iron oxide nanomaterials, however, is thought to be low due to natural occurrence of iron oxides in the environment.

**Metal nanoparticles**

Metallic nanoparticles can be synthesized in many shapes, sizes and surface chemistries, each resulting in specific properties that are tailored to fit a myriad of applications [49]. Typically, metallic nanomaterials are synthesized chemically through a bottom up approach, using controlled reduction reactions in the presence of a reducing agent and metal salts. Common reducing agents are sodium borohydride, citrate and heat. Examples of metallic nanomaterials include gold and silver nanoparticles. These NMs have received special attention due to their surface plasmonic resonance properties. Plasmonics describes the collective movement and oscillation of conduction electrons in a metallic nanoparticle [51]. This movement of the free electrons along the surface of gold and silver nanoparticles can be excited when in the presence of light in the visible spectrum. It is this property that allows gold nanoparticles to be vibrant red under incandescent lighting and for silver nanoparticles to appear yellow in coloration. Gold colloids have been used for centuries as a dye or additive in stained glass for the brilliant red coloration. Often, these color characteristics correlate with nanoparticle size. As size increases or decreases, a shift in color occurs due to slightly changing the plasmonic resonance. Through size manipulation, shape and surface chemistry, these visible properties can be adjusted or tuned for specific applications [49,50]. These applications
include optical sensors, targeted cellular delivery and single molecule detection systems [49,50]. Because elemental gold is generally inert in biological systems, the use of gold nanoparticles in biomedical imaging, cellular delivery and use as a contrasting agent have been investigated. A recent study by Jang et al. [52] found that gold nanoparticles coated with dextran could be tuned to deliver the anticancer drug doxorubicin to the nucleus of cancer cells. It was found that doxorubicin conjugated with gold nanoparticles was more efficient in delivering the drug, resulting in higher cancer cell mortality rates and more successful drug delivery and treatment.

Silver nanomaterials by far are the most commonly used and produced metallic nanomaterial [53, www.wilsoncenter.org]. Metallic silver nanoparticles are produced by the reduction of silver salt in the presence of a reducing agent such as citrate, sodium borohydride, ascorbate or even glucose [54]. Products such as baby bottles to blood clotting agents have taken advantage of the antimicrobial properties expressed by these particles [55]. In a review of silver compounds, Ratte [56] introduced the silver ion as one of the most toxic forms of heavy metals second only to mercury. This classifies silver as a hazardous substance [57]. Silver toxicity has been reported in numerous publications, and silver ions are toxic to bacteria and green algae. Das et al. [58] observed the toxicity of silver nanoparticles to bacterial activity in natural surface waters, and found that lowest observed effect concentrations range from 8 to 66 µg Ag/L,
indicating that in natural systems silver present in the microgram per liter could negatively impact the bacterial community.

These antimicrobial properties have given rise to silver nanomaterials use in cosmetics, fabrics and medical instruments to reduce bacteria growth or keep items sterile. More novel items that utilize silver nanoparticles are shoes, socks, cutting boards, dietary supplements and even children’s toys. Because silver is oxidized more easily than gold, silver is likely to undergo catalytic oxidation. The release of silver ions contributes to the bactericide effect [59]. Although the exact mechanism of action is still under scrutiny, there are three accepted mechanisms of toxicity silver nanomaterials elicit. The first is that the silver nanoparticles undergo dissolution, and the free ionic silver disrupts ATP production and DNA replication. The second is that silver nanoparticles create free radicals (ROS), and the third is that silver nanomaterials directly act to damage the cell or plasma membrane [59]. Because of the diversity of products that utilize silver nanomaterials, concern over the introduction of silver into the environment is valid.

**Environmental Implications of Nanomaterials**

Many concerns still exist about ecosystem health with regards to NMs release into the environment [6,60,61]. There is a significant information gap understanding the fate of NMs and their lifecycle. Several recent review papers have addressed these knowledge gaps associated with understanding the
implications of NMs and the challenges in correctly approaching environmental risk assessment and management [6,9,60-63].

Natural nanoparticles exist in the environment. Examples of such compounds include minerals, clays, fine particulate matter and even organic macromolecules. Previous study of these natural colloids has prompted similar fate and toxicity studies with regards to engineered nanomaterials. It is clear however from literature that a paucity of information still exists with regards to engineered NMs fate, toxicity and transformation. Research is headed in the direction to determine the potential toxicity effects of these nanomaterials to both human and ecological receptors. Oberdoster et al. [64] outlined general screening strategies to determine potential toxic response to nanomaterials. These included physiochemical characterization, followed by in vitro testing and in vivo testing. Physiochemical characterization is important in determining size, shape, agglomeration state, charge and other features of the nanomaterials being tested. This characterization is crucial in the development of qualitative structure activity relationships (QSARs). The QSAR method is commonly used to predict toxicity of a chemical compound based on its physiochemical properties. This technique has been used to predict the cytotoxicity of various metal oxide nanomaterial to bacteria as well as predicting nanomaterial ability to act as oxidants or antioxidants [65,66]. Because of the rapid expansion and diversity of nanomaterials used, the application of QSAR models may be able to predict potentially hazardous nanomaterials reducing the number of in vitro and
in vivo testing by identifying nanomaterials that fall into specific categories, such as the ability to create reactive oxygen species. It has been suggested that oxidative stress is one of the principle mechanisms of biochemical injury resulting from engineered nanomaterials [62]. Kahru et al. [67] indicated that there is already a large amount of data on nanomaterials exposure to various biological levels, ranging from in vitro cell culture assays to in vivo studies on rodents. The need and challenge is linking this existing ecological data to synthesize new knowledge and approaches in what is collectively called nanoecotoxicology [67]. As of 2011, there were no specific standardized protocols or certified reference materials for nanomaterials testing [68]. However, this has changed with the introduction of National Institute of Standards and Technology (NIST) nanomaterial standards. Currently, only a few NIST standards are available. These include gold nanomaterials of size 10, 30 and 60 nm, as well as titanium dioxide and single-walled carbon nanotubes (https://www.nist.gov). Handy et al. [69] released a critical review investigating practical methods of nanomaterial testing. Nanomaterials research continues to be refined, and methodologies and frameworks utilized for standard organismal testing have proven to be efficient in the study of nanomaterial. However, issues of nanomaterials exposure include nanomaterial characterization such as particle size and aggregation state, as well as the need for accuracy in determining mass concentrations and dispersion or suspension methodology [69].
**Sources of nanomaterials in the environment**

Entry of nanomaterials into the environment includes both point and nonpoint sources. Examples of point source entry comprise of accidental spillage, as well as direct and intentional release. Intentional release of iron oxide or zero-valent iron nanoparticles have occurred in remediation efforts to sequester carbon dioxide by blooming algae and phytoplankton. Also, the zero-valent iron particles act as a reducing agent in the presence of dissolved oxygen to facilitate the breakdown of nitrates from fertilizers, organic pesticides and can act as chelators to immobilize metal ions such as chromium, arsenic and mercury [70]. Point source release can also occur from effluent discharge sites at wastewater treatment plants (WWTPs). Kiser et al. [71] identified titanium dioxide in the tertiary effluent from an Arizona wastewater treatment plant, and indicated that the TiO$_2$ that was able to pass through was generally less that 0.7 µm in diameter. Nanomaterials that enter with raw sewage into the WWTP that do not pass through have been observed to associate with sewage sludge [71-75]. Nanomaterials associated with WWTPs and sludge will be discussed in greater detail below. Sources of nonpoint nanomaterial release include the degradation and breakdown of products containing nanomaterial, runoff from land and roads (especially during rain and storm events) as well as from agricultural application of sewage sludge that contain nanomaterials. These runoff pathways of nanomaterials are a source of loading to aquatic environment [76].
Currently, it is difficult to quantify the environmental concentrations of engineered nanomaterials. Not only is this due to numerous entry points, but is also confounded with lack of product labeling indicating the use of nanomaterials, and the need for technological advancements in areas such as analytical chemistry. Maurier-Jones et al. [59] reported some of the difficulty and the knowledge gaps that exist in the prediction of environmental concentrations of nanomaterial. The authors point out several difficulties that could be solved with major advances in analytical chemistry such as nanomaterial transformation, dissolution, surface charge and surface chemistries, aggregation tendencies, interaction with macromolecules and sedimentation. Besides measuring environmental concentrations, the transformation of nanomaterial can also be important in determining fate, toxicity and bioavailability. It is for these reasons that adequate particle characterization is needed for accurate and relevant nanomaterials research.

Although it is difficult to measure concentrations of nanomaterials in the environment, predicted environmental concentrations have been modeled in three major environmental pathways. These pathways include surface water, effluent from wastewater treatment plants and sludge. In the review by Maurier-Jones et al. [59] current literature was mined for modeled environmental concentrations for four major types of nanomaterials. The lowest and highest predicted environmental concentrations were given for the most widely used nanomaterials in consumer products as listed in the Woodrow Wilson Project of
Emerging Nanotechnologies (http://www.nanotechproject.org). Of these, predicted surface water concentrations are as follows for silver, TiO$_2$, ZnO and carbon based nanomaterials: 0.088-10,000 ng/L, 21-10,000 ng/L, 1- 10,000 ng/L and 0.001-0.8 ng/L, respectively. Interestingly, it was discovered that sludge concentrations were found to be the highest with concentrations for silver, TiO$_2$, ZnO and carbon estimated at 1.29-39 mg/kg, 100-2000 mg/kg, 13.6-64.7 mg/kg and 0.0093-0.147 mg/kg, respectively. From these data, the authors concluded that the large concentration range for individual nanomaterial types is due to the complexity in measuring accurate concentrations due to complex environmental matrixes, lack of speciation data and the transformation state of the nanomaterials. Further nanomaterials characterization will ultimately lead to determination of more realistic environmental concentrations as well as better predictions of potential toxicological responses.

Effluent from wastewater treatment plants has been a recognized source of nanomaterials entrance into the aquatic environment. Not only can nanomaterials pass through the WWTP, but NMs can also partition to sludge. Limbach et al. [74] and Kiser et al. [71] have shown that metallic oxide (CeO$_2$, SiO$_2$, ZnO, and TiO$_2$) NMs entering wastewater will partition to sewage sludge and biofilms in wastewater treatment plants. Limbach et al. [74] investigated the removal of oxide nanomaterials by studying a model wastewater treatment plant. They observed that a high stabilization effect occurred, which kept the nanoparticles from aggregating. The authors concluded that this effect was due
to the particle adsorption onto bacteria and other constituents of the sludge. This adsorption reduced clearance of the nanomaterials. For example, only 6 wt.% of cerium oxide was found in the effluent. In 2009, Kiser et al. [71] reported for the first time the occurrence and characterization of TiO$_2$ nanomaterials at full scale WWTPs. They identified that raw sewage contained 100-3000 µg Ti/L. The authors observed that Ti particles larger than 0.7 µm were effectively removed and the majority of Ti in the biosolids consisted of Ti particles >0.7 µm. Effluent concentrations ranged from <5-15 µg Ti/L and Ti found in the effluent were measured in the <0.7 µm size range. Images from this study indicated that single nanoparticles in the 50-100+ nm size range existed in effluent and biosolids, indicating that nano Ti was present in WWTP effluent and biosolids [71].

More recently, an article by Hendren et al. [75] modeled the fate of silver nanoparticles using Monte Carlo simulation. Monte Carlo simulations are often applied when the inputs are uncertain, and it is difficult to impossible to obtain a closed form expression. The results of the study by Hendren et al. [75] indicated that silver nanoparticle surface chemistry influenced their removal. In this study, bare silver nanoparticles, polyvinylpyrrolidone-coated nanoparticles (PVP nano-Ag), an aqueous suspension of citrate-coated nanoparticles and an aqueous suspension of gum arabic-coated nanoparticles were studied. They estimated that gum arabic-coated nanoparticles would be most likely to pass through the WWTP, and that bare silver nanoparticles would be most likely to associate with
the sludge. Results of previous studies indicate that 90-96% of all nanomaterials that enter into a WWTP partition to the sludge [71,74,77]. Here, Hendren et al. [75] determined that 95% of the estimated concentrations for the effluent of WWTPs for gum arabic-coated silver nanoparticles (the coating most likely to pass through the effluent) will fall below 0.24 µg/L and that 95% of the estimated sludge concentrations for bare silver nanoparticles (the most likely to associate with sludge) will be below 13 µg/kg. Hendren et al. [75] concluded that the nanoparticle coating directly influenced the environmental fate, and highlighted the need for greater analytical advances to determine more realistic predictions.

**Land application of Sewage Sludge**

Exposure to NMs can occur through three basic media: air, soil and water. Colvin [9] indicated that waterborne exposures are of most interest with nanomaterials, as it is known that many compounds often elicit the most significant environmental effects in water.

In 2009 the U.S. EPA released the Targeted National Sewage Sludge Survey (TNSSS) statistical analysis reports that investigated the contaminants present in sewage sludge ranging from pharmaceuticals to metals [78-81]. These reports evaluated the concentration of impurities in sewage sludge from a subset of the more than 3,500 large-scale WWTPs in the United States. The subset consisted of 74 full scale public municipal WWTPs that met a one million gallon per day treatment, and had secondary or better treatment processes (physical, biological and chemical). Virtually every sludge sample contained 27
different metals and of these metals, silver was present in each of the plants sampled.

Sludge, which is a byproduct of wastewater treatment, is a mixture of organic matter and solids. For the WWTPs to operate efficiently, excess sludge is removed and is often land applied as a soil amendment [82]. Land application of sewage sludge is an alternative to either landfill disposal or incineration. Land application has potential benefits, such as introducing macronutrients, use as a soil conditioner due to slightly acidic pH, recycling nitrogen and phosphorous, as well as a source of organic carbon [82]. Addition of sewage sludge to agriculture land has increased the growth of crop plants [82]. However, if toxic compounds, non-essential trace metals, or other organic pollutants exist, land application of sewage sludge can be a source of contamination introduced unintentionally to the environment. Among the risks of heavy metal contamination, as well as a myriad of personal care products, prescription medications and potentially toxic organic compounds, nanomaterials are also likely to be found in WWTP sludge.

If NMs are present in the sludge, release to the environment could result. Further analyzing the silver concentrations discovered by the TNSSS reports, Kim et al. [83] reported silver nanoparticles ranging from 5 to 20 nm present in the sewage sludge from municipal wastewater plants on the TNSSS sampling report. Silver nanomaterials are by far the most abundantly used NMs in consumer and manufacturer products. These products utilize the broad-spectrum anti-microbial properties of silver ions that are released from silver
nanoparticles when they are introduced into an oxidizing environment [53,84,http://www.nanotechproject.org].

**Econanotoxicity**

The term econanotoxicology is a combination of nanotoxicology and ecotoxicology. It describes the branch of science that is concerned with the effects that nanomaterials may have on the environment. Due to the large-scale use and production of engineered nanomaterials and their likely presence in the environment, many studies have investigated the toxicity that nanomaterials may elicit. Because of the lack of standardized testing, there is much debate about the econanotoxicity of nanomaterials within the literature. Batley et al. [85] indicated that the overall findings of environmental risk of nanomaterials should give regulatory agencies reassurance. The authors reach this conclusion due to the non-relevant tests that use extremely high concentrations to elicit a response. Granted, it is important for effects testing to occur at environmentally relevant exposure concentrations; however, it is also important to develop acute datasets that may require high concentrations not only to develop no observable effect concentrations, but also for the formation of future nanomaterial regulations. Regardless, within the current literature many uncertainties exist between nanomaterial fate and ecological effects. In the review by Maurier-Jones et al. [59], gaps in econanotoxicity studies indicated the need for econanotoxicity data in the area of analytical chemistry, including detection, speciation and transformation. Advancements in analytical chemistry will begin answering
questions about nanomaterials behavior in different mediums, and will help nanotoxicological research progress in a direction of mechanisms and modes of action.

Autotrophic species are a particularly relevant starting point for answering questions that exist with regard to econanotoxicology. Because plants make up the base of the food chain and interact closely with air, water and soil, they are likely to come in contact with nanomaterials. This not only allows plants to be used in nanomaterials uptake and toxicity studies, but also in potential nanomaterial trophic transfer studies. While some studies have been performed with terrestrial plants, no studies beside our own have examined aquatic plant-NM interactions.

**Potential mechanisms of nanomaterial uptake in plants**

Nel et al. [62] indicated that the generation of reactive oxygen species is the main mechanism by which nanoparticles illicit cellular damage and toxicity. Reactive oxygen species (ROS) are generated due to the high surface area to volume ratio of nanoparticles. As particles become smaller the percentage of surface molecules grows exponentially. This inverse relationship increases the reactivity of these particles, with the most reactive particles being the smallest.

