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Bioavailability of Manufactured Nanomaterials in Terrestrial Ecosystems

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BIOAVAILABILITY OF MANUFACTURED NANOMATERIALS IN TERRESTRIAL ECOSYSTEMS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

BY
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Lexington, KY

Director: Dr. Paul Bertsch
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2013

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ABSTRACT OF DISSERTATION

BIOAVAILABILITY OF MANUFACTURED NANOMATERIALS IN TERRESTRIAL ECOSYSTEMS

Manufactured nanomaterials (MNMs) from the rapidly increasing number of consumer products that contain MNMs are being discharged into waste streams. Increasing evidence suggests that several classes of MNMs may accumulate in sludge derived from wastewater treatment and ultimately in soil following land application as biosolids. Little research has been conducted to evaluate the impact of MNMs on terrestrial ecosystems, despite the fact that land application of biosolids from wastewater treatment will be a major pathway for the introduction of MNMs to the environment. To begin addressing this knowledge gap, we have conducted a series of experiments designed to test how bioavailable MNMs are to terrestrial ecoreceptors when exposed through a variety of pathways.

First, we used the model organisms Nicotiana tabacum L. cv Xanthi (tobacco) and Triticum aestivum (wheat) to investigate plant uptake of 10, 30 and 50 nm diameter gold (Au) MNMs coated with either tannate (T-MNMs) or citrate (C-MNMs). Both C-MNMs and T-MNMs of each size treatment bioaccumulated in tobacco, but no bioaccumulation of MNMs was observed for any treatment in wheat.

In a second exposure, we investigated the potential for bioaccumulation of MNMs from contaminated plant surfaces by a terrestrial secondary consumer, tobacco hornworm (Manduca sexta). We found that hornworms bioaccumulate Au MNMs, but that the assimilation efficiency of bioaccumulation was low. Hornworms eliminate ingested Au MNMs rapidly from 0-24 h, but very slowly from 1 d to 7 d.

Finally, we used the model organisms tobacco and tobacco hornworm to investigate the potential for trophic transfer of Au MNMs. Biomagnification of Au MNMs was observed in the hornworms.

We have demonstrated that MNMs of a wide range of size and with different surface chemistries are bioavailable to plants, that MNMs resuspended by wind, rain, biota, and mechanical disturbance from soil onto plant surfaces are bioavailable to terrestrial consumers, and that trophic transfer and biomagnification of plant accumulated MNMs
can occur. These results have important implications for risks associated with nanotechnology, including the potential for human exposure.

Keywords: nanotechnology, nanomaterials, nanoparticle, nanotoxicology, ecotoxicology.

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BIOAVAILABILITY OF MANUFACTURED NANOMATERIALS IN TERRESTRIAL ECOSYSTEMS

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Chapter 1: Manufactured nanomaterials in terrestrial ecosystems: Emerging contaminant?

Advances in nanotechnology have led to the development and introduction of many new consumer products containing manufactured nanomaterials (MNMs). These products employ a wide range of MNM compositions, including Ag, Au, TiO$_2$, ZnO, CeO$_2$, C, and CdSe quantum dots. Silver (Ag) MNMs are used extensively as an antimicrobial agent in textiles and food containers, and are also used as a conductive ink.$^{1-4}$ Gold (Au) MNMs are being used in the detection and imaging of cancer cells,$^5$ pharmaceuticals designed to combat HIV,$^6$ catalysis in fuel cells,$^7$ and in the development of organic memory module technology.$^8$ Zinc oxide (ZnO) and titanium dioxide (TiO$_2$) MNMs are both widely used in sunscreens and pharmaceuticals$^9-10$. TiO$_2$ MNMs are also used in paint pigments and UV protective coatings$^{10-11}$. In Europe, cerium dioxide (CeO$_2$) MNMs are used to catalyze diesel fuel combustion and thereby reduce NO$_x$ emissions.$^{12}$ Carbon (C) MNMs are being implemented in composite materials, sensors, and hydrogen fuel cells.$^{13-15}$

One pathway by which MNMs from consumer products are expected to enter the environment is through the land application of biosolids. MNMs from consumer products are released into waste streams during use and through wastewater treatment, MNMs have been shown to concentrate in the sludge,$^{16-19}$ 60% of which is applied to agricultural land as biosolids in the U.S. and the majority of Europe.$^{19}$ One early modeling study predicted that between 2008 and 2012 the concentrations of MNMs in sludge-treated soil in the U.S. will increase from 0.1 to 0.5 mg kg$^{-1}$ for TiO$_2$ MNMs, from 6.8 to 22.3 μg kg$^{-1}$ for ZnO MNMs, and from 2.3 to 7.4 μg kg$^{-1}$ for Ag MNM.$^{19}$ However, this study estimated these concentrations by simply dividing the estimated
mass of MNMs discharged into the soil compartment within biosolids by the mass of soil (calculated as agricultural soil area times 0.2 m mixing depth plus urban soil times 0.05 m mixing depth and converted to mass using an estimate for bulk density) in the U. S., without considering regional and local differences in sludge application rates or practices. Despite the fact that this study predicts exponential increases in soil MNM concentrations, these predictions are likely unrealistically low for many regions of the U. S that receive biosolid amendments, since they typically represent agroecosystems in the vicinity of urban areas that receive applications annually. Our conclusion that this is an overly conservative estimate is reinforced by the EPA Guide to Biosolids Risk Assessment for the Part 503 Rule document which stipulates using a 1:1 soil/sludge mixture for ecotoxicity testing of biosolids.20 Thus as a first pass for defining environmentally realistic concentrations for evaluating risk in agroecosystems, it might be more meaningful to use the predicted concentrations of MNMs in biosolids, which are 137, 23.2, and 1.55 mg kg\(^{-1}\) for TiO\(_2\), ZnO, and Ag MNMs, respectively.\(^{19}\)

There is concern that over time, MNMs supplied to terrestrial ecosystems in this manner may accumulate to concentrations that affect soil productivity or biota. Furthermore, the degree to which soil-accumulated MNMs may bioaccumulate in various ecoreceptors, migrate into groundwater, or be carried into surface waters via runoff is unknown. Despite this, the vast majority of nano-ecotoxicological research has focused on aquatic species.\(^{21}\) As a result, our fundamental understanding of the mobility, fate, bioavailability and potential toxicity of sludge-accumulated MNMs to biota in terrestrial ecosystems is poorly developed.\(^{21}\)
Complicating the ability to develop unifying principles for predicting the interactions with and toxicity of MNMs to biota has been the great diversity of MNM composition and surface functionality, as well as the diversity of species which may be potentially be exposed. Each MNM has a set of intrinsic properties, such as composition, crystal structure, size, shape and surface chemistry\textsuperscript{22-25} that may affect mobility, bioavailability, and toxicity. In most cases, these properties will also vary within a single sample of MNMs. Furthermore, each exposure environment will introduce various extrinsic factors that can transform the MNMs, which also affect mobility, bioavailability, and toxicity. Ultraviolet light, ionic strength, pH, and the presence and concentration of natural organic matter (NOM) and other ligands such as sulfide (S\textsuperscript{2-}) have all been shown to have an impact on the bioavailability and/or toxicity of MNMs.\textsuperscript{26-31}

1.1 Environmental transformations of MNMs: In many cases, nanomaterials introduced into wastewater streams will be modified after release from consumer goods as a result of biogeochemical transformations and/or interactions with organic and inorganic ligands.\textsuperscript{30,32} The resulting aged MNMs (a-MNMs) have properties that are different from pristine MNMs. The environmental transformations of MNMs are discussed in detail in a recent review by Lowry \textit{et al.} (2012).\textsuperscript{33}

1.1.1 Dissolution: Gold, TiO\textsubscript{2} and CeO\textsubscript{2} MNMs are highly insoluble under almost all conditions and will strongly resist dissolution.\textsuperscript{34-36} However, Ag, ZnO, and Cu/CuO MNMs are relatively soluble and will at least partially dissolve over time once introduced into many environments.\textsuperscript{37-38} Transformations of these more soluble MNMs will be controlled by the pH, Eh and the presence of inorganic and organic compounds important for the chemical speciation of the specific MNM species. For example, the
concentrations of oxygen (O), chloride(Cl) and organic S\(^{2-}\) ligands are predicted to be highly influential in driving transformations of Ag MNMs.\(^{33,39}\) In aerobic, relatively high Eh and low sulfur (S) environments, AgCl and Ag-thiol complexes are likely to form.\(^{39}\)

Initially, MNMs will enter wastewater treatment suspended in wastewater.\(^{17,40}\) Impellitteri \textit{et al.} (2009) examined how tap water would affect the speciation of Ag MNMs and reported that when clothing treated with Ag MNMs is washed using tap water and household bleach (sodium hypochlorite) AgCl formation is favored.\(^{41}\) Once partitioned to biosolids at a wastewater treatment facility, MNMs will encounter very high concentrations of S and P relative to what is present in most natural environments. For example, a study characterizing six different Australian biosolids reported S concentrations ranging from 6.9 and 14.4 g kg\(^{-1}\) and P concentrations between 8.0 and 53.0 g kg\(^{-1}\).\(^{42}\) In soils, the concentration of S is dramatically lower. David \textit{et al.} (1989) found concentrations of S in a variety of soils from Minnesota, Michigan and Wisconsin to vary from 99.4 to 843.3 µg kg\(^{-1}\).\(^{43}\) In the anaerobic, low Eh and high S conditions of wastewater treatment settling tanks, formation of Ag\(_2\)S is predicted.\(^{39,44}\) The formation and persistence of Ag\(_2\)S is favorable under many conditions due to its extremely low solubility (\(K_{sp} = 5.92 \times 10^{-51}\)).\(^{39}\) Several studies have demonstrated experimentally that Ag MNMs will transform to Ag\(_2\)S MNMs during wastewater treatment.\(^{32,45-46}\) Evidence of this transformation was first reported by Kim \textit{et al.} (2010), who identified and characterized Ag\(_2\)S MNMs found within municipal sewage sludge using transmission microscopy (TEM) based analyses, although it was not known whether the Ag\(_2\)S MNMs formed from reaction of S\(^{2-}\) with dissolved Ag\(^+\) or from the sulfidation of Ag MNMs.\(^{46}\) Formation of Ag\(_2\)S MNMs from Ag\(^0\) was first confirmed through experimentation using
Ag foils and subsequent SEM by Liu et al (2010) and later in another study by Kaegi et al. (2011) who investigated the transformations of Ag MNMs in a pilot wastewater treatment plant. Using TEM and x-ray absorption spectroscopy (XAS), this study demonstrated that the majority of the Ag MNMs in the sewage sludge had been transformed to Ag2S. Another study by Levard et al. (2011) demonstrated how sulfidation of Ag MNMs changed the physiochemical properties of the MNMs. These researchers reported that sulfidation of Ag MNMs resulted in the loss of the organic capping agent, altered surface charge and dramatically lowered MNM dissolution rate compared to pristine Ag MNMs. Liu et al. (2011) reported that sulfidation of MNMs is dependent on the presence of dissolved oxygen and is strongly influenced by pH and organic matter. The environmental transformations of Ag MNMs are reviewed in detail elsewhere.