Engineered nanomaterials do not have to be internalized within the plant cell to elicit a response. Nanomaterials that aggregated onto roots can affect hydraulic conductivity and reduce water uptake [86]. At the present time, cellular uptake and penetration is the accepted mode of action on how nanomaterials
interact with plants, although, the mechanism of uptake has not been fully defined [87-91]. Nanomaterials likely follow a similar absorption path as moisture and nutrients by the plant roots [89]. Nanomaterials may enter the roots and be transported within plants via three possible routes. The first is entrance into the cell wall pores. These pores are defined as the overlapping microfibrils network that is formed by the building of the cell wall. These openings or gaps between the microfibrils vary in size and thickness dependent on plant species. The pore structures have been measured in literature and are commonly accepted to be in the 5-10 nm range, and have even been reported up to 50 nm in diameter [90,92-96]. The second route of transport is through apoplastic transfer. The apoplast is defined as the cell wall and the intercellular space between the cell wall and the plasma membrane. This pathway would allow nanomaterials to enter into the plant, but not cross the plasma membrane. Lin et al. [97] confirmed the presence of ZnO nanomaterial on the root surface of rye grass using electron microscopy. The authors then used transmission electron microscopy to determine that ZnO nanoparticles were sorbed with the root apoplast and the cytoplasm. For these particles to have entered the cytoplasm, they must have crossed the plasma membrane and entered into the symplast region of the plant. The symplastic pathway, which is defined as the intracellular region of the plant, is the third entrance and transport pathway. If nanomaterials entered into the symplast, they would be inside the plasma membrane and able to move intracellularly through channels called plasmodesmata that connect adjacent cells. Plasmodesmata
are typically around 40 nm in diameter. Although current research is beginning to answer some of the questions regarding nanomaterial interaction and transport within higher vascular plants, it is still important to better define pathways of uptake, translocation and bioconcentration. As plants are a main route of nanomaterials exposure to higher species, including humans [96,98].

**Toxicity to algae and higher plants**

Little is known about the potential impacts nanomaterials could pose to higher plant species [96,98]. From current literature, it is clear that significant gaps understanding fate, toxicity, transformation and possible risk still exist with regards to nanomaterials and plants [15,61,99]. Interaction of nanomaterials with photosynthetic organisms and in particular, higher plants, have grown over the last four to five years. These interactions include transport and uptake studies as well as toxicity responses.

Anthropogenic sources of engineered nanomaterials pose a risk due abundant reactive sites, high surface area and mobility [99,100]. In 2008, Handy et al. [61] indicated that much of the ecotoxicological data gathered for nanomaterials was limited to standard freshwater organisms. This has led to a lack of data on bacteria, terrestrial species, marine species and higher plants. Although data sets have grown since, it is still recognized that nanotoxicity studies with higher plants are few [68]. In 2008, Navarro et al. [101] investigated the potential risks engineered nanomaterials pose to algae, plants and fungi. Because plants and algae interact very closely with their surrounding
environment, these primary producers are susceptible to interaction and possible consequences of nanomaterial exposure [101,102].

Several endpoints exist to measure the phytotoxicity response of plants after exposure to potential toxicants. These phytotoxicity responses include genotoxicity and cytotoxicity endpoints, as well as seed germination and seedling growth. Many studies have evaluated both growth and cyto/genotoxicity endpoints in higher plants. Ma et al. [103] noted that exposure to 7 nm CeO$_2$ nanoparticles at 2000 mg/L had no effect on seed germination and root elongation in *Cucumis sativus*, however, *Lactuca sativa* exposed to the same particles was observed to have inhibition of root elongation. This study represents one of many examples of species dependent responses after exposure to nanomaterials. Investigating the genetic response of plants exposed to nanomaterials, Ghosh et al. [104] exposed *Allium cepa* and *Nicotiana tabacum* to 100 nm TiO$_2$ at a concentration of 2 -10 mM. The authors observed that *A. cepa* showed signs of DNA damage, as indicated by a comet assay, most notably at the 4 mM concentration. Although the mitotic index indicated no significant effect, chromosomal aberration of anaphase-telephase bridges were observed and micronuclei were detected. In *N. tabacum*, DNA damage was the highest at 2 mM concentration.

Concerning toxicity, both direct and indirect effects of nanomaterials have been observed. Hund-Rinke and Simon [105] observed size dependent toxicity of TiO$_2$ exposed to the green algae *Desmodesmus subspicatus*. The authors
observed that the smallest particles elicited the highest toxicity response, which implies that size and surface area of the NM plays an important role [105]. Indirect responses have also been observed. In 2007, Franklin et al. [106] investigated the toxicity effects of nanoparticulate ZnO, bulk ZnO and ZnCl$_2$ to the freshwater algae Pseudokirchneriella subcapitata. The toxicity response could be attributed to the dissolved zinc fraction and not the nanoparticle itself. Lee and An [107] observed toxicity to Pseudokirchneriella subcapitata exposed to ZnO and TiO$_2$ nanoparticles. In their study, the effects of UV radiation were investigated as these NMs are known to be photoreactive; inhibitory effects on growth were observed, but pre-irradiation did not impact growth rate. Free metal ions could predict the toxicity of the NMs, indicating the indirect effects of NMs, such as the dissolution and release of toxic metal ions [101,107]. Lee et al. [108] indicated that Arabidopsis thaliana showed developmental toxicity when exposed to metal oxide nanomaterials, specifically ZnO. This study observed a drastic decline in seed germination, as 400 mg/L ZnO (14.6 mg/L soluble Zn) resulted in 6% germination. It was also observed that Zn hindered root elongation by 75% compared with control treatments [108].

Studies on the interaction of NMs with higher plants are focused on crop plants grown for human consumption [59,109]. Investigating the effects of nanomaterials with crop plants, Rico et al. [98] highlighted interactions and scenarios of potential food chain implications. They focused on the uptake, bioaccumulation, biotransformation and risks (toxicity to plant) associated with
exposure [98]. From Rico et al. [98], it is clear that a paucity of information still exists about NMs interactions and factors that influence NMs bioavailability to the plant. Rico et al. [98] defined factors such as nanomaterial size and shape and suggest they impact the route of uptake into plants, and ultimately concluded the exact mechanisms of uptake are unknown. Factors that influence translocation within the plant are also unknown. Although research is moving in the direction to determine how NMs interact with food plants, currently little is understood.

Studies investigating uptake and bioaccumulation of NMs in higher plants have used traditional toxicity endpoints such as seed germination and root elongation [68]. These endpoints have been measured in many crop and edible plant species to determine potential effects that NMs may have on growth related endpoints. Not all plants exposed to nanomaterials are adversely affected. Interestingly several positive effects have been documented, such as increased root/shoot ratios in soybean, tomato, corn, alfalfa, and spinach [110-113]. These positive effects have been observed in treatments with silica and titania nanomaterials, as well as carbon nanotubes. Because negative, neutral and positive effects of nanomaterials have been observed, no defining mechanism of toxicity has been identified. It is unknown if intracellular uptake is a requirement for causing a toxicity response [108]. It is possible that physical interactions between nanomaterial and roots could alter normal function of the root cells. These functions include uptake of nutrients, water and minerals. Each of which would change the hydraulic conductivity and nutrient uptake ability of the root
systems [86]. It has also been suggested that visual toxicity endpoints such as root and shoot length may not be a good indicator of potential nanomaterial related toxicity responses. Studies at the proteomic, genomic and metabolic levels might reveal more information according to Rico et al. [98].

Although studies with terrestrial plants have grown in number, studies using aquatic plants are still sparse. In 2008, Navarro et al. [101] stated that studies on aquatic plants do not exist. However, in the last four years, some studies with aquatic plants and many more terrestrial plant studies have been published [114].

**Current literature on aquatic macrophytes**

Although studies have focused on terrestrial plants, important information can be applied to aquatic plants. Many similarities exist between aquatic and terrestrial plants. Similarities include conservation of basic plant physiology such as vascular systems, photosynthetic structures and plant organ systems (roots, leaves). A difference, however, is that fully submerged aquatic macrophytes can absorb nutrients from both roots and leaves in the water column [115]. This difference in exposure and nutrient acquisition warrants further investigation into parameters that influence uptake of NM in aquatic vascular plants. Root structure and function also warrants further investigation with regard to nanomaterial uptake. In aquatic species, the entire plant including roots can be free floating in the water column. In fully submerged growth, the major drivers of water and nutrients uptake are concentration gradients and transporters;
evapotranspiration is no longer a major force. Because water loss is no longer a concern, cuticular structures can be minimized in submerged aquatic plants. These factors should be considered when investigating the interactions of NMs with aquatic plants.

Physiological processes are conserved between aquatic and terrestrial plants, such as basic photosynthesis, cell wall morphology and root function [91,96,114,116-118]. However, structural differences exist among aquatic plant species. For example, the leaves of *E. densa* only contain a single longitudinal vascular bundle and the leaf blade consists of only two cell layers, which means all of the leaf cells are in direct contact with the external environment [117]. *Egeria densa*’s leaves occupy about 5% of the cross sectional leaf surface area [116-117]. Also, root air spaces occur at the junctions of cortical cells which make up about 20-28% of the root’s cross sectional area [116].

At present, few studies have evaluated the effects of NM exposure to aquatic plant species. The most common aquatic plants used in toxicity testing are *Lemna spp.* In 2010, Kim et al. [83] investigated the growth inhibition in *Lemna paucicostata* following exposure to TiO$_2$ (2 to 3 nm) and Ag nanoparticles (50 nm). From this study, effective concentrations for growth inhibition were calculated for each particle type. They reported that AgNPs (EC$_{50}$ 13.8 ppm) were much more effective at decreasing growth than TiO$_2$ (EC$_{50}$ 538.5 ppm). Interestingly, LOECs for both particle types were calculated and AgNPs caused growth inhibition as low as 1 ppm, followed by titania at 125 ppm. A possible
explanation for the observed toxicity could be due to the build up of ROS produced from dissolution of Ag ions, or by photoactivation of TiO$_2$. Oukarroum et al. [119] observed a decline in *Lemna gibba* growth when exposed to 50 nm AgNPs at concentrations up to 10 mg/L. It was noted that an increase of ROS was detected after exposure to 1 and 10 mg/L of AgNP. Reduction in plant viability was strongly correlated with the increased ROS, most likely from the intercellular release of Ag$^+$ from AgNPs. Other biochemical endpoints have been observed in the literature. Mishra et al. [120] indicated that a decline in chlorophyll levels as well as increased catalase activity was observed in *H. verticillata* in response to exposure to semiconducting nanomaterials such as ZnO and CdS.

Aquatic plants may also contribute to the stabilization of nanomaterials in the aquatic environment. It has been documented that natural organic matter (NOM) influences the behavior and fate of nanomaterials in suspension [121-124]. It has been observed that the association of nanomaterials with NOM has protective effects against aggregation, salinity and changes in pH, which is due to the ability of NOM to interact with the surface of the NMs and allow for both steric and charge repulsion to minimize aggregation response. Unrine et al. [125] investigated the fate and toxicity of AgNPs in mesocosms and observed that *Egeria densa* might have released exudates in response to Ag$^+$. These plant-derived exudates may have been released as a result of Ag$^+$ toxicity or in an attempt to reduce toxicity. In a related paper, Bone et al. [126] focused on the
media contained within these mesocosm setups. It was noted that the mesocosms that contained *Egeria densa* exposed to AgNPs had approximately four times more dissolved organic carbon (DOC) than the control mesocosm, which suggested that *E. densa* contributed to the DOC levels in response to AgNPs exposure. Both Unrine et al. [125] and Bone et al. [126] discussed the complexity of nanomaterials interactions with abiotic and biotic factors. These studies highlighted the complexity of NM exposure scenarios with aquatic macrophytes.

Most toxicity studies using aquatic macrophytes have focused on growth, free radical production and enzymatic endpoints for measuring toxicity response. Studies focused on toxicity responses are needed, but perhaps as critical is the need to define the initial interactions NMs have with primary producers, including size and species dependent uptake, the role of roots and shoots in NM transport and factors that reduce/enhance bioavailability. Developing these resources will increase the accuracy of plant exposure estimates and facilitate screening of NMs with characteristics known to interact with plants. Initial NM/plant interactions have been shown to be complex and need to be further investigated [125]. In order to begin answering these questions, the goal of my dissertation is to characterize the factors, both biotic and abiotic, that influence the bioavailability and translocation of gold nanoparticles to aquatic macrophytes.

**Dissertation goals**

To gain insight on factors that influence the bioavailability of NMs to
aquatic macrophytes, I proposed an extensive research project to evaluate factors that influence nanomaterial absorption. These factors included nanomaterial size, presence of dissolved organic matter, growth habitat, species, root structure and function, as well as humidity levels and evapotranspiration rate. The growth habitats of aquatic plants included fully submerged, free-floating within the water column or dimorphic with both submerged and emergent growth phase (Figure 1.1). By using hydroponic growth chambers, natural conditions were mimicked in the laboratory and greenhouse. Not only did this make the aquatic macrophyte studies more environmentally relevant, but also allowed for factors such as temperature, humidity, nanomaterial concentration and nutrient levels to be easily monitored and controlled to reduce variability.

Gold nanoparticles (AuNPs) with citrate surface chemistry served as a model NM for tracking NM movement within the plant (Figure 1.2). Due to their ease of synthesis in various sizes, stability in suspension and ability to be visualized, AuNPs were a good model NM for tracking studies [23,49,50,127]. Further, AuNPs are considered relatively non-toxic; therefore it was possible to study bioconcentration into plants with minimal chance of tissue damage. It is important to note that while it is generally accepted that bulk gold is safe, nano-sized gold is still under scrutiny [9,23,114,128]. However, my own studies have not observed toxic effects with different aquatic plants species [114]. Finally, it was possible to synthesize gold nanoparticles in the size range needed in our lab that facilitated accomplishing the goals and objectives of this research. All of
these attributes made AuNPs ideal for delivery, imaging and transport studies within aquatic macrophytes.

The overall goal of this research was to characterize the factors that influenced the bioavailability and translocation of gold nanoparticles to aquatic macrophytes. This was met by investigating each of the objectives below:

1. Determine the particle characteristics and plant characteristics that influenced the sorption of NM with aquatic plants with pristine and “modified” gold nanoparticles coated with natural organic matter.
2. Determine potential mechanisms of AuNP uptake in aquatic plants based upon root structure and function.
3. Determine the ability of gold nanoparticles to translocate within aquatic plants and factors that influenced the translocation.
Figures:

Figure 1.1: Photographs of aquatic macrophytes
(A) *Azolla caroliniana*, free floating aquatic, vascular fern with scale like leaves. (B) *Myriophyllum simulans*, fully submerged, aquatic, vascular plant with needle like leaves. (C) *Egeria densa*, fully submerged, aquatic, vascular plant with whorls of 4 leaves. (D) *Myriophyllum aquaticum*, submerged, aquatic vascular plant that has a dimorphic growth phase that becomes emergent before flowering. Emergent phase shown.
Figure 1.2: Cartoon of citrate capped gold nanoparticles
Cartoon representation of atoms forming the gold core of a gold nanoparticle. Surface oxidation of gold (Au$^0$ to Au$^{3+}$) attracts citrate surface chemistry, forming a disorganized citrate multi-layer that aids in suspension and stability in water.
References:


CHAPTER 2:

INTERACTIONS OF GOLD NANOPARTICLES WITH FRESHWATER AQUATIC MACROPHYTES ARE SIZE AND SPECIES DEPENDENT

Introduction

Industry research and development have been the driving force behind producing and applying many types of engineered nanoparticles (ENPs). These ENPs are used in a wide spectrum of applications including enhancing existing products, advancing micro-sized technology, and developing new medical and imaging techniques. The Woodrow Wilson Center on Emerging Nanotechnologies reports that more than 1,000 products now contain or use nanomaterials (http://www.nanotechproject.org/) according to their manufacturers.

Although many types of nanomaterials exist, metallic nanoparticles (NPs) are useful in that they are easily visualized, easily modified with different surface chemistries, and easily made into a wide variety of shapes and sizes [1,2]. Metallic NPs include particles made from Au, Ag, Pt, Fe, Cu, as well as semi conducting metal oxides such as ZnO and TiO$_2$ particles. Of these particles, gold nanoparticles (AuNPs) are used most opportunistically because of their ease of synthesis, stability in suspension, and unique optical properties [1,3–5]. For centuries, people have taken advantage of the optical properties of AuNPs in various applications from stained glass windows to art glass. These optical properties make AuNPs easy to visualize because the light scattered by AuNPs is in the visible range [4–6]. These unique properties of AuNPs make them ideal for delivery, imaging, and transport studies. Also important is that AuNPs are considered relatively nontoxic. While it is generally accepted that bulk gold is safe, nano-sized gold is still under scrutiny [4,7,8].
Because of the increased production of nanomaterial and compounds containing nanomaterial, concern exists about the eventual release of nanomaterial into the environment. Although natural nanomaterials are present in the environment, these NPs lack the specific size range and shapes that are characteristic of ENPs [9]. These natural particles, often referred to as fine particulate matter, also lack specific surface chemistry modifications that can enhance bioavailability, stability, and, in some cases, toxicity. Because ENPs may interact with the environment differently than natural particulates, understanding their fate is a vital first step in proactive research and possible future regulations. Limbach et al. [10] and Kiser et al. [11] hypothesized that engineered nanomaterials entering wastewater will partition to sewage sludge and biofilms. Sludge is often land applied as a soil amendment, and if nanomaterials are present, eventual release of these nanomaterials to the environment could result [12]. Nanomaterials may be released into the aquatic environment by rain events, leaching, runoff, or direct release of nanomaterials in treated wastewater.

Proactive research on the fate of NPs is important in understanding where other types of nanomaterial may partition or interact once exposed to a freshwater aquatic system. Aquatic plants were chosen for the present study because they comprise a major portion of aquatic primary productivity; and as such, they represent the base of the aquatic food web. Several studies have examined exposure of NPs to terrestrial plants such as carbon nanomaterials translocation in rice plants [13], copper nanomaterial exposure to wheat and mung bean [14], and several other plants [15,16], but a paucity of information exists on aquatic plant exposure. A recent study by Ferry et al. [17]
looked at partitioning of gold nanorods (65 nm length x 15 nm diameter) in an estuarine mesocosm. They reported little partitioning of nanorods into the salt marsh cord grass, *Spartina alterniflora*. These results should not be universally applied because *S. alterniflora* is an emergent halophyte, is able to tolerate high salinity concentrations and is not always submerged. *Spartina alterniflora* is in the Poaceae family and like other grasses absorbs nutrients through its roots. Also, because *S. alterniflora* is an emergent species, it has a thicker waxy cuticle on the leaf and stem surface to help reduce water loss [18]. The thick cuticle on the leaves of *S. alterniflora* acts as a barrier (Figure 2.1a), and the primary absorption mechanism for nutrients are the roots, where uptake from soil/sediment occurs. All of these characteristics make it a poor representative species for freshwater aquatic plants.

Aquatic macrophyte species differ in leaf morphology and nutrient acquisition mechanisms. In *Egeria densa* for example, the cuticle is much thinner and the leaves are only two cell layers thick (Figure 2.1b and 2.1c). Submerged aquatic plants can absorb nutrients through their roots directly from the water column, as they are often not anchored to sediment. Some species can also acquire nutrients via their shoots [19]. For example, *Ceratophyllum spp.*, a rootless aquatic plant, uses shoots for nutrient uptake directly from the water column [20].

In the present study, 4 and 18 nm citrate-coated gold nanospheres were used to investigate the influence of particle size on the interactions of NPs with aquatic plants. The objective of the present study was to determine if particle size influenced AuNP uptake among different plant species. Secondary objectives included visualizing which plant tissue sorbed nanomaterials,
quantifying tissue concentrations, and determining whether NP sorption is related to macrophyte surface area. To my knowledge, this is the first study investigating the interaction of citrate-AuNPs with freshwater aquatic plants.

**Materials and Methods**

*Nanoparticle characterization and preparation*

Four and eighteen nanometer AuNP spheres (obtained from Catherine Murphy, University of Illinois, Urbana-Champaign) were characterized for size and morphology via transmission electron microscopy (TEM) in both stock and exposure media before and after exposure (Clemson University Electron Microscope Facility, Anderson, SC). Zeta potential measurements (Malvern Zetasizer) of 4 and 18 nm AuNP suspensions (nominal concentration of 250 µg/L) in exposure media were taken to determine stability of AuNPs in suspension. This concentration was chosen as a working concentration so that imaging of AuNPs interacting with tissues could be performed. This is not an environmentally relevant concentration at this time. Nanoparticle suspensions were made in filtered well water (Pall Corporation type A/E Glass Fiber Filters). Well water (alkalinity 80 mg/L CaCO₃; hardness 107 mg/L CaCO₃; pH 7.1; TOC 0.56 mg/L; salinity 0.10 g/L; conductivity 210 µS/cm³) was chosen to limit nutrients and to restrict periphyton growth during experimental exposures. For the 4 and 18 nm AuNP suspensions, each size was suspended independently in one batch before being aliquoted to test chambers, to insure concentration uniformity. Samples were collected to verify initial (0 h) and final (24 h) concentrations.
Test plant species

Three aquatic plants were chosen for the aqueous exposures to AuNPs. These species were chosen due to their distinct leaf morphology and location within the water column. *Azolla caroliniana* is an emergent, free-floating aquatic fern that floats atop the water column and has roots that extend downward about 3 cm, with scale-like leaves. *Myriophyllum simulans* is a submergent species with finely dissected needle-like leaves, and *Egeria densa* is a submergent species with whorls of broad leaves. Both submergent species can reside floating free in the water column or can be rooted in the sediment via adventitious roots. Aquatic macrophytes were cultured in a double-layer, polyethylene-covered greenhouse at the Clemson University Institute of Environmental Toxicology, Pendleton, SC. The greenhouse was maintained at 25° ± 2°C, with a photoperiod of 14:10 h light/dark. Original specimens of *Egeria densa* were collected from Lake Issaqueena, South Carolina. *Myriophyllum simulans* was ordered from an online pet supply store, and *Azolla caroliniana* was obtained from constructed wetlands in Cairo, Georgia. All plants selected for this study appeared healthy and had limited or no periphyton growth. Plant cultures were not axenic. Plants were rinsed vigorously with distilled water before experimental use. Experimental exposures occurred in the greenhouse during July 2010.

Experimental design

The experiment was a 2 x 3 x 6 factorial with two AuNP sizes, three plant species, and six harvest periods. Each aquatic macrophyte was grown separately in a 70 mL glass test tube. Each treatment had three replications (*n* = 3) with three sub-replications that were combined to ensure sufficient dry
mass for gold detection. Each sub-replicate plant was approximately 1.0 ± 0.2 g fresh weight. Plants were harvested at 1, 3, 6, 12, 18, and 24 h, allowed to drip dry, patted with a KimWipe™, and then dried overnight at 60°C in a drying oven. Plants were not rinsed in this experiment so that any AuNPs adsorbed with the surface of the macrophyte were retained. The final dry weight (g) was recorded after 24 h of drying.

Treatment suspensions were sampled and gold concentration remaining in the test chamber was determined. Water samples from each sub-replicate were combined to form one replicate for a total n = 3 for each harvest period per AuNP size. Collected water samples were acidified to achieve 5% final acid concentration using full-strength aqua regia (1:3 nitric acid to hydrochloric trace metals grade acids) and then allowed to sit for 24 h before analyzing for gold (see below). Test chambers were acid rinsed with 10% Aqua Regia to recover any gold adsorbed to the chamber wall and the rinsates were analyzed. The concentration of Au in the rinse was minimal; therefore, the results were not included.

Gold analysis

Tissue digestion was performed based on a modified method from Anderson et al. [21]. After drying, tissue was then transferred to cooled, cleaned, pre-weighed 20 mL ceramic crucibles. Crucibles were cleaned before each digestion by heating to 450°C for 4h in a muffle furnace. Dried plant material within the crucible was dry-ashed at 530°C for 14 h to facilitate breakdown of plant cellulose and lignin components. Once cooled, plant ash was weighed, digested with 0.652 mL of full-strength Aqua Regia, and then diluted to 6 mL to achieve 5% acid concentration for analysis using inductively coupled plasma
mass spectrometry (Thermo Scientific X series 2). All samples were centrifuged at 3,000x g for 9 min after acidification, so that particulates, if present, did not interfere with analysis. Water samples were analyzed using inductively coupled plasma mass spectrometry without further manipulation.

**Colorimetric surface area analysis**

We modified the colorimetric method developed by Cattaneo and Carignan [22] to estimate aquatic macrophyte surface area. For each species, 0.150 g of fresh tissue was used to determine surface area. In modifying the method, we used 0.2 g/L of methylene blue dye mixed with a 50:50 liquinox: ultrapure water surfactant mix. Plants were dipped into the dye mixture, shaken 30 times, and rinsed in 200 mL of ultrapure water. This procedure was repeated three separate times using the same plant section. Each time the ultrapure water was replaced and reserved. The surfactant effectively breaks the water surface tension on the plant and facilitates a uniform coating of dye across the entire plant. The dye was measurable at an absorbance of 664 nm to quantify the amount of dye in each rinse. The absorbance value of the rinsate was compared to a standard curve of terrestrial leaves with a known surface area, measured using a LI-3100C leaf surface area meter (LI-COR).

**Scanning electron microscopy and TEM preparation**

Plant root tissue samples were prepared for TEM and scanning electron microscopy (SEM), and scanning transmission electron microscopy (STEM) by overnight fixation in 3.5% glutaraldehyde. After samples were fixed, both TEM and SEM samples were washed in phosphate buffer at pH 7.1 three times, for 30 min each time, with a final buffer wash overnight. Samples were washed with ultrapure water three times, and dehydrated with increasing ethanol
concentrations (35, 50, 70, and 95, and three times at 100%) for 10 min each step.

For SEM, after samples had been dehydrated, they were rinsed with a 50:50 mixture of 100% ethanol:hexamethyldisilazane, allowed to evaporate then rinsed with only hexamethyldisilazane and allowed to evaporate. When the samples had been dried, they were mounted onto the appropriate grid or microscopy stub.

For TEM and STEM samples, after the ethanol dehydration step, samples were embedded into a 50:50 mixture of 100% ethanol: L.R. White resin for 15 minutes, embedded into 100% L.R. White resin, refrigerated overnight, and polymerized at 60°C in a drying oven overnight. Samples were sectioned (90–100 nm thick) using a Leica Ultra-microtome. Microscopy samples were viewed on a Hitachi HD-2000 STEM at 200 kV or on a Hitachi 4800 SE with TE detector at 25 kV (Clemson University Electron Microscope Facility, Anderson, SC). Samples were imaged on 200-mesh silicon-free copper grids. Energy dispersive X-ray spectroscopy was performed on the HD-2000 or Hitachi 4800 to confirm gold presence.

Data analysis

Statistical analyses were performed on data using SAS (SAS Institute, Cary, NC). Slopes for sorption rate were checked for homogeneity of intercepts to determine if slope or sorption rate had any significant differences. Analysis of variance was performed using PROC GLM to test for treatment differences. Least Significant Difference post hoc test was performed to quantify differences between treatments and controls (α = 0.05).
Results and Discussion

Characterization of AuNPs and Treatment Suspensions

Transmission electron microscopy revealed that stock AuNPs did not undergo visual changes in size or morphology during the 24 h exposure (Figure 2.2). The zeta potential measurements were -14.1 mV and -9.73 mV for the 4 and 18 nm AuNPs, respectively. Positive controls, or vials containing AuNP suspensions but no plant tissue, were run for each treatment. No significant change in concentration was detected for the 4 nm AuNPs positive control treatment after 24 h, when compared to 0 h. Hour 0 positive control represents the initial concentration of the suspension in Figure 2.6. However, a significant decrease in concentration occurred in the 18 nm AuNPs positive control treatment (206 µg/L to 123.5 µg/L), suggesting aggregation and settling of the 18 nm AuNPs from the water column. Although aggregation occurred in the 18 nm AuNPs treatments, a significant decrease in gold concentration was observed in the treatments with aquatic plants compared to the positive controls. After the 12 h time point, aqueous gold concentrations (Figure 2.6) were reduced by approximately 38 and 32% in the 4 and 18 nm treatments containing plants, respectively, compared with positive controls.

Sorption of Gold Nanoparticles with Aquatic Macrophytes

In the present study, I aimed to determine if AuNPs adsorbed, absorbed, and potentially bioconcentrated within or onto aquatic macrophyte tissue. If trophic transfer of nanomaterial can occur in the environment, NP partitioning either within or on tissue surfaces is irrelevant. Tissue concentration is important, however, when determining the sorption rates and factors that influence particle sorption. In all treatments, no visual phytotoxic effects were
evident. Gold accumulated in the tested plant tissue over the 24 h exposure (Figure 2.7). These data suggest that while NP sorption by *E. densa* did not differ between particle sizes, 4 nm AuNPs sorbed with *A. caroliniana* and *M. simulans* at a higher rate and number than 18 nm particles. This difference in sorption rate and concentration may be due to differences in plant species as well as AuNP size. Plant cells have complex cell walls that support the plant and protect cells from damage and pathogens. Because nutrient uptake and cell signaling is necessary, cell walls have pores. Any particle > 40 angstroms or 4 nm would have difficulty passing through a pore [23]; however it has been proposed that pore uptake could be one of multiple transport pathways [24]. Larger particles not able to pass through pores in cell walls may be able to enter through foliar structures such as stomata. Particle assimilation through stomata was reported in the terrestrial plant *Vicia faba* [24]; however, high humidity and conditions that favored stomata opening were necessary. Stomata may or may not be present in aquatic plants [25]. In the case of *A. caroliniana*, a floating vascular fern, stomata are present on the top and underside of the floating leaves [26], although plant uptake of NPs via roots seems the most plausible route, as various other studies have supported [13,16,27,28].

The presence of AuNPs in the root tissue of these aquatic macrophytes was affirmed using electron microscopy; TEM, SEM or STEM was utilized to image sections of root tissue from all species for AuNP presence. *Azolla caroliniana* images indicated that both 4 and 18 nm AuNPs were absorbed into the root, although 4 nm AuNPs were present in higher numbers (Figure 2.8). Using elemental dispersive X-ray (EDX) spectroscopy, we confirmed AuNPs were present in all images (Figure 2.3-2.5). Unlike the other
aquatic species studied, *A. caroliniana* roots possess root hairs that help the plant acquire nutrients. Root uptake of AuNPs by *A. caroliniana* is consistent with other reports of NP absorption by terrestrial plant roots, which also have root hairs [13,27–28]. *Myriophyllum simulans* and *E. densa* root tissue was also sectioned. In these species, 18 nm AuNPs were adsorbed on the surface of the root cells, but no uptake into cells was observed (Figures 2.9 and 2.10). However, *M. simulans* absorbed 4 nm AuNPs into root cells (Figures 2.9), but no uptake into *E. densa* tissue was observed (Figure 2.10). Based on these results, I conclude that AuNP uptake and partitioning is both a function of particle size and plant species.

When comparing absorption of AuNPs among these three species, a possible mechanism of AuNP exclusion could be salinity tolerance and membrane structure. Plants have developed a wide range of adaptations for salinity stress. Adaptation mechanisms include biochemical pathways that exclude ions or actively transport ions to vacuolar storage areas and physical adaptations such as cell wall modification or altering the membrane structure [29]. These adaptations can alter membrane permeability in specialized tissues such as roots. Previous studies have documented the salinity tolerance of *E. densa* and *M. simulans* up to 8 g/L [30–31]. *Azolla caroliniana* is not a salinity tolerant species. Studies documented a decrease in *A. caroliniana* biomass and growth when salinity levels increased above 0.05 g/L [32]. The adventitious root structures of *E. densa* emerge from stem nodes and may influence its salinity tolerance [33]. *Egeria densa*’s roots are not as efficient at transporting ions and acquiring nutrients in general compared to other aquatic macrophytes [19]. The adaptation mechanisms that *M. simulans* and *E. densa* use for survival in saline
environments may include enhanced exclusion mechanisms, which may account for the limited AuNP uptake noted in our study. In contrast, *A. caroliniana* is not salinity tolerant and exhibited increased absorption of AuNPs into tissues, regardless of NP size. This further supports my hypothesis that plant salinity tolerance may influence AuNP uptake and partitioning.

*AuNP sorption rate of the three aquatic macrophytes*

When comparing the 18 nm AuNP sorption rate among the three species, *M. simulans, E. densa,* and *A. caroliniana,* I detected no significant difference between the slopes, or sorption rate, of each treatment over the 24 h exposure (Figure 2.11). However, the sorption rate of 4 nm AuNP by *A. caroliniana, E. densa,* and *M. simulans,* differed from that of *E. densa.* These data indicate that absorption of the 4 nm AuNPs is likely for these two species. This is supported further by tissue concentration and electron microscopy images. Although the rate of AuNP sorption among the three species does not differ in the 18 nm AuNP treatment, the initial concentration sorbed by each plant at the 1 h time point differed significantly among each species. This difference in initial sorption rate is attributed to the difference in surface area among the macrophytes and to their distinctive leaf morphologies. *Myriophyllum simulans* has finely dissected, needle-like leaves; *E. densa* has whorls of leaves; and *A. caroliniana* has small, compact leaves that fold over one another multiple times. The colorimetric assay revealed that *A. caroliniana* (35.7 ± 1.8 cm$^2$) had the highest surface area to mass ratio compared with the other two species. *Myriophyllum simulans* and *E. densa* were very similar in surface area (31.1 ± 0.27; 31.1 ± 0.51 cm$^2$; respectively). *Azolla*
*caroliniana* also had the highest initial concentration of gold measured for both 4 and 18 nm AuNPs.

**Environmental relevance**

Upon harvest, experimental plants were not rinsed, but rather were allowed to drip and then patted dry. This was to prevent removal of loosely adsorbed AuNPs. In the present study, I wanted to quantify both adsorbed and absorbed AuNPs. If trophic transfer of nanomaterial can occur in the environment, it does not matter whether NPs are within the tissue or on the surface. However, tissue concentration is important in determining sorption rates and the factors that influence particle sorption. Nanoparticle surface chemistry may also play a vital role in the bioavailability of NPs. The AuNPs investigated here had a citrate surface chemistry. Citrate is an organic acid, and many plants exude organic acids (typically malate or citrate) into the rhizosphere, and thus alter the environment surrounding their rhizosphere to increase nutrient availability or to decrease metal availability in the case of aluminum [34–35]. Because plants normally export organic acids, it is possible that the citrate coatings on the nanoparticles hinder uptake. These variables should be considered in future uptake studies using nanomaterials with organic surface chemistries.

**Conclusions**

This study investigated the potential of 4 and 18 nm citrate capped AuNPs to sorb with plant tissue. No visual toxicity symptoms to macrophyte tissue were evident over the 24 h of exposure. Both NP sizes adsorbed to the surface of all three macrophytes. The 4 nm AuNPs were found internalized within the root tissue by *A. caroliniana* and *M. simulans*. Transmission electron microscopy,
SEM, and STEM micrographs support this conclusion. Differences in adsorption and absorption of AuNPs are likely due to the difference in particle sizes and differences among the three plant species. These differences include salinity tolerances and surface area. Plant uptake could be an important factor when considering NP fate in aquatic ecosystems and the potential for trophic transfer. Further, bioassays that have longer exposures to gold nanoparticles may provide insight into transport within the plant and potential sequestration.
Figure 2.1: Cuticle and structural comparison of *Egeria densa* and *Spartina alterniflora*.