Research has also shown that ZnO MNMs will rapidly transform after entry into wastewater treatment. Reed et al. (2012) examined the dissolution of ZnO MNMs in deionized water (DI), moderately hard water, Dulbecco’s modified Eagle’s medium (DMEM) and Rosewell Park Memorial Institute medium (RPMI). The concentration of dissolved Zn in the moderately hard water was very low compared to DI. The authors speculated that this was likely due to the formation of insoluble Zn-carbonate mineral phases. However, again, ZnO MNMs discharged from consumer goods into wastewater streams will encounter high concentrations of S and P during wastewater treatment. Lv et al. (2012) examined the transformations of ZnO MNMs in the presence of varying concentrations of phosphate as a function of time. The ZnO MNMS were rapidly transformed to zinc phosphate mineral phases and the release and persistence of Zn2+ was...
dramatically reduced.\textsuperscript{49} Recently, Lombi \textit{et al.} (2012) used XANES to investigate the speciation of ZnO MNMs after anaerobic digestion and post-wastewater treatment processing as a function of time.\textsuperscript{50} Resulting speciation was examined in biosolids taken directly out of the digestors as well as in biosolids that had been subjected to a 2 month period of wetting and drying, as biosolids are stockpiled prior to land applications in many areas. Although the ZnO MNMs were rapidly transformed in both fresh and aged biosolids, likely through dissolution and precipitation, the ultimate Zn speciation was strongly influenced by the aging of the biosolids. In the fresh biosolids, ZnS was the dominant Zn species whereas Zn was complexed with phosphate and Fe oxy/hydroxides in the aged biosolids.

Similarly, Mudunkotuma \textit{et al.} (2012) reported that copper based MNMs readily oxidize at room temperature and that MNM size, bulk composition and surface properties were altered dramatically by MNM aging in the presence or organic acids.\textsuperscript{51} These reactions were strongly influenced by pH and concentration of organic acids. Donner \textit{et al.} (2011) demonstrated that dissolved Cu in biosolids predominately forms Cu-humic acid complexes and chalcocite (Cu\textsubscript{2}S), cavelite (CuS), and cubanite (CuFe\textsubscript{2}S\textsubscript{3}), mineral phases, although this Cu was not introduced as MNMs.\textsuperscript{42} Furthermore, evidence suggest that in some cases, an oxidized outer shell may form with a metal core, possibly protecting the metallic core from further oxidation.\textsuperscript{33,37}

Research that has examined the transformations of MNMs in tap water, moderately hard water and in biosolids has provided interesting and environmentally relevant information.\textsuperscript{32,41,48} However, considering that the bulk of MNMs released from consumer products will first be exposed to tap water of varying hardness and then subsequently to
the high S, high P and sometimes anaerobic conditions of wastewater treatment, it is unclear what the ultimate speciation of MNMs will be once exposed to the sequence of conditions that MNMs would encounter during wastewater treatment. This is an interesting area for future investigations.