(A) Transmission electron micrograph image of *S. alterniflora* cuticle and cell wall. Scale bar = 0.5 µM. (B) Scanning transmission electron microscope image of *E. densa* leaf cuticle and cell wall. Cuticle appears as dark line marked with arrows. Scale bar = 1 µM. (C) Light microscopy image of *E. densa* transverse section of leaf tissue. Light micrograph stained with Saffron O and Fast Green. Scale bar = 25 µM. CP = cuticle proper; CW = cell wall; RL = reticulate layer. Image (A) reproduced with permission from Maricle et al. [18].
Figure 2.2: Size distribution of gold nanoparticles.
Transmission electron micrograph of (A) 4 nm gold and (B) 18 nm gold nanoparticle stock used in the present study. Both particle sizes were imaged before and after exposure. Images shown represent stock gold nanoparticle suspension (40 mg/L) before exposure. Histograms represent particle distribution. No change in size was observed over the duration of exposure.
A) Azolla root tissue, 18 nm AuNP Treatment

<table>
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<th>Spectrum</th>
<th>In stats.</th>
<th>C</th>
<th>O</th>
<th>Al</th>
<th>Si</th>
<th>Cu</th>
<th>Ge</th>
<th>Sn</th>
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<td>3.70</td>
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Max. | 79.10 | 6.01 | 0.32 | 0.40 | 9.82 | 0.73 | 0.57 | 4.64 | 7.31 |
Min. | 71.85 | 4.61 | 0.21 | 0.15 | 8.17 | 0.73 | 0.39 | 3.70 | 2.80 |

All results in weight %

B) Azolla root tissue, 4 nm AuNP Treatment

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<td>Au L</td>
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<td>Totals</td>
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Figure 2.3: Energy dispersive X-ray analysis (EDAX) data, Azolla caroliniana root. (A) Represents a transmission electron (TE) micrograph of A. caroliniana root tissue exposed to 18 nm gold nanoparticles, with individual spectrum scans highlighted. Nanoparticles were imaged at 25 kV using a TE detector on a Hitachi FE-4800 electron microscope. (B) Elemental contrast micrograph of the EDAX scanned area for A. caroliniana root tissue exposed to 4 nm AuNPs. EDAX was performed on Hitachi HD-2000 at 200kV. Each chart indicates the weight% of each element listed. K and L refer to the electron shell spectra excited. Gold weight% is highlighted with a rectangle.
A) Myriophyllum root tissue, 18 nm AuNP treatment

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B) Myriophyllum root tissue, 4 nm AuNP treatment

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Figure 2.4: Energy dispersive X-ray analysis (EDAX) data, Myriophyllum simulans root. (A) Elemental contrast micrograph of the EDAX scanned area for M. simulans root tissue exposed to 18 nm AuNPs (B) Elemental contrast micrograph of the EDAX scanned area for M. simulans root tissue exposed to 18 nm AuNPs. EDAX was performed on Hitachi HD-2000 at 200kV. Each chart indicates the weight% of each element listed. K and L refer to the electron shell spectra excited. Gold weight% is highlighted with a rectangle.
A) Egeria root tissue, 18 nm AuNP treatment

![Elemental contrast micrograph of the EDAX scanned area for E. densa root tissue exposed to 18 nm AuNPs.](image)

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<th>Element</th>
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*note no AuNPs are present in this micrograph

B) Egeria root tissue, 4 nm AuNP treatment

![Elemental contrast micrograph of the EDAX scanned area for E. densa root tissue exposed to 4 nm AuNPs.](image)

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Figure 2.5: Energy dispersive X-ray analysis (EDAX) data, *Egeria densa* root. (A) Elemental contrast micrograph of the EDAX scanned area for *E. densa* root tissue exposed to 18 nm AuNPs. (B) Elemental contrast micrograph of the EDAX scanned area for *E. densa* root tissue exposed to 4 nm AuNPs. EDAX was performed on Hitachi HD-2000 at 200kV. Each chart indicates the weight% of each element listed. K and L refer to the electron shell spectra excited. Gold weight% is highlighted with a rectangle. Note that for (A) no gold was observed present in the 18 nm treatment.
Figure 2.6: Gold nanoparticles remaining in suspension.
Gold concentration remaining in suspension in (A) 4 nm and (B) 18 nm gold nanoparticle treatments. Error bars represent the standard deviation of the mean. ◇ represent control suspensions with no plant tissue present. First ◇ is time 0, representing initial gold concentration. Each additional ◇ shows concentration remaining for corresponding time point.
Figure 2.7: Tissue concentration of gold over time.
Total tissue concentration of gold using whole plant. (A) 4 nm gold nanoparticle treatment. (B) 18 nm gold nanoparticle treatment. Error bars represent the standard deviation of the mean. Treatments (and positive controls after 12 h) are significantly different from hour 0. (P < 0.05)
Figure 2.8: *Azolla caroliniana* electron micrographs.
Electron micrographs of *Azolla caroliniana* sectioned root tissue. Arrows indicate gold nanoparticles. (A) Elemental contrast micrograph of *A. caroliniana* root tissue indicating the presence of absorbed 4 nm gold nanoparticles (where electron dense material appears as bright white). (B) Transmission electron micrograph of *A. caroliniana* root tissue indicating absorption of 18 nm gold nanoparticles present (where electron dense material appears dark).
Figure 2.9: *Myriophyllum simulans* electron micrographs.
Elemental contrast micrographs of *Myriophyllum simulans* sectioned root tissue. Gold nanoparticles are indicated by arrows and appear white. 
(A) Indicates 4 nm gold nanoparticle absorbed by *M. simulans* root tissue. 
(B) Indicates 18 nm gold nanoparticles only adsorbed to the surface of the root cell wall. Scale bar = 800 nm
Figure 2.10: *Egeria densa* electron micrographs.
Elemental contrast micrographs of *Egeria densa* sectioned root tissue. Gold nanoparticles are indicated by arrows and appear as white. (A) Indicates 4 nm gold nanoparticles adsorbed to *E. densa* cell wall. (B) Indicates 18 nm gold nanoparticles adsorbed to *E. densa* cell wall. Scale bar = 800 nm.
Figure 2.11: Slope comparison of gold nanoparticle sorption.
Comparison of slopes for the sorption rates of gold nanoparticles in *Azolla caroliniana*, *Myriophyllum simulans* and *Egeria Densa* during 24 h of exposure to (A) 4 nm or (B) 18 nm gold nanoparticles. Error bars represent standard deviation of the mean.
References:


CHAPTER 3:
ABiotic and Biotic Factors That Influence the Bioavailability
of Gold Nanoparticles to Aquatic Macrophytes

Introduction

The consequences of environmental release of nanomaterials (NMs) are uncertain. Uncertainty with regard to the fate and effects of NMs in the environment have been identified in several recent papers as critical knowledge gaps that prevent the quantitative assessment of environmental risk and limit the management of NMs in aquatic ecosystems [1-9]. Studies focused on the release of NMs in the environment range from modeling exposure through life cycle assessments for predicting surface water concentrations [10,11] to characterizing the effects of NMs on crop plants and terrestrial plant health such as wheat, squash, pumpkin, tobacco and rice [12-17]. At this time, detailed studies of the interactions of NMs with aquatic plants are limited [18,19]. Since plants represent a large interface that interacts closely between the environment and biosphere in both terrestrial and aquatic systems, this interface is in need of further investigation with regards to NMs and their distribution, fate and potential effects.

Recent review papers by Rico et al. [20] and Miralles et al. [21] have inventoried the interactions that NMs have with terrestrial plants. The common endpoints for measuring plant-nanoparticle interactions include germination and root/shoot elongation. While these measurements provide valuable datasets, additional studies that address more in-depth modes of action are needed. From
Rico et al. [20], it is clear that a paucity of information exists about NM interactions with plants, and factors that influence their bioavailability. These factors include NM size and shape, route of uptake, bulk NM composition and surface chemistry. Miralles et al. [21] advances the need for more information and understanding of mechanism of NMs uptake and transport within plants, including bioavailability.

Terrestrial plants may come in contact with NMs through atmospheric deposition, improper disposal of NM-containing compounds, and from land applied sewage sludge, a known sink for NMs [16, 22, 23]. Through storm events, runoff, erosion and improper or the lack of best management practices, the aquatic environment will then serve as a sink for NMs. Although much literature is focused on the impact of NMs on terrestrial crop species, some of the results obtained to date are relevant to aquatic plants. Many similarities exist between aquatic and terrestrial plant physiology, such as vascular systems, photosynthesis structures and plant organ systems (roots, leaves). A difference, however is that aquatic macrophytes can absorb nutrients from both roots and leaves [24]. This difference in nutrient acquisition warrants further investigation into parameters that influence absorption of NM in aquatic vascular plants, as roots may not be the only site of NM absorption. Studies have shown NMs are capable of penetrating into the roots of many different species of plants, typically limited to pores present in the microfibril network structure of the cell wall. These pores (gaps in microfibril layers) limit the diameter of structures that are capable
of penetrating through to the cell membrane. Typical pore structures of higher plants range from 3-20 nm, exhibiting the differences among plant species [16, 20, 21, 25, 26]. Contradicting this exclusion mechanism, however, studies have published absorption of larger NMs including carbon nanotubes and AuNPs that are larger than the pore structures present [20, 26]. This evidence suggests another route of absorption; either through wounding of tissue, creation of new pore structures, stomatal absorption or even active transport across cell membranes [20, 21, 27]. As progress is made in understanding NM interactions with terrestrial plants, additional studies are needed with respect to aquatic plants; however, probable mechanisms of uptake, translocation, and toxicity are scarce for all plant species. Further, research into biotic and abiotic factors that influence NM bioavailability to aquatic plants is also needed.

Because aquatic plants are often fully submerged, water quality parameters become critical in understanding bioavailability of NMs to aquatic macrophytes. Water hardness plays a well-known role in aggregation of NMs. pH can also dictate speciation of NMs and aggregation state. The presence of natural organic matter (NOM) can also influence bioavailability. Natural organic matter may vary in concentrations and chemical composition but is considered ubiquitous in water bodies [19]. Natural organic matter can influence the stability, charge and surface chemistry of NMs [28-30]. Stankus et al. [28] observed similar properties between AuNPs exposed to NOM, regardless of initial AuNP surface chemistry, which indicated that initial AuNP surface chemistry was no
longer the driving force of particle stability when in the presence of NOM. Diegoli et al. [29] proposed NOM replaces the citrate surface chemistry of the AuNPs, stabilizing the AuNPs from aggregation. This replacement of surface chemistry resulted in more stable suspensions of particles, which did not aggregate even in extreme pH ranges. Nanoparticle and NOM interactions do vary, however, between NOM isolates, and the higher molecular weight humic acids were found to give the citrate coated AuNPs the greatest stability in the presence of NOM [30]. Natural organic matter consists of humic and fulvic acids, both known to have the ability to chelate metal ions and organic pesticides [31-33] and to stabilize AuNPs [28-30]. This interaction of NOM with AuNPs is hypothesized to reduce the absorption of AuNPs by aquatic plants due to AuNPs binding with the NOM and forming a larger, more stable association.

To address AuNP bioavailability to aquatic plants, biotic and abiotic factors were investigated. The effect of plant species, root presence, natural organic matter and nanoparticle size were studied to determine which factors most strongly influence bioavailability and absorption of AuNPs. Gold nanoparticles served as a model NM for tracking within the aquatic plant due to their ease of synthesis in various sizes, stability in suspension, capacity to be visualized and no toxicity visually observed in aquatic plants [18, 34-38]. The interaction NMs have with aquatic plants was important in understanding potential for NM fate, transfer and possible escape from the aquatic ecosystem.
Materials and Methods

Nanoparticle synthesis and characterization

Four, 18, and 30 nanometer (nm) gold nanoparticle spheres (AuNPs) were synthesized following the Turkevich and related Frens method [37, 39, 40] at the Clemson University Institute of Environmental Toxicology, Pendleton, SC. The 4 nm AuNPs were synthesized by combining 0.5 mL of 0.01M chloroauric acid with 19 mL of Milli-Q water, and 0.5 mL of 0.01M sodium citrate tribasic dihydrate. After, 0.6 mL of 0.1M sodium borohydride was added to reduce the chloroauric acid completing the process. The 18 nm AuNPs were synthesized by combining 2.5 mL of 0.01M chloroauric acid with 97.5 mL Milli-Q water. The solution was brought to a boil, and 3 mL of 1% by weight sodium citrate reduced the chloroauric acid, forming the AuNPs. Lastly, for the 30 nm AuNPs, 2.5 mL of 0.01M chloroauric acid was combined with 100 mL of Milli-Q water. The solution was then boiled and 10 mL of 1% by weight sodium citrate solution was added to reduce the chloroauric acid.

Gold nanoparticle size and morphology characterization was performed with transmission electron microscopy (Hitachi H-7600) in both stock and exposure media at the Clemson University Electron Microscope Facility, Anderson, SC. Electrophoretic mobility measurements were measured on a Malvern Zetasizer Nano of stock and treatment suspensions.
Nanoparticle suspensions

Nanoparticle suspensions were made in 0.45 μm filtered well water (PALL type A/E glass fiber filters). For the dissolved organic carbon (DOC) treatments, Suwannee River aquatic reference NOM (1R101N) was purchased from the International Humic Substances Society (IHSS). Natural organic matter (NOM) was added to filtered well water (0.45 μm) at the nominal concentration of 5 mg/L, stirred overnight, and then filtered with a 0.45 μm, glass fiber filter (PALL type A/E) to capture the DOC fraction. After filtration, organic carbon content (mg C/L) was analyzed using a Shimadzu total organic carbon analyzer (TOC-V, Pendleton, SC).

Plant species

*Egeria densa* Planch., *Azolla caroliniana* Willd. and *Myriophyllum simulans* Orch. were cultured in a double-layer, polyethylene-covered greenhouse in Pendleton, SC (25° ± 2 °C, 14:10 h light/dark). All plants selected for this study appeared healthy and had no visible periphyton growth. Plant cultures were not axenic. Plants were rinsed vigorously with distilled water before experimental use. Experiments were conducted in the greenhouse during July and August 2012.

Microscopy

Gold nanoparticle suspension samples were captured on 200-mesh, carbon formvar™ copper grids (type FCF200-Cu). The Hitachi 7600 (120kv) and Hitachi 9500 (300kV) transmission electron microscopes (TEM) were used to
image nanomaterials in bright field micrographs. The Hitachi HD-2000 Scanning transmission electron microscope (STEM) was used to image dark field micrographs. All electron microscopy was performed at the Clemson University Electron Microscope Facility, Anderson, SC.

**Experimental design**

The experimental setup was a 24 h static exposure with a 3 x 2 x 3 x 2 randomized complete factorial design. Factors included plant species (\textit{M. simulans}, \textit{E. densa} and \textit{A. caroliniana}), plant roots (R+, present or R-, absent), particle size (4, 18 or 30 nm AuNPs) and SR-NOM (DOC+, present or DOC-, absent) for a total of 36 treatments. All roots were removed at the plant base via razor blade prior to exposure. Each treatment combination was performed in triplicate. Gold nanoparticle suspensions were made at a nominal concentration of 250 µg Au / L then subdivided for each treatment replicate.

\textit{Myriophyllum simulans} and \textit{Egeria densa} were exposed in 70 mL glass test tubes. \textit{Azolla caroliniana} was exposed in 30 mL glass beakers. Before exposures, glassware was acetone and acid washed (10% nitric). After harvest, plants were vigorously rinsed in distilled water by submerging 10 times, removing loosely associated surface AuNPs. After rinsing, the final dry tissue weight (g) was recorded after 24 h of drying at 60ºC in a drying oven.

**Gold analysis**

Tissue digestion was performed based on a modified method from Anderson et al. [41]. Dried tissue was transferred to 20 mL cleaned ceramic
crucibles and was heated to 530 °C for 14 h in a muffle furnace to ash, and facilitate breakdown of plant cellulose and lignin components. Crucibles were cleaned with aqua regia. Once cooled, tissue was digested with aqua regia (1:3 nitric to hydrochloric trace grade acids), and then diluted to achieve 5% volume acid for analysis using ICP-MS (ThermoScientific Xseries2). Water samples were acidified to achieve 5% volume acid using aqua regia.

**Data analysis**

Analysis of variance indicated a significant difference in main effects for gold nanoparticle size distribution and electrophoretic mobility. Due to large sample size ($n = 250-1200$) in the particle size analysis, Tukey’s HSD was used to separate main effects, revealing significant difference between treatments ($P<0.0001$). Fisher’s LSD post hoc test was used to separate means for gold nanoparticle electrophoretic mobility measurements ($n = 3, P=0.0205$). A full factorial, standard least squares analysis of main effects (plant species, plant roots, particle size and SR-NOM) was conducted using JMP v10.0 (SAS Institute Inc. Cary, NC). Interactions of main effects were significant ($p < 0.0001$) and were separated using Fisher’s LSD post hoc test ($\alpha = 0.05$). The interaction profiler was used to characterize interactions of main effects (Appendix A-1).

**Results and Discussion**

**Particle characterization**

Each size of gold nanoparticles in stock and treatment suspensions (initial and 24 h time points) was characterized for size by transmission electron
microscopy (TEM) and for stability with zeta potential. The same samples were used in each characterization analysis. Gold concentration remained consistent throughout the exposures, at 249 ± 11.0 µg Au/L. Dissolved organic carbon for each treatment was normalized to carbon (C) content. Well water only treatments (DOC-) had 0.80 ± 0.02 mg C/L, and well water with DOC (DOC+) had 2.0 ± 0.4 mg C/L. The well water used in this study had a pH of 7.1, hardness of 100 mg/L CaCO₃, and alkalinity of 80 mg/L CaCO₃. The nominal AuNP sizes of 4, 18 and 30 nm were determined from the stock suspensions (5.2 ± 2.0 nm, 18.1 ± 5.6 nm and 27.0 ± 8.0 nm, respectively (Figure 3.1). Figure 3.1 and 3.2 show all of the data points gathered for AuNP characterization and Table 3.1 summarizes these characterization results. For all AuNPs, a loss in stability was observed in treatment suspensions (Figure 3.2). The 4 nm AuNPs stock suspension had an electrophoretic mobility of -24.4 ± 8.2 mV, suggesting the 4 nm particles are moderately unstable. The measurement is likely due to the high ionic strength, as these particles where not purified to remove excess sodium that results from the addition of sodium borohydride. In the 4 nm AuNP well water treatment the zeta potential was -16.7 ± 1.8 mV, which can be explained by the increase in water hardness of 100 mg/L as CaCO₃. After 24 h, however, the zeta potential indicated a gain of stability to -25.8 ± 0.9 mV. The increase in the 4 nm AuNP treatment occurred simultaneously with a gain in particle size mean from initial 8.0 ± 6.0 nm to 24 h size of 10.0 ± 5.0 nm. This size increase was mitigated with the addition of DOC. In the well water with DOC treatment
the initial zeta potential was -19.4 ± 2.1 mV shifting to -25.7 ± 1.1 mV after 24 h. Particle size in the DOC+ treatment showed reduced particle aggregation with initial size being 6.6 ± 3.6 nm to 5.2 ± 1.8 nm after 24 h in suspension. Compared to the 24 h values for the well water, DOC- treatment (10.0 ± 5.0 nm) and well water DOC+ treatment (5.2 ± 1.8 nm) indicated the potential effect DOC had in reducing particle aggregation.

The 18 nm AuNPs stock suspension was moderately stable (-35.2 ± 4.4 mV) whereas the well water treatment suspensions showed a reduction in initial AuNP stability (-17.8 ± 2.9 mV) that shifted to -26.8 ± 4.1 mV after 24 h. This shift in stability correlated with an increase average particle size, initial 17.5 ± 2.4 nm to 24 h 23.3 ± 12.5 nm, indicating the onset of aggregation in the well water treatment. The addition of DOC did not enhance particle stability (initial -18.5 ± 3.5 mV to 24 h -19.2 ± 4.7 mV); however, when comparing the mean core diameter of the 18 nm AuNPs in both the well water treatment (23.3 ± 12.5 nm) and DOC well water treatment (18.4 ± 8.4 nm), the core diameter becomes more similar to that of the stock suspension (18.1 ± 5.6 nm). When comparing these results to the 18 nm well water only treatment, it appeared that DOC had an effect mitigating AuNP aggregation and polydispersity.

The 30 nm AuNP stock suspension had the highest stability of all particles (-37.2 ± 1.6 mV; Table 3.1). When well water treatment suspensions were made, the zeta potential initially declined to -23.7 ± 4.2 mV, then after 24 h remained the same at -24.9 ± 3.4 mV. With the addition of DOC, no change was observed in
particle stability. Comparing particle size for the well water treatment, the onset of aggregation was observed (initial 25.0 ± 7.0 nm to 24 h of 39.1 ± 8.0 nm). With the addition of DOC, similar results were observed, where the onset of particle aggregation was still observed in the well water DOC treatment (initial 34.1 ± 7.8 nm to 24 h 42.3 ± 8.3 nm). In this treatment, the addition of DOC did not reduce the average particle core diameter.

*Gold nanoparticles and aquatic macrophytes*

The absorption of AuNPs by aquatic plants is AuNP size and plant species dependent [18]. Several studies reported that NM absorption by terrestrial plants was specific to NM characteristics, as well as the plant species [16,26,42-44]. However, the current mechanisms of NM absorption and translocation in plants are presently unknown [16,19,20,21,43]. In order to better address factors that influence the absorption of AuNPs from suspension to aquatic plants, 2 biotic (plant species and root presence) and 2 abiotic factors (DOC and AuNP size) were investigated.

The three specific plant species utilized had unique characteristics. *Azolla caroliniana* is a free-floating aquatic fern with scale like leaves. It has many slender roots that protrude from the floating leaf bottoms, and resides in the water column with an average length of 4-6 cm. These roots contain root hair structures that increase the surface area of the roots in contact with the water column. Stomata are also present on the leaf surface [45]. Because *A. caroliniana* is free floating, it has an evapotranspiration stream present.
*Myriophyllum simulans* is fully submerged and can be rooted in the sediment or free floating in the water column. It may or may not have roots present. The leaf structure is needle-like, and leaves grow in whorls around each node. *Egeria densa* is also fully submerged. This species has broad whorls of 4 leaves around each node. *Egeria densa* is either rooted into the sediment or free floating. The root structure of *E. densa* may or may not be present. Roots propagate from a root crown node, typically one or two roots at a time [46]. These roots are adventitious, which develop to anchor the plant into the sediment.

**Factors that affect bioavailability of AuNPs**

Figure 3.3 shows each species broken down into the individual treatment factors of root presence (R+ or R-), DOC (DOC- or DOC+), AuNP size (4, 18 and 30 nm) and plant species (*A. caroliniana, M. simulans* and *E. densa*). Four way interactions among factors influencing gold concentration in plant tissues were significant (*P > 0.0001*). AuNPs absorption by aquatic plants is species dependent, as shown by previous research [18].

These results suggest that tissue concentrations observed in *E. densa* were due to surface adsorption only. Previous research indicated that *E. densa* did not absorb 4 or 18 nm AuNPs from suspension [18]. Considering tissue concentration of Au in *E. densa* by treatment, results in the DOC-, R+ treatment for 4, 18 and 30 nm AuNPs had tissue concentrations of 6.3 ± 1.4, 11.3 ± 2.4 and 6.3 ± 4.2 mg Au/kg, respectively. This treatment indicated that root or shoot absorption is not a driver of AuNP uptake, since similar Au tissue concentrations
were observed in the DOC-, R- treatment, with the 4, 18 and 30 nm AuNPs tissue concentrations being $10.0 \pm 7.2$, $21.1 \pm 7.0$ and $15.1 \pm 5.4$ mg Au/kg, respectively. In the DOC-, R- and R+ treatment, no AuNP size dependence was observed in treatment tissue concentrations, supporting that only surface adsorption of AuNPs occurred.

Comparing the DOC+ and DOC- treatment for *E. densa* the addition of DOC resulted in a trend of declined tissue gold concentration. In the DOC+, R- and R+ treatment, no significant effect of roots was found to correlate with tissue concentrations. For the DOC+, R+ treatment tissue gold concentrations observed were $8.6 \pm 1.9$, $5.3 \pm 1.7$ and $5.1 \pm 2.8$ mg Au/kg for 4, 18 and 30 nm AuNPs, respectively. The DOC+, R- treatment resulted in similar tissue gold concentrations of $10.2 \pm 1.5$, $7.0 \pm 2.3$ and $6.6 \pm 2.5$ mg Au/kg for 4, 18 and 30 nm AuNPs, respectively.

Results for *Myriophyllum simulans* indicate higher gold tissue concentrations than *E. densa* for each AuNP size. For *M. simulans*, no differences in tissue gold concentrations were observed across AuNP sizes. Examining tissue gold concentration results by treatment, the DOC-, R+ treatment had concentrations for the 4, 18 and 30 nm AuNPs of $23.9 \pm 16.8$, $27.8 \pm 5.0$, and $30.3 \pm 12.2$ mg Au/kg compared to the DOC-, R- treatment for the same sizes with tissue concentrations of $14.5 \pm 2.9$, $33.4 \pm 11.0$, and $28.3 \pm 13.0$ mg Au/kg, respectively. Comparing the tissue gold concentrations for the R+
and R- treatments for the DOC- treatment above also indicated that shoots were not a driver of AuNP absorption.

The addition of DOC resulted in a general decline in Au tissue concentration in *M. simulans*. Tissue concentrations for the DOC+, R+ treatment observed for the 4, 18 and 30 nm AuNPs were 16.8 ± 2.3, 12.9 ± 3.8, and 14.0 ± 4.5 mg Au/kg, respectively, were similar to the tissue gold concentrations for DOC+, R- treatment of 14.8 ± 4.6, 15.6 ± 8.3, and 8.7 ± 7.0 mg Au/kg for the same size AuNPs.

Roots and DOC played an important role regulating *A. caroliniana* gold tissue concentrations. With the 4 nm AuNP, DOC- treatment, the removal of roots (R-) resulted in the largest decrease in tissue concentration from the R+ treatment (145.5 ± 45.5, to 9.0 ± 1.5 mg Au/kg) indicating roots are the primary absorption mechanism of 4 nm AuNPs in this species, (Figure 3.3A and 3.3D). When only *A. caroliniana* shoots were exposed to each size AuNP in the DOC- treatment, tissue concentrations do not significantly change, representing non-size specific adsorption to the leaf surfaces (9.0 ± 1.5, 15.8 ± 4.7, and 13.8 ± 1.0 mg Au/kg for 4, 18 and 30 nm AuNPs, respectively). For both the 18 and 30 nm AuNPs in the DOC- treatment, a decrease in tissue concentration can be observed with the absence of roots. The R+ treatment resulted in concentrations of 47.1 ± 5.3 mg Au/kg and 36.4 ± 5.3 mg Au/kg, which were significantly higher than the R- treatment (15.8 ± 4.7 mg Au/kg and 13.8 ± 1.0 mg Au/kg) for 18 and 30 nm AuNPs, respectively.
Comparing the DOC- and DOC+ concentrations for *A. caroliniana* (Figure 3.3A and 3.3D), the addition of DOC resulted in decreased Au tissue concentration, even with roots present. In the 4 nm AuNP, DOC+, R+ treatment for *A. caroliniana*, the addition of DOC resulted in a decreased tissue concentration from the DOC- treatment, 145.5 ± 45.4 mg Au/kg to 33.8 ± 11.4 mg Au/kg. In the DOC+, R+ treatment, AuNP size effect on tissue concentration was mitigated by the presence of DOC, as tissue gold concentrations were not significantly different for the DOC+, R+ treatment (33.8 ± 11.3, 52.4 ± 7.5, 48.1 ± 19.1 mg Au/kg) for the 4, 18 and 30 nm treatments, respectively. In the DOC+, R- treatment a further decrease in tissue concentration occurred with the removal of roots to 20.3 ± 1.0, 20.3 ± 6, and 14.8 ± 2.0 mg Au/kg for the 4, 18 and 30 nm treatments, respectively. This difference in tissue gold concentration was most likely due to the decrease in adsorption sites for the AuNPs to attach. Comparing only the 18 and 30 nm AuNPs the presence of DOC did not reduce tissue concentrations of gold (Figure 3.3A and 3.3D). The highest concentrations of gold in *A. caroliniana* tissue were reported in the DOC- and R+ treatment.

*Organic matter interactions*

Further evaluating these factors, it is important to note that aquatic plants are typically found in shallow, high productivity, eutrophic water systems high in natural organic matter. Investigating the interactions between AuNP sizes and dissolved organic carbon, it was found through TEM characterization that the 4
nm AuNPs associated with DOC in the highest numbers followed by the 18 nm AuNPs. The 30 nm AuNPs did not associate closely with the DOC (Figure 3.4C).

Comparing the size measurements of the 4 nm AuNPs in the DOC- (10.0 ± 5.0 nm) and DOC+ (5.6 ± 1.8 nm) results indicated that the presence of DOC reduced particle core aggregation after 24 h. Even though the particle core aggregation was reduced, the DOC/AuNP association became much larger than individual AuNPs (Figure 3.4). In the 18 nm AuNP treatment, comparing the size range in the DOC- (23.3 ± 12.5 nm) and DOC+ (18.4 ± 8.4 nm) treatments, a reduction in particle polydispersity and an average particle core size was observed (Figure 3.1).

The presence of NOM can reduce particle aggregation by sterically hindering particle-particle interactions as a result of pH change, increased ionic strength, or from the presence of divalent cations, all causing a decrease in particle stability [28-30]. The absorption of compounds by organic matter is not uncommon. Organic matter is comprised of various aliphatic and aromatic components that give rise to hydrophobic, and hydrophilic regions. Organic matter primarily consists of polysaccharides and peptides as well as fulvic and humic acids that are derived from the breakdown of plant material and microbial degradation products [19]. Organic matter has been shown to act as a chelator reducing toxicity of metal ions and pesticides. Organic matter has also been shown to decrease the toxicity of silver nanoparticles to bacteria [31]. Also organic matter is a known chelator of organic molecules, altering the toxicity and
bioavailability of many pesticides [32,33]. The DOC/AuNP association that is formed is hypothesized to be responsible for the decreased tissue gold concentrations observed.

Mechanism of tissue concentration reduction

The presence of DOC reduced the bioavailability of the 4 nm AuNPs nearly four-fold in *A. caroliniana* (Figure 3.3D). For *A. caroliniana*, the presence of DOC mitigated AuNP size effect on tissue gold concentration. Hence, the presence of DOC drastically reduced the ability of the 4 nm AuNPs to be absorbed due to the increased size of the AuNP/DOC association (Figure 3.4). The surface adsorption of AuNPs was expected, as indicated by the presence of gold in all species, but the significant decrease that resulted from the addition of DOC to the *A. caroliniana* treatment further supports decreased AuNP absorption. Although addition of DOC resulted in the largest change in tissue gold concentration for the 4 nm *A. caroliniana* treatments, the general trend in treatments with DOC indicated a reduced tissue gold concentration for each treatment.

Figure 3.5 shows a high-resolution electron micrograph detailing the surface interactions 18 nm AuNPs have in the DOC+ treatment. Note that the presence of organic carbon is closely associated with the AuNP core surface. Supporting this interaction, Figure 3.6 indicates that the DOC completely encapsulates the AuNPs. Using STEM microscopy, the same sample was observed under scanning electron and elemental contrast microscopy
simultaneously. AuNPs were present and visible as white structures within the DOC+ treatment using elemental contrast, however under scanning electron microscopy, observing only the surface, the AuNPs are observed covered in a layer of DOC (Figure 3.6). The formation of this larger association of AuNPs and DOC resulted in decreased tissue concentrations due to the association being too large for absorption.

**Conclusions**

These results indicate that AuNPs interact with aquatic plants and bioaccumulate in the tissues. This bioaccumulation is not only affected by water quality parameters, such as ionic strength, hardness and pH, but also by the presence of natural organic matter. The absorption of AuNPs is species specific, and dependent on the presence of roots for *A. caroliniana*. In any case, significant gold tissue concentrations for all treatments were found. Regardless of absorption, the need for ecotoxicity and transport studies of NMs in the aquatic environment is needed. The mechanism of uptake, factors that influence the bioavailability, and the potential for trophic transfer of AuNPs in and potentially out of the aquatic ecosystem still need more attention.
Tables:

**Table 3.1: Characterization averages for gold nanoparticles in each exposure treatment.**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>ζ –suspension * (mV)</th>
<th>TEM diameter ** (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock</strong></td>
<td>Initial/ (24 h)</td>
<td>Initial/ (24 h)</td>
</tr>
<tr>
<td>4 nm</td>
<td>-24.4 ± 8.2 / (n/a)</td>
<td>5.2 ± 2.0 / (n/a)</td>
</tr>
<tr>
<td>18 nm</td>
<td>-35.2 ± 4.4 / (n/a)</td>
<td>18.1 ± 5.6 / (n/a)</td>
</tr>
<tr>
<td>30 nm</td>
<td>-37.2 ± 1.6 / (n/a)</td>
<td>27.0 ± 8.0 / (n/a)</td>
</tr>
<tr>
<td><strong>Well water</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 nm</td>
<td>-16.7 ± 1.8 / (-25.8 ± 0.9)</td>
<td>8.0 ± 6.0 / (10.0 ± 5.0)</td>
</tr>
<tr>
<td>18 nm</td>
<td>-17.8 ± 2.9 / (-26.8 ± 4.1)</td>
<td>17.5 ± 2.4 / (23.3 ± 12.5)</td>
</tr>
<tr>
<td>30 nm</td>
<td>-23.7 ± 4.2 / (-24.9 ± 3.4)</td>
<td>25.0 ± 7.0 / (39.1 ± 8.0)</td>
</tr>
<tr>
<td><strong>Well water / DOC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 nm</td>
<td>-19.4 ± 2.1 / (-25.7 ± 1.1)</td>
<td>6.6 ± 3.6 / (5.6 ± 1.8)</td>
</tr>
<tr>
<td>18 nm</td>
<td>-18.5 ± 3.5 / (-19.2 ± 4.7)</td>
<td>20.2 ± 6.6 / (18.4 ± 8.4)</td>
</tr>
<tr>
<td>30 nm</td>
<td>-28.4 ± 3.6 / (-29.0 ± 7.0)</td>
<td>34.1 ± 7.8 / (42.3 ± 8.3)</td>
</tr>
</tbody>
</table>

*Zeta n=3, (mV ± std dev) pH 7.1, ** n=250-1200 particles
Figures:

**Figure 3.1: Gold nanoparticle characterization: gold core size distribution in each treatment over initial and 24 hour time period.**