1.1.2 Aggregation: MNMs of all compositions have the potential to aggregate, either during manufacture or during wastewater treatment, which evidence suggests may dramatically reduce their bioavailability, mobility and toxicity.\textsuperscript{52} The aggregation behavior of MNMs will be largely dependent on how the interaction of many variables, including pH, ionic strength, natural organic matter (NOM) concentration, and surface chemistry of the MNMs, affect the electrostatic and steric repulsion between individual nanoparticles.\textsuperscript{53-54} The ionic strength and NOM concentrations of most natural waters favor aggregation.\textsuperscript{54} High ionic strength, specifically high concentrations of divalent cations such as Ca\textsuperscript{2+}, increases the likelihood of MNM aggregation.\textsuperscript{53-54}

MNM aggregation occurs in two forms: homoaggregation and heteroaggregation.\textsuperscript{33} Homoaggregates consist of solely of aggregated MNMs whereas heteroaggregates consist of both MNMs and other particles.\textsuperscript{33} In the environment, heteroaggregation will be dominant due to the ubiquitous presence of environmental particulates.\textsuperscript{33}

1.1.3 Interactions with biologic macromolecules: MNM surfaces can be modified in the environment by adsorption of biologic macromolecules such as NOM, carbohydrates and proteins.\textsuperscript{33,55} Studies have shown that in natural waters, NOM can have an effect on the surface charge of suspended many different MNM types including SWCNTs, C\textsubscript{60}, ZnO, NiO, TiO\textsubscript{2}, Fe\textsubscript{2}O\textsubscript{3}, Fe\textsubscript{3}O\textsubscript{4}, and SiO\textsubscript{2}.\textsuperscript{28-29,53-54,56} Furthermore, adsorption of NOM to nano oxides is pH dependent and the amount of NOM adsorbed is
related to surface hydrophilicity and magnitude of negative surface charge.\textsuperscript{28} At lower concentrations, NOM will adsorb to active sites on the nanoparticle surface, reducing the overall surface charge of the particle. However, at higher concentrations the highly charged NOM will form a coating of organic matter with a high negative charge density as well as large molecule size, which creates additional steric hindrances to aggregation.\textsuperscript{29} Humic materials can also increase the solubility of a MNM solution by enhancing dispersion as a result of humic acid adsorption.\textsuperscript{26}

1.2 Bioaccumulation, toxicity, and trophic transfer of MNMs: The bioavailability and toxicity of a-MNMs and MNMs to terrestrial ecoreceptors is not well understood. Many of the studies in this area have provided inadequate MNM characterization data, failed to consider MNM dissolution during exposure or used environmentally unrealistic MNM concentrations. Many studies have also provided seemingly contradictory results for reasons that are not entirely clear, but likely related to the experimental inadequacies described above. However, accumulating evidence suggests that under certain circumstances, MNMs can be bioavailable and toxic to many different organisms and that MNMs can be transferred from one organism to another through dietary exposure. In this section, we review what is known about uptake and toxicity of MNMs to plants, soil bacteria, fungi, and soil invertebrates.

1.2.1 Mechanisms of toxicity: Toxicity as a result of MNM exposure could be the result of the action of one or a combination of several mechanisms.\textsuperscript{21} Traditionally, the acute ecotoxicity of some metals has been described by models such as the Biotic Ligand Model (BLM).\textsuperscript{57} The BLM describes metal toxicity as the accumulation of a critical concentration of free metal ion-biotic ligand complexes, where the biotic ligand is a
generic site of action for metal ion binding (e.g. sodium and calcium channel proteins in a fish gill; carboxylic ligands associated with membrane surfaces), in the presence of competing ions and organic matter. Some MNMs, such as Ag, Cu, and ZnO, may exert toxic effects via dissolution and release of free metal ions within the soil pore water or the organism. Insoluble MNMs, such as Au, CeO₂, and TiO₂, are unlikely to induce toxicity as a result of free ion activity under most environmentally relevant exposure scenarios.

However, this does not necessarily mean that insoluble MNMs will be non-toxic as MNMs may also exert specific effects unrelated to release of metal ions. One such mechanism is the generation of reactive oxygen species (ROS). When reactive oxygen species such as oxyradicals are produced inside an organism, they can cause damage to cellular components including proteins, lipids, and DNA. This effect is influenced by UV and visible light, as well as possibly the presence of transition metals. MNMs might also induce toxicity through mechanical disruption of membranes and cell walls.

Due to the large number of extrinsic and intrinsic factors that might influence MNM transport, fate, bioavailability, and potential toxicity, it is impractical to test all possible combinations of these variables and it is necessary to develop guiding principles with which to develop a predictive framework. To this end, we have previously proposed a Particle Biotic Receptor Model (pBRM) as a new conceptual construct that defines toxicity based upon both the interactions of free metal ion, pristine MNMs, and a-MNMs with the biotic receptor (e.g. cell membrane or membrane bound macromolecule).

1.3 Toxicity and bioaccumulation of MNMs by terrestrial plants: As plants comprise the base of many terrestrial food webs, the degree to which MNMs are
bioavailable and toxic to plants will have a major impact on the risks to humans and terrestrial ecosystems posed by MNMs.

1.3.1 Plant root uptake of MNMs: Barriers exist in plant root physiology that will affect the movement of MNMs from the soil into the plant root. Directly interior to the root cortex is the endodermis. The cell walls of endodermal cells contain hydrophobic layers of suberin and lignin which are collectively referred to as Casparian bands. This band of cells, which is not fully developed near immature root tips, prevents the apoplastic movement of ions from the root stele back out into the root cortex. Most angiosperms also have an exodermis, which is a layer of cells exterior to the cortex which also have Casparian bands.64-65 The Casparian bands in the exodermis and endodermis will inhibit the apoplastic movement of water, most solutes and MNMs into the root stele.64-66 Therefore, for MNMs to enter the root stele they will have to either cross the cell wall and plasma membrane of an endodermal or exodermal cell, or cross a root cell wall either at or exterior to the endodermis or exodermis and move into the stele symplastically.

1.3.1.1 Uptake via cell wall pores: To cross an intact cell wall, it has been hypothesized that MNMs will have to passively move through a cell wall pore, suggesting that plant uptake should be highly size selective. The diameter of these pores has been estimated using a variety of techniques. Carpita et al. (1979) used solute exclusion experiments to estimate cell wall pore diameters for root hair cells of *Raphanus sativus* to be 3.5 to 3.8 nm and 4.5 to 5.2 for palisade parenchyma cells from leaves of *Xanthium strumarium* and *Commelina communii*.67 Adani et al. (2011) recently used gas
adsorption isotherms to demonstrate that although the majority of cell wall pores are smaller than 8 nm, a population of mesopores exist with diameters as large as 50 nm.68

1.3.1.2 Uptake via endocytosis. Alternatively, cellular transport studies indicate that endocytosis may be an important pathway by which MNMs enter plants.69 A MNM with the appropriate surface chemistry could theoretically activate membrane receptors and induce endocytosis. Liu et al. (2009) investigated the role of endocytosis in cellular uptake of MNMs by exposing Nicotiana tabaccum L. bright yellow (BY-2) cells to single walled carbon nanotubes labeled with fluorescein at 4 °C and 26 °C.69 Confocal fluorescence microscopy revealed that the MNMs were readily taken up at 26 °C, but were not taken up at 4 °C even after prolonged incubation. As plant endocytosis has been shown to be dramatically reduced at low temperatures,70 this result indicates that endocytosis may be an important mechanism for plant uptake of MNMs.

1.3.1.3 Hydroponic studies examining root uptake. Plant root uptake of MNMs was first demonstrated in a study by Zhu et al. (2008), who reported evidence of uptake of magnetite MNMs by pumpkin plants.71 Subsequently, there have been many studies published examining uptake of a range of MNMs to a variety of plant species in hydroponic exposures. Early work in this area includes a study by Lin et al. (2008) that demonstrated accumulation of the ZnO MNMs in the protoplast of the endodermal cells but found no evidence that the particles were translocated into the shoots and leaves, possibly the result of the observed MNM aggregation the authors observed of the MNMs in the exposure media.72 The authors reported that virtually all of the MNM had aggregated, likely due to the high ionic strength of the nutrient media, which likely
reduced bioavailability. These studies have been reviewed comprehensively recently elsewhere.73-74

Unfortunately, many of the studies examining plant uptake of MNMs have not provided adequate MNM characterization data, considered MNM dissolution, employed appropriate controls, and/or conducted spatially resolved analysis confirming actual particle uptake. This, coupled with the immense number of possible combinations of MNM treatments and plant species, has produced many seemingly contradictory results and limited progress in our understanding of the factors that control the bioavailability of MNMs under environmentally realistic scenarios.

Additionally, a likely factor contributing to the observed contradictory results between studies is the lack of standard MNM exposure protocols, which often leads to comparisons across studies that were conducted using vastly different protocol, e.g., media, exposure times, and analytical techniques. For example, although most studies have been conducted in solution hydroponics, some studies have investigated uptake of MNMs suspended in solid agar-like media.75-76 How this might affect uptake and comparisons between studies using these different media is unclear, but one study examining uptake of 2.8 nm TiO\textsubscript{2} MNMs from both solid and liquid media observed uptake in the liquid media, but not in the solid media.77 Another example is the use of different exposure time, which varies from hours78-79 to months.80 The impact of these variations in largely unclear.

1.3.1.4 Root uptake from soil. Only a handful of studies have examined the bioavailability of MNMs to plants from soil and thus, we do not have a good understanding of how environmental variables such as soil type, soil biota, and soil
chemistry may affect uptake. Zhu et al. (2008) reported no measureable uptake of Fe$_3$O$_4$ MNMs by pumpkin in either soil or sand.\textsuperscript{71} Birbaium et al. (2010) also reported no uptake after exposing 3-5 week old corn plants to 37 nm diameter CeO$_2$ MNMs.\textsuperscript{81} Doshi et al. (2008) reported no uptake of MNMs with an Al$^0$ core and either a carboxylate or an Al$_2$O$_3$ shell by red kidney beans, but did report uptake in ryegrass. However, the researchers in this study made no attempt to distinguish uptake Al ions from bioaccumulation of MNMs.\textsuperscript{82} Du et al. (2011) exposed Triticum aestivum to TiO$_2$ (predominately 20 ± 5 nm) MNMs and slab-like ZnO (predominately 40 ± 10 nm) nanomaterials from seed until maturity, a period of approximately 7 months.\textsuperscript{80} The plants were exposed in a loamy clay medium with a pH of 7.4. The researchers reported that the TiO$_2$ MNMs aggregated within the soil medium. TiO$_2$ MNMs were observed adsorbed to the root surfaces, but were not visible within the root cells. No ZnO MNMs were observed within the wheat root cells and the authors speculated that the majority of the ZnO dissolved during the exposure period. Taken together, there is little evidence suggesting that MNMs will be bioavailable under environmentally relevant conditions. However, considering the number of intrinsic MNM and environmental variables, such a small collection of studies is inadequate to fully explore this issue and further study is required.