Box graphs of gold nanoparticle core diameter in nanometers overlaid with water treatment and sampling time. \( n = 250-1200 \) particles per treatment. Outlier points represent higher particle polydispersity. Polydispersity increased in 30 nm, DOC, 24h treatment. Core diameter measured with transmission electron microscopy (Hitachi H-7600), and core diameters analyzed using ImageJ particle analysis tool. DOC = contains dissolved organic carbon, WW = well water, stock = undiluted nanoparticle stock suspension, initial= time 0 suspension, 24h= 24 hours post suspension. Tukey’s HSD post hoc test was used to separate the means. Means not connected by the same letter indicate significant differences (P<0.0001).
Figure 3.2: Gold nanoparticle characterization: electrophoretic mobility measurements in each treatment over initial and 24 hour time period. Gold nanoparticle electrophoretic mobility measurements of each size, water treatment (pH 7.1) and sampling time (n=3). Initial and 24h measurements indicate all AuNP treatment suspensions were moderately stable over 24h exposure. Stock 18 and 30 nm AuNPs were most stable. DOC = contains dissolved organic carbon, WW = well water, stock = undiluted nanoparticle stock suspension, initial= time 0 suspension, 24h= 24 hours post suspension. Fisher’s LSD post hoc test was used to separate means. Means not connected by same letter are significantly different (P=0.0205).
Figure 3.3: Tissue concentration graphs of complete factorial design aquatic plant study. The graph headings indicate plant species. DOC – (A, B, C) indicates exposure suspension in only well water (0.80 ± 0.02 mg Carbon/L). The DOC + (D, E, F) indicates the addition of Suwannee river dissolved organic carbon (2.0 ± 0.4 mg Carbon/L). X-axis shows the gold nanoparticle sizes in nanometers for each treatment. Gray bars indicate the presence of roots (R+) and white bars indicate roots removed (R-) treatments. Error bars indicate standard deviation of the mean (n=3). Statistical analysis letters are only comparable between same plant species graphs, A and D, B and E, C and F. Fischer’s LSD post hoc test was used to separate the means. Means not connected by the same letter within each species are significantly different (P<0.0001).
Figure 3.4: Transmission electron micrographs of gold nanoparticles associated with Suwannee River dissolved organic carbon.  (A) Represents 4 nm gold nanoparticles, (B) Represents 18 nm gold nanoparticles and (C) Represents 30 nm gold nanoparticles. Scale bar represents 100 nm.
Figure 3.5: High-resolution transmission electron micrograph of 18 nm gold nanoparticles. (A) Shows 18 nm gold nanoparticles associated with Suwannee River dissolved organic carbon. (B) Shows 18 nm gold nanoparticle stock suspension without any dissolved organic carbon present. Note the white arrows indicate the close association of dissolved organic carbon with the particle core. Scale bar = 5 nm.
Figure 3.6: Interactions of 18 nm gold nanoparticles with dissolved organic carbon. Micrographs (A) and (B) were taken of 18 nm gold nanoparticles exposed to Suwannee River dissolved organic matter in the same sample and location using two microscopy techniques. (A) Represents scanning electron microscopy (surface only) and (B) shows elemental contrast microscopy (penetrates through sample). (A) Shows dimensional structure of dissolved organic matter, encapsulating the 18 nm gold nanoparticles as indicated by the circle. (B) Shows the elemental contrast of the same area, showing the gold nanoparticles within the dissolved organic matter. Note that images are superimposable, and indicate the DOC/AuNP association is much larger than individual particles.
References:


CHAPTER 4:

ROOT STRUCTURE AND FUNCTION: AQUATIC MACROPHYTE’S ROOTS PLAY KEY ROLE IN DETERMINING SIZE DEPENDENT GOLD NANOPARTICLE ABSORPTION

Introduction

The implications that engineered nanomaterials (NMs) may have within the environment have been the focus of many research studies. Engineered NMs can be found in many consumer products, and as a result their entrance into the environment is unavoidable. Several researchers have documented the potential for nanomaterials to enter the aquatic environment [1-3]. Engineered nanomaterials have been detected in wastewater effluent and are present in sewage sludge [4,5]. Kim et al [4] documented the presence of silver nanomaterials in sludge from a large-scale wastewater treatment plant (WWTP) indicating that WWTPs are the main link between anthropogenic Ag and the environment. Metal oxide nanomaterials such as TiO₂ and ZnO have also been detected in WWTP sewage sludge [6]. As a part of routine operation of WWTPs, excess sludge is removed. In a Federal Register Notice entitled Standards for the Use or Disposal of Sewage Sludge [7], the United States Environmental Protection Agency indicates that 33.3% of annual sludge generation is disposed through land application, 16.1% is incinerated, 33.9% is disposed in landfills and 10.3% is placed in surface disposal lagoons. Other estimates for the land application of sludge as soil amendments have been reported over 60% [6].
Although NMs have been observed to enter the aquatic and terrestrial ecosystems through effluent and sludge, only recently have studies looked at tracking NMs fate within higher plants [8-12]. Current literature indicates that NMs interact with terrestrial and aquatic species, with varying results. Glenn et al. [13] observed size and species dependent absorption when investigating exposure of gold nanoparticles (AuNPs) to aquatic macrophytes. Glenn et al. [13] exposed *Azolla caroliniana*, *Egeria densa* and *Myriophyllum simulans* to 4 and 18 nm AuNPs with a citrate surface chemistry. Results indicated that *Azolla caroliniana* had the highest tissue concentrations of 4 nm AuNPs, while *E. densa* had the lowest tissue concentrations. Electron microscopy confirmed the presence of 4 nm AuNPs within the roots of *A. caroliniana*; however, it was not determined if these AuNPs were located within the cell wall apoplast or if they were intracellular. Glenn et al. [13] suggested that salt tolerance of each species might correlate with AuNP absorption by influencing the cell wall and membrane permeability. Although this correlation may exist, results from this current study indicate that it is likely that NMs passively diffused into the apoplastic region of the root tissue through the microfibrils network “pore” structures.

The pore structures are defined as the overlapping space created by the layering of cellulose microfibrils. These pore sizes can be estimated by several methods including solute exclusion techniques, NMR spectroscopy, and direct visualization using various electron microscopy techniques [14-16]. These
openings or gaps in the microfibrils network range from 3 to 20 nm in diameter. In some cases, such structures have been measured near 50 nm [17].

Sabo-Attwood et al. [2] investigated the uptake and distribution of 3.5 and 18 nm AuNPs with citrate surface chemistry in hydroponically grown *Nicotiana xanthi* seedlings. Uptake of AuNPs was size dependent, with only the 3.5 nm AuNPs detected in leaves and roots. Clusters of nanoparticles were discovered in the cell cytoplasm, indicating that true uptake of NMs occurred in the 3.5 nm exposure. These results are not surprising, however, as the exposure concentrations (48 mg/L) most likely drove the diffusion of these AuNPs across the cell wall and plasma membrane. However, 18 nm AuNPs did not penetrate into the root tissues, even at an exposure concentration of 76 mg/L, which indicated that plant root structures can be size selective, even if concentration gradients would favor the diffusion of AuNPs from suspension.

The mechanisms of non-specific NMs uptake and/or exclusion are still uncertain [11]. Glenn and Klaine [18] characterized AuNP absorption in roots of *A. caroliniana, E. densa* and *M. simulans* and observed that plant species, NM size, dissolved organic carbon, and the presence of roots all contributed to the overall Au tissue concentration measured. Because roots played a vital role in the absorption of AuNPs by *A. caroliniana*, further investigation of root structures was needed to determine how AuNPs interact with the cell walls of each of the three aquatic plant species.
The objective of this study was to identify and characterize the role of root structure in the size and species dependent absorption of AuNPs. This was achieved by electron microscopy imaging, as well as measuring tissue gold concentrations after exposure to 4, 18, and 30 nm AuNPs in two treatments, which, consisted of control or wounded root organs. Although electron microscopy sample preparation can cause loss of NMs due to extensive sample preparation, it is still an important tool for understanding NMs interactions with plant samples [18,19]. Further, by utilizing solute exclusion methods proposed by Carpita et al. [14] the porosity of A. caroliniana roots was further investigated, giving indication that root structure is key in defining size and species dependent AuNP absorption.

**Materials and Methods**

*Nanoparticle synthesis and characterization*

Four, 18, and 30 nm AuNP spheres were synthesized following the Turkevich and related Frens method at the Clemson University Institute of Environmental Toxicology, Pendleton, SC [20,21]. Detailed synthesis methods are described in the methods of Chapter 3.

*Plant species*

Plant species were chosen based off of leaf morphology and growth habitat as outlined in Chapter 2. Aquatic macrophytes used were cultured in a double-layer, polyethylene-covered greenhouse in Pendleton, SC (25º ± 5º C, natural light/dark cycle). All plants selected for this study appeared healthy and
had no visible periphyton growth. Plant cultures were not axenic. Plants were rinsed vigorously with distilled water before experimental use. Experiments were conducted in the greenhouse during June and July 2013.

Microscopy

Twenty-millimeter root tip sections were removed from each plant species and placed into a 3.0% buffered glutaraldehyde (pH 7.1) solution overnight in 1.5 mL centrifuge tubes. After roots were fixed, they were rinsed in 0.2M cacodylate buffer at pH 7.1 for 15 minutes, dehydrated in ethyl alcohol diluted with ultra pure water stepwise at 50, 70, 80, and 95% for 15 minutes each, then 100% ethyl alcohol for 30 minutes. Sections where rinsed with a 1:1 ethyl alcohol and L.R. White resin for 15 minutes on a nutating mixer. After rinsing with diluted resin, sections were placed into fresh L.R. White resin and placed back on the nutating mixer. After 15 minutes of mixing, sections were transferred into BEEM® capsules with fresh L.R. White resin and polymerization occurred at 60ºC in a drying oven overnight.

After the embedding process was complete, samples were sectioned using a DiATOME diamond knife on a Reichert Ultramicrotome 90–100 nm thick and captured on 200-mesh carbon formvar™ copper grids (type FCF200-Cu). To enhance contrast of root cell walls, samples were post-stained with a two-step procedure of uranyl acetate and lead citrate. Microscopy samples were viewed on Hitachi 7600 transmission electron microscope (TEM) at 120 kV (Clemson University Electron Microscope Facility, Anderson, SC).
**Experimental design**

The experimental design was a 3 x 2 x 3 randomized complete block design. Three aquatic plant species (*M. simulans, E. densa* and *A. caroliniana*) with roots wounded or intact, were exposed to three AuNP sizes (4, 18 and 30 nm) in a static exposure over 48 h with a nominal exposure concentration of 250 µg Au/L. In order to wound the root structure, roots were cut at a 45° angle prior to exposure. The control roots remained uncut in their intact, normal structure.

*Myriophyllum simulans* and *E. densa* were exposed in 70 mL glass test tubes. *Azolla caroliniana* was exposed in 30 mL glass beakers. Before exposure glassware was acetone and acid washed (10% nitric). After harvest, plants were rinsed 10 times via submersion in distilled water. Roots and shoots were carefully separated and the final dry tissue weight (g) was recorded after 24 h of drying at 60°C in a drying oven. Only the concentration of Au in root tissues are presented, as root uptake was of primary interest in this study.

**Gold analysis**

Root tissues were digested using a modified method from Anderson et al. [22]. Dried tissue was transferred to 20 mL cleaned ceramic crucibles and was heated to 530°C for 14 h in a muffle furnace to ash samples. Crucibles were cleaned with full strength aqua regia (1:3 nitric to hydrochloric, trace metal grade acids). Once cooled, tissue was digested with aqua regia, and diluted to achieve 5.0% volume acid for analysis using ICP-MS (ThermoScientific Xseries2).
Solute Assay setup

The capillary diameters of living *A. caroliniana* root tissue were measured following the methods of Carpita et al. [14]. *Azolla caroliniana* root cells were observed for signs of cytorrhysis due to increasing molecular size of polyethylene glycols in solution. Solutions of 0.2 M sucrose and polyethylene glycol (PEG) 200, 400, 600, 1000, 1450, 4000 and 6000 were used. Sucrose molecular diameter was 1 nm, and PEG diameters are 1.3, 2.2, 2.9, 3.5, 3.8, 4.5 and 5.2 nm, respectively. *Azolla caroliniana* roots and cytorrhysis response were imaged using a Nikon LV-UDM light microscope (Clemson Light Microscopy Facility, Clemson, SC USA.)

Data analysis

A standard least squares analysis of main effects (plant species, plant root treatment, and particle size) was conducted using JMP v10.0 (SAS Institute Inc. Cary, NC). Analysis of variance indicated a significant difference between main effects in tissue gold concentrations, root treatment (cut or wounded) and gold nanoparticle size treatment (4, 18 or 30 nm) $P<0.0005$. Fisher’s LSD post hoc test was used to separate means, indicating that root wounding only, had no significant effect on tissue gold concentrations, $P=0.6391$. Size dependent absorption still existed among each species, $P<0.0001$. 
Results and Discussion

Root Wounding

Root wounding was proposed as a likely pathway for NMs entry into plant roots [23,24]. Large openings that disrupted the cell wall seemed a plausible mechanism for root absorption of NM. However, in this study root wounding did not enhance Au uptake into roots (Table 4.1). Root wounding had no effect on tissue concentrations. Tissue concentrations between wounded and normal roots were not significant ($p >0.1075$) indicating wounding had no effect on bulk nanomaterial concentration ($\alpha = 0.05$). However, it is possible, that in isolated instances larger NM may enter the wounded area of root tissue.

Species dependent response was maintained with exposure to AuNPs. *Azolla caroliniana* exposed to 4 nm AuNPs had the highest average tissue concentrations of all species tested, at 50.2 ± 19.0 mg Au/kg dry tissue, *M. simulans* (20.6 ± 12.2 mg Au/kg dry tissue) and *E. densa* had the lowest tissue concentrations at 21.3 ± 11.5 mg Au/kg dry tissue (Table 4.1). In *A. caroliniana*, tissue concentrations in the 4 nm AuNP intact and cut roots (44.5 ± 14.6 and 55.9 ± 23.4 µg Au/kg dry tissue) were higher than those of the 30 nm AuNP (25.9 ± 8.69 and 26.2 ± 9.68 µg Au/kg dry tissue). Comparing the wounded and normal root treatments between each size AuNP and each species, revealed that wounding the root tissue did not enhance tissue concentrations (Table 4.1). Cutting or wounding the root did not disrupt the size dependent absorption of AuNPs and did not increase absorption of AuNPs via bulk flow.
Plants respond to root wounding events in several ways, however, a common response is the release of root exudates. Plant root exudates encompass a wide variety of compounds. These compounds include amino acids, organic acids, sugars, glycosides, vitamins, enzymatic nucleotides, as well as inorganic ions [25,26]. Citrate, an organic acid, can stabilize and bind to nanomaterials. Glenn and Klaine [18] reported that 4 and 18 nm AuNPs associate very strongly with organic matter, which is comprised of several complex fulvic and humic acid components, as well as complex sugar moieties. The AuNPs sorb to the organic matter creating a larger association that reduces the bioavailability of AuNPs to aquatic macrophytes. Plants typically heal quickly after initial injury via lignification and suberization of the injured cell [27]. Root injury could potentially limit uptake of compounds by root tissues by either binding NMs at the wound entrance with organic acid exudates or the scar tissue forming less penetrable tissue.

**Observations of NMs absorption**

True uptake is defined when a material encounters the cell wall, travels to the plasma membrane and then enters into the symplastic region (intracellular). A nanomaterial could enter extracellular cell wall space of the root, however, and never cross the plasma membrane and enter into cells. The xylem is also non-living tissue, and NMs can enter into the xylem from the apoplastic region and undergo translocation without ever entering into living cells. Miralles et al. [12] indicated that NMs are likely absorbed with moisture and nutrients. This
observation has also been documented in many plant studies [8,28,29,30]. However, it is unlikely that these NMs follow the exact pathway of traditional nutrient and water absorption. For example, typical diameters of Aquaporins are 3.8 angstroms [31], and highly selective membrane bound transporters for nutrients are not a likely pathway for bulk non-specific nanomaterial movement unless the NM posses surface chemistry that would be selective to specific transporters.

It is important to note that surface chemistry has long been known as an important factor with regards to the cell localization of gold colloids. The importance of AuNP surface chemistry has been documented and used as contrast agents in electron microscopy during sample preparation. The development of immunolabelling gold colloids has been used since the early 1970s. In this process 1-15 nm spherical gold particles are coated with immunoglobulin proteins that are specific for binding regions within the cell structure [32,33]. Although this technique is applied after initial sample preparation, it would be beneficial to investigate NM/plant interactions of particles with specific surface chemistries to target endocytic pathways, or intracellular structures to determine size dependent uptake properties unique to plants.

*Complexity of the cell wall & cell wall porosity*

The most commonly accepted mode of action for NM entrance into plant cells is cellular penetration [12]. Although there is much uncertainty around this uptake pathway [11], I hypothesized that the root cell wall acts as a non-specific
The cell wall is the first line of defense between the environment and plasma membrane, acting as a barrier as well as supporting structural and organizational roles. The cell wall is composed of cellulose, hemicellulose, pectins and polysaccharides [31]. Generally, cellulose microfibrils have a net negative charge [34]; yet, some studies reported that the cell wall structure has pockets of both charged and neutral areas [34]. The charge of these pocket areas may be either positive or negative, depending on the redox state of the polysaccharides or organic molecules that are present [36].

Cellulose is the primary constituent of the cell wall, and consists of long chains of D-glucose units joined together by β-1,4-glicosidic bonds that are tightly linked by hydrogen bonding to form a linear homobiopolymer that is referred to as a microfibril [35]. Microfibrils can be imaged utilizing high magnification electron microscopy, where the darker (more electron dense) areas of the cell wall micrograph indicate the presence of thicker, denser microfibril structures. The cell wall structure of intact roots of *A. caroliniana, M. simulans* and *E. densa* were imaged to investigate if morphological differences existed among species, and the density gradients of the external cell wall for each plant species are shown in Figure 4.1. The cell walls of *A. caroliniana* are the least dense and thinnest width of each species, followed by *M. simulans* and *E. densa*. *Egeria densa* contained the most dense microfibril network, perhaps due to the root structure being adventitious and developed for support and attachment rather than nutrient uptake [37]. After exposure to AuNPs for 24 h, the root
structure of each species was investigated using electron microscopy (Figure 4.2). Each size AuNP interacted closely with the external cell wall. Cell wall structure limited the uptake of larger particles (30 nm AuNP) with all species. Four nanometer AuNPs have passed through the cell wall of _A. caroliniana_ as indicated by the white arrow in Figure 4.2, A-4.

**Size and species dependent uptake of AuNPs**

A study by Judy et al. [24] investigated _Nicotiana tabacum_ L. ‘Xanthi’ (tobacco) and _Triticum aestivum_ (wheat) exposed to 10, 30 and 50 nm AuNPs with a citrate or tannate surface chemistry. Particle size limited NM bioavailability, as no 10, 30 or 50 nm AuNPs penetrated the root structure of wheat. The authors also reported that the surface chemistry rather than AuNP diameter determined its capacity to translocate within the tobacco plant and accumulate in the leaf tissues. In fact, 50 nm AuNPs with a citrate surface chemistry were present in leaf tissues at higher concentrations than the 50 nm AuNPs with tannate surface chemistry. These data conflicted with reported pore values believed to determine NM sizes that are capable of penetrating root cells. However, the factors that contributed to Au detected in the leaf tissue remain uncertain, including exposure concentrations. It is possible that further investigating tobacco plant root structure would indicate the potential for larger sized AuNPs to penetrate through the cell wall and be absorbed and translocated by these tobacco plants.
Azolla caroliniana has previously been observed to selectively absorb 4 nm AuNPs from suspension, but exclude 30 nm AuNP [13,18]. To investigate this size dependent absorption, root cell wall porosity was measured using the solute exclusion technique [14]. Cell wall exclusion limits are assigned by the ability of progressively larger solutes to osmotically induce cytorrhysis (cell wall collapse). By adding solutes of known molecular weights and diameters, the diameter of the pores can be estimated by observing the shape change of the plant cell. If the molecules in the solute become too large to penetrate the cell wall, osmotic pressure will collapse the cell wall, known as cytorrhysis. When A. caroliniana roots were placed in a solution of PEG 4000, cytorrhysis occurred (Figure 4.3D). As determined by Carpita et al. [14], this collapse correlated with the solutes being too large to cross the cell wall, resulting in the collapse of the cell from water leaving the cell structure. If the solutes can diffuse across the cell wall to the plasma membrane, then the structure will reach equilibrium and no collapse will be observed. Although Chara corilliana, the original plant upon which this size exclusion technique was studied, had much smaller pore structures (2.1 nm) [38], results from A. caroliniana were similar to those reported by Carpita et al. [14]. This solute exclusion technique can be used to estimate the maximum size molecules that can easily diffuse across a plant cell wall by observing qualitative changes in cell wall configuration. The collapse and shrinkage of the A. caroliniana root structure after exposure to PEG 4000 correlate with an approximant pore size of 4.5 nm. These results concur with the
observed size dependent uptake of 4 nm AuNPs and exclusion of larger AuNPs as seen with the current study. Thus it was likely that the cell wall defined the size dependent uptake observed in the Glenn et al. [13] study when salinity tolerance was hypothesized as the mechanism driving AuNP uptake.

**Conclusions**

Although root wounding may allow larger NM to enter plant cells in isolated instances, it did not explain bulk flow of NMs absorption. Absorption of NM was limited by NM size and plant species, even when root wounding occurred. This size selective filtering correlated with the structure of the cell wall, with the least dense cell wall (*A. caroliniana*) showing the highest tissue concentrations for the 4 nm AuNP treatment. This size selectivity is further supported by the solute exclusion of PEGs larger than 4.5 nm. Electron microscopy was beneficial for observing differences among the three aquatic plant cell wall densities and thickness. Differing cell wall density helped to explain the species dependent absorption observed due to the physical/structural differences in the construction of cell walls. Further research is needed to determine the driving force behind absorption of NM and to determine the factors that influence the bulk flow of NMs within aquatic plants.
Figures:

Table 4.1: Tissue concentration of gold present in normal and wounded roots.

<table>
<thead>
<tr>
<th>Species*</th>
<th>AuNP Size (nm)</th>
<th>Root Normal (mg Au/kg dry tissue)</th>
<th>Root Cut** (mg Au/kg dry tissue)</th>
<th>Root Normal / Cut Comparison (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. caroliniana</td>
<td>4</td>
<td>44.5 ± 14.6</td>
<td>55.9 ± 23.4</td>
<td>0.2179</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>41.4 ± 23.7</td>
<td>27.8 ± 8.4</td>
<td>0.1468</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>25.9 ± 8.7</td>
<td>26.2 ± 9.7</td>
<td>0.9744</td>
</tr>
<tr>
<td>M. simulans</td>
<td>4</td>
<td>30.5 ± 13.7</td>
<td>10.8 ± 3.7</td>
<td>0.0693</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>15.04 ± 7.4</td>
<td>10.5 ± 1.0</td>
<td>0.6703</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>9.0 ± 3.0</td>
<td>17.2 ± 3.0</td>
<td>0.4427</td>
</tr>
<tr>
<td>E. densa</td>
<td>4</td>
<td>25.0 ± 13.3</td>
<td>17.6 ± 9.7</td>
<td>0.4817</td>
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<tr>
<td></td>
<td>18</td>
<td>19.6 ± 7.5</td>
<td>18.0 ± 4.6</td>
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<tr>
<td></td>
<td>30</td>
<td>31.77 ± 12.7</td>
<td>38.6 ± 19.9</td>
<td>0.5236</td>
</tr>
</tbody>
</table>

*n = 5, **Roots were cut prior to exposure at 45°
Figure 4.1: Transmission electron micrographs of the external root cell wall membranes of aquatic plants. Micrographs show varying thickness and density of microfibrils. The thinnest, least dense is (A) *Azolla caroliniana* followed by (B) *Myriophyllum simulans*, and (C) *Egeria densa* the densest of the species. IE= internal environment. CW= cell wall. EE= external environment. Scale bar = 100 nm.
Figure 4.2: Transmission electron micrographs of gold nanoparticles associated with the external root cell wall. (A) Azolla caroliniana, (M) Myriophyllum simulans and (E) Egeria densa exposed to (-4), (-18) or (-30) nanometer citrate capped gold nanoparticles as indicated by the white arrows. EE= External environment; CW=Cell wall; IE= Internal environment. Scale bars are equal to 100 nm.
Figure 4.3: Light micrographs of solute pore analysis study: *Azolla caroliniana* root tissue. Morphological changes were monitored for each window. Each window represents a separate living *A. caroliniana* root after exposure to the following treatments: (A) Indicates control root with no solutes present. (B) Represents root structure exposed to 0.2M solution of sucrose. (C) Represents root structure exposed to 0.2M solution of polyethylene glycol (PEG) 200, 2.1 nm diameter. (D) Represents root structure exposed to 0.2M PEG 4000, 4.5 nm in diameter. (D) Represents root structure cell wall collapse (cytorrhysis) due to PEG molecules being larger than cell wall pore structures. Scale bar = 20 µm.
References:


CHAPTER 5: EVAPOTRANSPIRATION EFFECTS ON GOLD NANOPARTICLE ABSORPTION INTO THE ROOT APOPLAST

Introduction

Many researchers have investigated the fate of nanomaterials (NMs). Current production of nano TiO$_2$ has been estimated to be 250,000 metric tons per year, however, production is expected to exceed 1 million metric tons by 2023 [1]. TiO$_2$ nanoparticles have been detected in effluent, sludge and surface waters [2]. With rising production of NMs, there is no doubt that release of NMs into the environment will occur and increase. Although several studies have evaluated the interaction of NMs with terrestrial plants, very few studies investigated their interactions with aquatic macrophytes. Although terrestrial plant research serves as a risk assessment dataset for potential human exposure through food, a more robust dataset is needed with regards to ecotoxicological fate and effects that NMs may have within the aquatic environment.

In 2008, the need for NM and plant based studies was documented by multiple researchers [3-5]. Since then, only a handful of aquatic studies have been published, most focused on toxicity endpoints such as growth, reactive oxygen species production and other biochemical pathways [6,7]. Few studies exist that are focused on the fate, rather than toxicity, of NMs within plant systems. The use of gold nanoparticles (AuNPs) as a model for tracking distribution within a plant system, however, has been documented [8]. Although studies on toxic end points are important in understanding plant response and for grounding development of regulations, understanding the factors that control NM
uptake and fate will be critical in determining classes and characteristics of NMs that may pose higher risks.

Glenn and Klaine [9] investigated how NM size, plant species, the presence of roots and dissolved organic carbon controlled AuNP bioavailability to aquatic plants. Roots played an important role in the uptake of 4 nm AuNPs, and the presence of dissolved organic carbon reduced the bioavailability of AuNPs. Aquatic macrophyte roots were considered support structures only and thought to have little function in nutrient movement. However, the current paradigm is that the major source of nutrients for the aquatic plant is from the sediment [10]. Because aquatic plant roots are functional, the translocation of nanomaterials warrants further investigation as bioaccumulation can give rise to nanomaterial cycling and potential movement into higher trophic level organisms. Judy et al. [11], observed the transfer of AuNPs into higher trophic levels. Hornworms that fed on tobacco plants exposed to 5, 10 or 15 nm AuNPs accumulated gold into their tissue, indicating the ability of NMs to bioaccumulate.

Nanomaterial size plays a critical role in their bioavailability to plants. *Azolla caroliniana*, a freshwater aquatic fern, has been documented to absorb and bioconcentrate 4 nm AuNPs from suspension [13]. It has also been shown that 5 nm TiO\textsubscript{2} nanoparticles have penetrated the cell walls of *Arabidopsis thaliana* [12]. Koelmel et al. [8] investigated the uptake and distribution of 2 nm AuNPs with three distinct surface chemistries (positive, neutral and negatively
charged) in the roots and shoots of rice, and reported that rice bioaccumulated AuNPs with organ level distribution, dependent on surface chemistry.

A recent study by Paulik et al. [14] investigated the translocation potential of 4 nm AuNP-exposed *Myriophyllum simulans* and *Azolla caroliniana*. Paulik et al. concluded that the presence of an evapotranspiration stream, or the movement of water from roots to shoots by evapotranspiration, might influence tissue concentrations in the shoots and leaves of the aquatic macrophytes, resulting in higher tissue concentrations as well as the potential for trophic transfer.

In order to better determine the effect of evapotranspiration, *A. caroliniana* and *M. aquaticum*, a species closely related to *M. simulans* was investigated. *Myriophyllum aquaticum* has a dimorphic leaf structure. The dimorphic leaf structure occurs when the plant shoot breaks the water surface and becomes emergent. At this time, the development of stomata occurs, resulting in the presence of an evapotranspiration stream [15]. *Azolla caroliniana* is a free-floating aquatic macrophyte that undergoes evapotranspiration [16].

The goal of my study was to investigate the effects of evapotranspiration rate upon AuNP root loading and translocation of 4 nm citrate AuNPs.

**Materials and Methods**

*Nanoparticle synthesis and characterization*

Four nanometer (nm) gold nanoparticle spheres (AuNPs) were synthesized following the Turkevich and related Frens method at the Clemson
University Institute of Environmental Toxicology, Pendleton, SC [17,18]. Four nanometer AuNPs were synthesized by combining 0.5 mL of 0.01M chloroauric acid with 19 mL of Milli-Q water, and 0.5 mL of 0.01M sodium citrate tribasic dihydrate. Afterward, 0.6 mL of 0.1M sodium borohydride was added to reduce the chloroauric acid completing the process. Size distribution of 4.86 ± 0.07 nm was measured utilizing transmission electron microscopy, \( n=1100 \) particles (Hitachi H-7600, 120kv, Clemson University Electron Microscope Facility, Anderson, SC). The particles used in this exposure were stable in suspension with a zeta potential of -25.8 ± 0.9 mV (Malvern Zetasizer Nano), consistent with the negative citrate surface chemistry at neutral pH.

**Plant species**

*Azolla caroliniana* was obtained from a constructed wetland in Cairo, GA. *Myriophyllum aquaticum* was obtained from a natural wetland in Anderson, SC. The aquatic macrophytes were cultured in a double-layer, polyethylene-covered greenhouse in Pendleton, SC (25º ± 5º C, 14:10 h light/dark cycle).

*Azolla caroliniana* were harvested from a mass culture. *Myriophyllum aquaticum* in the emergent phase were excised from rhizomes of mass culture plants. Individual uniform cuttings were grown in 70 mL test tubes in well water and were selected for exposure after rooting had occurred at approximately 21 days after cuttings were made. All plants selected for this study appeared healthy and had no visible periphyton growth. Plant cultures were not axenic.
Cultures were maintained, and experiments were conducted in the greenhouse during June and July 2013.

**Microscopy**

Twenty-millimeter root sections were removed from each plant species and placed into a 3.0% buffered glutaraldehyde (pH 7.1) solution overnight in 1.5 mL centrifuge tubes. After roots were fixed, they were rinsed in 0.2M cacodylate buffer at pH 7.1 for 15 minutes, dehydrated in ethyl alcohol diluted with ultra pure water stepwise at 50, 70, 80, and 95% for 15 minutes each, then 100% ethyl alcohol for 30 minutes. Sections were rinsed with a 1:1 ethyl alcohol and L.R. White resin for 15 minutes on a nutating mixer. After rinsing with diluted resin, sections were placed into fresh L.R. White resin and placed back on the nutating mixer. After 15 minutes, sections were transferred into BEEM® capsules with fresh L.R. White resin and polymerization occurred at 60º C in a drying oven overnight.

After the embedding process was complete, samples were sectioned using a DiATOME diamond knife on a Reichert Ultramicrotome. Samples were sectioned 90–100 nm thick and captured on 200-mesh carbon formvar™ copper grids (type FCF200-Cu). To enhance contrast of root cell walls, samples were post-stained with a two-step procedure of uranyl acetate and lead citrate. Microscopy samples were viewed on Hitachi H-7600 transmission electron microscope (TEM) at 120 kV (Clemson University Electron Microscope Facility, Anderson, SC).
Experimental design and setup

Experimental units were placed into three treatments of different relative humidity levels surrounding the plant. The three treatments consisted of fan, sealed, and controlled environments. In the fan treatment, experimental units were kept under constant wind generated by a circulating fan at 1550 rpm to increase evapotranspiration rate. The sealed treatment was kept covered with wax film to limit evapotranspiration. The control treatment was left uncovered, and was exposed to still open air over the course of the exposure. Experimental units were tested in triplicate, n=3.

Plants (M. aquaticum and A. caroliniana) were exposed to a nominal concentration of 250 µg Au/L over 16 days. Solutions were renewed every 48 h to maintain AuNP exposure concentrations. The AuNPs were suspended in 0.45 µm filtered (pall type A/E glass fiber filters) well water.

Myriophyllum aquaticum and A. caroliniana were exposed to AuNPs in 70 mL glass test tubes, referred to as experimental units. Only the roots of M. aquaticum were exposed to the AuNP suspension. Due to the growth habit of A. caroliniana, both roots and shoots were in contact with exposure suspension. Before experimental exposures, glassware was washed with acetone and acid (10% nitric). After harvest, plants were rinsed by submerging them in distilled water 10 times. Roots and shoots were carefully separated and the final dry tissue weight (g) was recorded after 24 h of drying at 60ºC in a drying oven.
Evapotranspiration measurements

The evapotranspiration rate was measured for each treatment and species ($n=4$) over the course of 96 h. Evapotranspiration is presented as mL H$_2$O per g of fresh tissue weight per day (24 h). Evapotranspiration was measured by recording the initial and final weight of the experimental units every 24 h to determine the amount of water lost in each treatment. Some water may have been lost due to surface evaporation from the individual experimental units, so results of this measurement were compared to repeated measurements of *M. aquaticum* evapotranspiration rates made using a potometer that consisted of a stem cutting, Tygon tubing and a burette. Water loss was recorded every 24 h and results from the potometer indicated similar evapotranspiration rates to those measured as a function of weight. Results represent average evapotranspiration in experimental conditions with low error associated with evaporation. Due to the structure of *A. caroliniana*, potometer measurements were not feasible.

Gold analysis

Tissue digestion was performed based on a modified method from Anderson et al. [19] that was observed to have high gold recovery from plant tissue. Dried tissue was transferred to 20 mL cleaned ceramic crucibles and was heated to 530°C for 14 h in a muffle furnace to ash and facilitate breakdown of plant cellulose and lignin components. Crucibles were cleaned with aqua regia. Once cooled, tissue was digested with aqua regia (1:3 nitric to hydrochloric trace metal grade acids), and diluted to achieve 5% volume acid for analysis using
ICP-MS (ThermoScientific Xseries2). Water samples were acidified to achieve 5% volume acid using *Aqua Regia*.

*Data analysis*

Analysis of variance revealed significant differences among main effects. To separate main effects, a full factorial, standard least squares analysis of plant treatments (Fan, Sealed, Control), tissue concentration and day was conducted using JMP v10.0 (SAS Institute Inc. Cary, NC). Fisher’s LSD post hoc test was used to separate treatment means (α < 0.05). Slope values and 95% confidence intervals were calculated using orthogonal contrasts of main effects (days 2 to 16).