\subsection{1.3.1.5 Effect of plant species on root uptake}

There are many physicochemical differences between plant species such as variations in hydraulic conductivity, cell wall pore size and root exudate chemistry that could be influence MNM bioaccumulation.\textsuperscript{67,83} Supporting this hypothesis are studies which have demonstrated uptake in one species but not another using the same exposure protocol. For example, Zhu et al. (2008) observed
accumulation of magnetite MNMs in *Vigna radiata* (mung bean) but did not observe bioaccumulation when conducting this same experiment using *Phaseolus lunatus* (lima bean). Currently, our understanding of how plant species affects plant uptake of MNMs is not well advanced and represents a ripe area for future work. However, we know, for example, that monocots and dicots exude fundamentally different chemicals into the rhizosphere and a reasonable hypothesis would be that these differences may fundamentally alter MNM stability and bioavailability.84

1.3.1.6 Effect of MNM stability on root uptake. An early hypothesis regarding the bioavailability of MNMs predicted that the degree to which MNMs are aggregated in the exposure environment will be a primary factor influencing the bioavailability of MNMs to plants and that bioavailability in the environment will be low due to environmental variables that would force MNMs to aggregate. This hypothesis is supported by a recent study that linked plant species dependant aggregation to bioavailability of Au MNMs to wheat and tobacco plants.85 However, since plant uptake of MNMs was first demonstrated, most studies employing hydroponic plant exposures have failed to characterize the stability of the MNMs used within the treatment medium or consider MNM aggregation over the course of exposure. As a result, studies may potentially be inaccurately attributing low MNM bioavailability to factors such as particle size and plant species, which themselves may be indirectly affecting bioavailability by influencing aggregation behavior. Characterization of the aggregation state of MNMs within the exposure media prior to and after exposure in future studies may provide information clarifying the bioavailability of MNMs to plants.
1.3.1.7 Importance of MNM intrinsic properties on root uptake. The impact that MNM intrinsic properties may have on the bioavailability of MNMs to root uptake by plants remains unclear. In many studies, properties that have been hypothesized to be factors that may influence uptake, such as particle size and electrophoretic mobility, are not measured or reported.

Particle size has often been hypothesized to be a primary factor affecting plant uptake, due largely to the assumption that the primary pathway by which MNMs would enter plants would be through pores in the plant cell wall. However, studies investigating this hypothesis have reported mixed results. Sabo-Attwood et al. (2011) used synchrotron based µXRF mapping and TEM to demonstrate uptake of 3.5 nm citrate coated Au MNMs by *Nicotiana tabaccum* whereas no uptake of 18 nm Au MNMs was observed, suggesting a size exclusion limit below 18 nm. 86 This result supports the hypothesis that the primary MNM uptake pathway is through passage through cell wall pores. However, another recent study reported data from bulk elemental and spatially resolved laser ablation ICP-MS analysis that indicated Au MNMs with particle size between 10-50 nm were taken up by *Nicotiana tabaccum* and that there were no significant differences in uptake as a function of size. 85 The reasons for the contradictory results between these two studies is unclear, but may be related to the lack of quantitative data in the former study. Regardless, uptake of MNMs larger than would likely be able to pass through these cell wall pores has been reported in other studies. 75,83,87-89 One of these studies examined the importance of size on uptake by exposing wheat plants to TiO₂ MNMs ranging from 14-655 nm. 88 MNMs greater than 140 nm were not taken up and MNMs greater than 36 nm were not translocated into the aerial portions of the leaves.
However, these MNM diameters do not correlate well with transport via cell wall pores and differences in MNM aggregation and settling as a function of size were not considered.

MNM surface chemistry is also likely to influence bioavailability of MNMs to plants. A suspended MNM with a positive surface charge moving apoplastically into the root could adsorb to the negatively charged carboxyl groups of galacturonic and glucuronic acids in hemicelluloses and pectins present in the walls of the cell wall pores of root cells, as metal cations demonstrate this behavior.90-91 Plants will differ in the amount of cation exchange sites that they have on their cell wall surfaces, and this CEC will decrease as pH decreases.92 Cell walls will also have a relatively small amount of anion exchange sites, which are theorized to be a result of organic cations fixed in the cell wall matrix.93-94 Both types of exchange sites are presumed to be near bioaccumulation sites on the plasma membrane and ostensibly facilitate the transport of nutrient cations across the plasma membrane.84 It is unknown whether these exchange sites might also facilitate transport of MNMs. Recently, the importance of surface charge to plant uptake of 6-10 Au MNMs in rice, radish, pumpkin, and perennial ryegrass was examined.95 Positively charged particles were taken up into the roots more readily whereas negatively charged MNMs were translocated into the plant shoots in higher concentrations.