*Results and Discussion*

*Evapotranspiration measurements*

The evapotranspiration rates of *A. caroliniana* were 3.9 ± 1.7 mL H₂O/g/24 h, 7.5 ± 3.9 mL H₂O/g/24 h and 0.4 ± 0.3 mL H₂O/g/24 h for the control, fan and sealed treatments, respectively (Figure 5.1). The highest evapotranspiration rates were recorded in the fan treatment for both *A. caroliniana* and *M. aquaticum*. Evapotranspiration rates for *M. aquaticum* were 3.9 ± 0.6 mL H₂O/g/24 h, 4.8 ± 2.4 mL H₂O/g/24 h and 0.2 ± 0.09 mL H₂O/g/24 h for the control, fan and sealed treatments, respectively. Wind from the fan disrupted the boundary layer of humidity around the plant foliage and increased water loss from stomata. By eliminating the boundary layer, a higher rate of water movement through the plant occurred.
Evapotranspiration effects on root Au concentration

Tissue concentrations of Au in A. caroliniana and M. aquaticum were separated by shoot and root (Figure 5.1). In all humidity treatments, the roots contained the highest concentrations of Au. For both plant species, concentration of Au in root tissues was highest for plants in the fan treatment.

The slope of each line in Figure 5.1 indicated the rate of change in Au concentration over time for both roots and shoots with the 95% confidence interval shaded around each line. Figure 5.1A shows the tissue concentrations observed in A. caroliniana. The slope of gold nanoparticle absorption was calculated for root tissue concentration measurements made over days 2 to 16. Each line equation is reported in Table 5.1. The slope, or rate of gold nanoparticle absorption by the roots per day (24 h), and the 95% confidence intervals, are as follows: 19.93 (13.31, 26.55) mg Au/kg dry tissue/24 h, 56.04 (49.42, 62.66) mg Au/kg dry tissue/24 h, and 36.52 (29.90, 43.14) mg Au/kg dry tissue/24 h in control, fan and sealed treatments, respectively. By comparing the root tissue accumulation rate to the control tissue accumulation rate in A. caroliniana, these data show that both the Fan > Control, and Sealed > Control. These rates of AuNP root loading indicate an increased accumulation rate of 18.83 (12.16, 25.50) mg Au/kg dry tissue/24 h for the fan treatment, and an increased accumulation rate of 5.00 (0.33, 9.67) mg Au/kg dry tissue/24 h for the sealed treatment, both significant with P<0.05. Although, it was expected to have an increased rate of AuNP accumulation for the fan treatment, the observed
increase in root loading for the sealed treatment is not consistent with results expected. For *Azolla caroliniana*, evapotranspiration rate alone did not explain the increased accumulation of Au within the root tissue. It is possible that root surface area is also important and should be considered when interpreting results. The higher surface area would allow for multiple binding sites. *Azolla caroliniana* roots played an important role in AuNP absorption, and have been documented by Glenn and Klaine [9]; thus it is likely that AuNP absorption depends on both root surface area and evapotranspiration. The association of 4 nm AuNPs with *Azolla caroliniana* root tissue is shown in Figure 5.2. Visually, the amount of detected 4 nm AuNPs associated with the root apoplast and cell wall correlated with each treatment. Although these micrographs yield only qualitative information, by day 8 of the fan treatment, a higher amount of AuNPs was observed to be associated with the root apoplast and cell wall of *A. caroliniana*.

Investigating root tissue for *Myriophyllum aquaticum* (Figure 5.1B), tissue gold concentrations are reported as the slope, or rate of gold accumulation by the roots per day (24 h). The rate was calculated from measurements made over days 2 to 16. The following rates were observed for each treatment, reported with 95% confidence interval. The rate of AuNP absorption by the roots are as follows: 1.54 (1.02, 2.06) mg Au/kg dry tissue/24 h, 3.69 (3.17, 4.21) mg Au/kg dry tissue/24 h, and 1.43 (0.91, 1.95) mg Au/kg dry tissue/24 h for the control, fan, and sealed treatments, respectively. These data indicated that AuNP
absorption for the Fan treatment > Control, and Control = Sealed. Comparing the Fan treatment to the Control treatment, an increased rate of absorption by the roots occurred, reported as an increase of 1.07 (0.71, 1.43) mg Au/kg dry tissue/24 h, \( P < 0.05 \). The sealed treatment rate, reported at 0.33 (-0.03, 0.69) mg Au/kg dry tissue/24 h, \( P > 0.05 \), indicated no difference in absorption rate from the control treatment.

Evapotranspiration rates correlated with root loading for *M. aquaticum* (Figure 5.1B). Qualitative observations of root loading of 4 nm AuNPs in each treatment are shown in Figure 5.3. Each humidity treatment influenced the quantity of nanomaterial associated with the cell wall and root apoplast. Although tissue concentrations were observed at a baseline level in shoots, no increased foliar Au content was measured after 16 days of exposure. The presence of an evapotranspiration stream did however increase the absorption of 4 nm AuNPs into the root apoplasm (Figures 5.1B and 5.3). Future research should focus on increased exposure concentrations and exposure length to determine if translocation of AuNP from roots to shoots could be achieved. Further, by separating tissues into upper and lower regions, biodistribution could be investigated to track the movement of AuNPs throughout the plant system.

*Evapotranspiration effects on the shoot concentrations*

The shoot tissue concentrations after exposure to the fan, sealed or control treatment for *M. aquaticum* were similar, indicating a baseline level of AuNPs movement into shoot tissues. This movement was observed to be
independent of evapotranspiration rate. The same trend held true for the sealed and control treatments for *A. caroliniana*. However, a positive increase in tissue Au concentration was recorded in *A. caroliniana* plants within the fan treatment; from day 2 to day 16, shoot tissue concentrations increased from 14.6 ± 3.29 mg Au/kg dry tissue to 140 ± 6.73 mg Au/kg dry tissue over 14 days. Concurrently, the day 16 concentration of Au in *A. caroliniana* root tissue were also the highest in concentration, 1265 ± 139 mg Au/kg dry tissue. Although positive slopes within all root treatments in both species indicated tissue concentrations continued to increase as duration of exposure to AuNP increased, no other treatment reached this tissue level concentration. This supports that translocation of AuNP is independent of evapotranspiration. These results suggest that root accumulation of AuNPs is dependent on evapotranspiration; however, translocation is driven by diffusion of AuNPs once concentrations in the roots are built up to a level high enough to drive passive diffusion.

These findings are consistent with results observed by Koelmel et al. [8] who investigated uptake and distribution of AuNPs with positive, negative and neutral surface chemistries in rice plants (*Oryza sativa*). Rice seedlings were exposed to two nanometer AuNPs and tissue concentrations were mapped using laser ablation ICP-MS. The authors observed that a lower exposure concentration of Au in the long-term (3 month) exposure resulted in no translocation to the shoots of the rice plants. They concluded that the mechanism for translocation might be dependent on an unknown threshold
concentration in the vascular tissue, where it cannot be removed until it exceeds a certain concentration threshold. The authors indicated that translocation could be dependent on exposure concentration, time and root concentration. The authors also note that bioaccumulation occurs over time, where Au concentrations were found to be significantly higher in roots than the shoots. These results concur with my current observations regarding tissue Au concentrations in the roots and shoots of the two aquatic macrophytes *A. caroliniana* and *M. aquaticum*. Tissue concentrations in *A. caroliniana* shoots did not increase until a much higher concentration was reached in the root tissues (by day 16). This suggested that translocation is independent of evapotranspiration, and is most likely driven by diffusion after the AuNPs have entered into the apoplastic region.

*Hyperaccumulation of metals mechanisms*

Glenn et al. [20] indicated that root structure played an important role in the size discrimination of AuNP absorption. The current results support the finding that xylem loading is not an efficient process. If 4 nm AuNPs were to follow the pathway of water, Au concentrations in shoot tissues should be similar to Au concentrations in root tissues.

The accumulation of ionic metals by plants, including aquatic plants has been studied for phytoremediation and geochemical prospecting purposes for decades. Two components of phytoremediation include 1) the ability of the plant to extract metals from the soils and surrounding environment and to concentrate
them within their tissues, and 2) the ability to utilize plants to immobilize metals; thus reducing the risk of metal migration to other areas [21]. To be a candidate for phytoremediation, a plant must have the capacity to accumulate metals. Hyperaccumulators are defined as plant species that concentrate metals and transport them to the shoot organs while avoiding toxic effects from high concentrations of heavy metals. Typically, a plant is considered a hyperaccumulator if, when grown in metal rich soils, shoots contain concentrations of greater than 10,000 mg/kg (dry weight) Zn or Mg, 1000 mg/kg Ni, Cu or Pb, or 100 mg/kg Cd [21,22]. Factors that influence the uptake of metals by plant roots have been studied extensively. Lu et al. [23] postulates that root to shoot translocation of Cd most likely occurred via the xylem and that the rate of transfer is dependent on the evapotranspiration stream. Hyperaccumulators typically exhibit high efficiency in the root to shoot transfer of metals; while in non-hyperaccumulators only a fraction of the absorbed metal may reach the shoots, while most remained in the root apoplast [23-28]. Although this study did not indicate that either A. caroliniana or M. aquaticum hyperaccumulate Au, the factors that controlled the movement of metals into the root are similar. Gold NPs concentrated in root tissues. Also, root loading occurred and root tissue concentrations consistently remained higher than shoot concentrations. It was observed that root tissue in A. caroliniana had nearly ten-fold the concentration before Au shoot tissue concentration became significant
(P<0.05). Koelmel et al. [8] have observed this similar root loading distribution in rice seedlings, as well as Sabo-Attwood et al. [29] in tobacco plants.

**Conclusions**

I hypothesized that the mechanism of root loading of AuNPs is based on diffusion of AuNPs into the root cortex of aquatic plants; this AuNP diffusion correlated with the movement of water into the roots. However, because shoot tissue concentrations did not increase in response to root loading, xylem transport was minimal. The AuNPs may have been sequestered in the root apoplast and blocked from moving into the vasculature by the Casparian strip. Although AuNPs could enter the vasculature directly at the root tip, by-passing the Casparian strip straight to into the symplast, it is not likely that this pathway represents the bulk flow of AuNPs in aquatic plants, as Glenn et al. [20] indicated that root wounding does not enhance uptake of AuNPs. Uptake of AuNPs appears to be initially based on the size of the AuNPs, as particles too large to diffuse across the cell wall remain adsorbed to the root surface. Once an AuNP reached the root apoplast, it binds to cell walls, possibly in pockets that contain charged surface groups such as sugars and polysaccharides. Increased evapotranspiration resulted in increased root loading, as indicating by tissue concentrations and electron micrographs. These results suggest that evapotranspiration rates and the movement of water through the plant are important in determining the absorption rate of AuNPs by aquatic macrophytes into the root tissue. Because shoot tissue concentrations did not correlate with
evapotranspiration rates, it was concluded that translocation is independent on root loading rate, which suggests that diffusion drives the movement of AuNPs from the roots to the shoots once AuNPs are absorbed into the root apoplast.
Table 5.1: Rate of gold nanoparticle absorption in roots and shoots of *Azolla caroliniana* and *Myriophyllum aquaticum*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Root/Shoot</th>
<th>Line Equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
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<td><em>A. caroliniana</em></td>
<td>Control</td>
<td>Root</td>
<td>$y = 19.93x - 21.9$</td>
<td>.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>$y = 3.37x + 17.9$</td>
<td>.72</td>
</tr>
<tr>
<td></td>
<td>Sealed</td>
<td>Root</td>
<td>$y = 36.52x - 165.8$</td>
<td>.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>$y = 2.25x + 5.7$</td>
<td>.60</td>
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<td>Fan</td>
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<td></td>
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<td>.86</td>
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<tr>
<td><em>M. aquaticum</em></td>
<td>Control</td>
<td>Root</td>
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<td></td>
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<td>$y = 0.03x + 0.0$</td>
<td>.20</td>
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<tr>
<td></td>
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<td>Root</td>
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<td>Shoot</td>
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Figures:

**Figure 5.1**: Tissue concentrations of *Azolla caroliniana* and *Myriophyllum aquaticum* under different evapotranspiration treatments. (A) Shows tissue concentrations for the roots and shoots of *A. caroliniana*. (B) Shows tissue concentrations for the roots and shoots of *M. aquaticum*. Fisher’s LSD post hoc test was used to separate means within each plant species; means not connected by the same letter are statistically different (*P*<0.05). Blue line indicates root tissue, and red line indicates shoot tissue concentrations. The shaded areas correspond with the 95% confidence intervals of the slope. Values under each humidity treatment presented as ml H$_2$O/g/24h represent average evapotranspiration rate.
Figure 5.2: Transmission electron micrographs of 4 nm gold nanoparticle root loading in *Azolla caroliniana* root apoplast. Top row correlates with 2 days of exposure. Bottom row correlates with 8 days of exposure. Columns define evapotranspiration treatments. Left column shows Fan treatment, middle column shows Control treatment and right column shows Sealed treatment. White arrows indicate the presence of 4 nm gold nanoparticles, appearing as dark structures. IE=internal environment, CW= cell wall, EE=external environment. Scale bars = 100 nm
Figure 5.3: Transmission electron micrographs of 4 nm gold nanoparticle root loading in *Myriophyllum aquaticum* root apoplast. Top row correlates with 2 days of exposure. Bottom row correlates with 8 days of exposure. Columns define evapotranspiration treatments. Left column shows Fan treatment, middle column shows Control treatment and right column shows Sealed treatment. White arrows indicate the presence of 4 nm gold nanoparticles, appearing as dark structures. CW= cell wall, EE=external environment. Scale bars = 100 nm
References:


CHAPTER 6: OVERALL CONCLUSIONS

Gold nanoparticle absorption is size and species dependent

1. Exposure to 4 and 18 nm gold nanoparticles revealed that 4 nm gold nanoparticles were absorbed by *Myriophyllum simulans* and *Azolla caroliniana*; however, only surface adsorption was observed in *Egeria densa*.

2. Absorption was correlated with salinity tolerance of each species, indicating that cellular structure may define size and species dependent absorption.

Gold nanoparticle absorption is a complex process based on several factors

1. Gold tissue concentrations revealed nanoparticle size dependent absorption in *Azolla caroliniana*, 4 nm > 18 nm = 30 nm. *Myriophyllum simulans* and *Egeria densa* did not show a significant size dependent tissue concentration, correlating with surface adsorption.

2. The addition of dissolved organic carbon reduced tissue concentration of 4 nm gold nanoparticles in *Azolla caroliniana*. The reduced tissue concentration occurred due to the formation of a gold nanoparticle/dissolved organic carbon association that was much larger than individual nanoparticles.
3. Both abiotic and biotic factors influenced the absorption of 4 nm gold nanoparticles in *Azolla caroliniana*. The presence of roots increased tissue concentration of Au, while the removal of roots decreased tissue concentration.

4. Dissolved organic carbon associated with 4 nm and 18 nm gold nanoparticles, but not with 30 nm gold nanoparticles.

*Aquatic macrophyte root structure played an important role in the size selectivity of gold nanoparticle absorption*

1. Gold nanoparticle size selectivity correlated with the density of microfibrils present in the external cell walls.

2. *Azolla caroliniana* had the thinnest and least dense root structures, while *Egeria densa* had the densest cell walls.

3. Measurements of cell wall porosity through defined solute exclusion methods indicate that *Azolla caroliniana* cell walls have the ability to absorb solutes <4.5-5 nm.

*High evapotranspiration rates increased the root loading of gold nanoparticles; however, translocation of gold nanoparticles is driven by concentration gradients.*

1. Increased evapotranspiration rate correlated with increased tissue concentrations in the roots for emergent species *Azolla caroliniana* and *Myriophyllum aquaticum.*
2. Shoot concentrations were independent of evapotranspiration rates. This suggested that translocation is driven by concentration gradients of AuNPs within the root apoplast.

3. Gold nanoparticle absorption by the roots correlated with known mechanisms of ionic metal accumulating ability of plants, but is not consistent with hyperaccumulators of heavy metals. The root loading is consistent with metal uptake, and results indicate that xylem transport is limited, keeping gold nanoparticles concentrating in the root apoplast until a passive diffusion drives movement of gold nanoparticles into the shoots.

The results observed in these studies indicate that aquatic macrophytes are able to bioconcentrate gold nanoparticles from waterborne exposure. Uptake of gold nanoparticles in aquatic systems is a complex interaction of several factors. Nanoparticle size is an important indicator in determination of absorption or adsorption. Plant species play a vital role in the uptake of gold nanoparticles from suspension, and root structure/morphology dictate the size of nanomaterials that can be absorbed. Water quality also influenced nanomaterials bioavailability. Water high in dissolved organic matter reduced nanomaterial bioavailability, as evidenced by reduced tissue gold concentrations. Evapotranspiration increased the movement of gold nanoparticles into the root apoplast and increased the association of gold nanoparticles with the cell wall. Translocation of 4 nm gold nanoparticles into shoot tissues did not occur in
Myriophyllum aquaticum and only occurred in Azolla caroliniana once high tissue Au concentrations were observed in the root tissue. This indicated that a threshold concentration must be reached in order for concentration gradients to drive movement of gold nanoparticles into the shoots. These findings are environmentally relevant in that parameters that influence the absorption of nanomaterials can be applied to future regulations involving the fate and effects that nanomaterials may have within our environment.
Appendix A-1:  

**Azolla caroliniana** interaction profiler, JMP v10.0.

JMP interaction profiler representing only *A. caroliniana* tissue concentrations. Each graphic shows four factors that influence tissue concentrations. Red letters indicate treatment parameters, and red concentration value show average tissue concentration and 95% CI for chosen parameters. Parameters are defined by dashed red line. *a=A. caroliniana, e=Egeria densa, m=Myriophyllum simulans.*

(A) Shows 4 nm, DOC-, R-, *Azolla caroliniana*. (B) Shows 4 nm, DOC+, R-, *Azolla caroliniana*. (C) Shows 4 nm, DOC-, R+, *Azolla caroliniana.*