The importance of MNM intrinsic properties on plant uptake of MNMs via endocytosis, an alternate non-destructive uptake pathway, has not been extensively studied and represents another important area for future research. Considering that endocytosis occurs in plants through stimulation of the cell wall by specific compounds such as sucrose or through the activation of a specific membrane receptor,96 a MNM
with the appropriate surface chemistry could theoretically activate membrane receptors and induce endocytosis. Evidence also exists from assays using intact animal and bacteria cells that MNM surface charge and size may be important factors in uptake via endocytosis.\textsuperscript{25,97}

1.3.1.8 Movement of MNMs in plant vasculature: Once within the plant symplast, MNMs must be able to move freely through the plant xylem to be transported into the plant shoots and leaves. Whether or not this is possible will likely be influenced by the size and surface chemistry of the MNMs, in addition to other factors such as geometry. Corredor \textit{et al.} (2009) investigated xylem transport of MNMs by injecting graphite coated iron MNMs into the pith cavity of the leaf petiole of pumpkin plants. A very homogenous population of ~46 nm MNMs was found in the xylem at a distance from the injection site, suggesting that MNMs larger than 46 nm might not be readily transported through the xylem.\textsuperscript{98}

Evidence also suggests that MNMs may be available for loading and transport via the phloem. Wang \textit{et al.} (2012) exposed corn to 20-40 nm CuO MNMs\textsuperscript{99} and analyzed the translocation of the MNMs through the plant vasculature. The CuO MNMs were observed in the xylem sap of the plants using TEM and confirmed to be CuO MNMs using electron diffraction. Interestingly, this study also demonstrated via separate split root exposures phloem loading and phloem transport of MNMs. In these exposures, plant roots were either placed in deionized water or in a suspension of CuO MNMs. Analysis indicated that CuO MNMs were present in the roots. This finding is supported by another study by Lin \textit{et al.} (2009), who demonstrated generational transfer of MNMs,
which would only be possible if MNMs could be translocated to the plant embryo via phloem.\textsuperscript{100}

1.3.1.9 Foliar uptake: Root uptake is not the only pathway by which plants may bioaccumulate MNMs. Resuspension of soil particles onto plant leaf surfaces as a result of rainfall, wind, biota and/or mechanical disruption is thought to be a pathway by which contaminants might enter terrestrial food webs.\textsuperscript{101} Once translocated onto leaf surfaces in this manner, MNMs could be available for either foliar uptake by the plant or dietary uptake by herbivorous consumers. Nanomaterials on plant surfaces could enter the plant vasculature by either cuticular diffusion or by transversing stomata. Cuticular diffusion can occur by two different pathways: hydrophilic and lipophilic. Non-polar substances are able to diffuse through the cuticle via the lipophilic pathway, and polar substances can be transported via the polar aqueous pores of the hydrophilic pathway.\textsuperscript{102-104} Therefore, it is possible that the hydrophobicity of the MNM coating molecule might affect how MNMs travel along this pathway. Cuticular diffusion is likely to be size selective, as cuticular pores in leaf surfaces of \textit{C. arabica} and \textit{Populus x canadensis} Moench averaged 4 and 4.8 nm.\textsuperscript{105} Other studies have measured cuticular pores to be as small as 0.6 nm in diameter.\textsuperscript{106} Different plants might have cuticular pores of different sizes, complicating assessment of the potential risks associated with this exposure pathway.\textsuperscript{105} The trans-stomatal pathway for plant uptake of material is not well understood. The pore diameter for this pathway is estimated to be greater than 40 nm for some plants, based on uptake of fluorescent polystyrene particles with a diameter of 43 nm. Evidence suggests that this pathway is dependent on environmental or circumstantial, rather than physiological, factors.\textsuperscript{107}
1.3.2 Phytotoxicity of environmentally relevant MNMs: TiO$_2$, ZnO, Ag, C and CeO$_2$

Phytotoxicity of MNMs has been investigated using a broad variety of MNM compositions. However, only a few of the MNM classes that have been used are likely to accumulate in the environment in concentrations likely to induce toxicity. As a result, we will divide our discussion of the research investigating the phytotoxicity of MNMs into two sections: one focusing on environmentally relevant MNM species and another section examining what has been learned from studies using other MNMs.

1.3.2.1 TiO$_2$ and ZnO: TiO$_2$ and ZnO MNMs are discharged into waste streams in larger masses than any other MNM due to widespread use in sunblocks and pharmaceuticals and as a result, the concentrations of these MNMs in biosolids and soil are increasing exponentially.$^9$-$^{10}$,$^{19}$ Some research has indicated that exposure to TiO$_2$ MNMs may be beneficial to plants. A series of related studies were conducted exposing spinach (Spinacia oleracea) to 4-6 nm TiO$_2$ (anatase) MNMs at 0.25% (w/v).$^{108}$-$^{110}$ Exposure to TiO$_2$ MNMs increased biomass, photosynthesis rate and enzyme activity. The researchers related this positive response to enhanced N$_2$ fixation from N photoreduction and the stimulation of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) activity. Increased biomass, photosynthesis rate and enzyme activity in spinach in response to the rutile TiO$_2$ MNMs has also been reported.$^{111}$ Alternatively, in another study when Arabidopsis thaliana seedlings were exposed to bare or sucrose coated TiO$_2$-50% alizarin red (ARS) MNMs no positive or negative effects on germination rate or reactive oxygen species production were reported.$^{77}$

Other studies have reported that TiO$_2$ MNMs can be phytotoxic. In one early study, excised corn (Zea mays) root apices were exposed to TiO$_2$ MNMs at 300 mg L$^{-1}$ and 1000
mg L$^{-1}$.$^{112}$ The TiO$_2$ MNMs inhibited transpiration, reduced root hydraulic conductivity, caused cell wall pores to constrict and resulted in minor inhibition to shoot and root growth. More recently, TiO$_2$ (predominately 20 ± 5 nm) MNMs were observed to induce a reduction in wheat biomass production. This study also reported that wheat biomass was reduced by exposure to and slab-like ZnO (predominately 40 ± 10 nm) MNMs.$^{80}$

Unlike TiO$_2$ MNMs, ZnO MNMs will dissolve to some degree under most conditions and as a result may induce toxicity via particle specific effects as well as free ion activity. Exposure to ZnO and Zn MNMs has been consistently reported to be phytotoxic. Lin et al. (2007) reported that soaking and incubating radish, rape, ryegrass, lettuce, corn, and cucumber seeds in 2000 mg L$^{-1}$ concentrations of Zn MNMs and ZnO MNMs reduced seed germination rate.$^{113}$ Root growth was slowed dramatically by seed soaking and incubation in suspensions of Zn and ZnO MNMs, with growth almost completely stopping by 200 mg L$^{-1}$. The concentrations at which 50% of the populations were inhibited, (IC$_{50}$) for both the Zn and ZnO MNMs was estimated to be 50 mg L$^{-1}$ for radish and 20 mg L$^{-1}$ for rape and ryegrass, and was lower than the IC$_{50}$ of Zn$^{2+}$, suggesting a MNM specific source of toxicity. Lin et al. (2008) demonstrated toxic effects such as biomass reduction, root tip shrinkage, and cortical cell collapse after exposing ryegrass to ZnO MNMs with a mean hydrodynamic diameter of approximately 20 nm at 10-1000 mg L$^{-1}$. Ionic zinc was measured in the exposure media, and although Zn$^{2+}$ was liberated from the MNMs, the concentration of Zn$^{2+}$ was still below the lower limit of toxicity, suggesting particle specific toxicity. L’opez-Moreno et al. (2010a) exposed soybean (Glycine max) to 8 nm hexagonal ZnO MNMs at 0-4000 mg L$^{-1}$. ZnO MNMs did not affect germination rate and increased root elongation at 500 mg L$^{-1}$, but decreased it
above 2000 mg L\(^{-1}\). Ionic Zn controls were not used and the relative contribution of free ion activity and particle specific effects to the observed toxicity are unclear.

1.3.2.2 Ag: Silver MNMs are used in more consumer products than any other MNM\(^{115}\). Studies have repeatedly reported phytoxicity as a result of Ag MNM exposure. In one study, onion (\textit{Allium cepa}) plants were exposed to <100 nm Ag MNMs.\(^{116}\) Plants were exposed for 4 h at 25, 50, 75, and 100 mg L\(^{-1}\) when roots reached 2-3 cm in length. A decrease in mitotic index in response to increased concentration was observed. At 50 mg L\(^{-1}\), chromatin bridging, stickiness, and disturbed metaphase were observed. At 75 mg L\(^{-1}\) chromosomal breaks observed. At 100 mg L\(^{-1}\), there was complete disintegration of cell walls in most of the cells. Hawthorne \textit{et al.} (2012) exposed zucchini plants to Ag MNM and reported concentration dependent reduction in biomass and transpiration starting at 250 mg L\(^{-1}\).\(^{117}\) Toxicity was alleviated by addition of humic acid. Little characterization data was provided and no attempt was made to either determine the extent of dissolution of the soluble MNMs or control for effects of dissolved metals using ionic controls, thus undermining the usefulness of the results.

El-Temsah \textit{et al.} (2010) exposed flax (\textit{Linum usitatissimum}, ryegrass (\textit{Lolium perenne}), and two-rowed barley (\textit{Hordeum vulgare}) to three different sizes of Ag MNMs at 0-100 mg L\(^{-1}\).\(^{118}\) Seeds were placed in a Petri dish on filter paper and 5 mL water or MNM suspension was added to each dish. In a separate exposure, seeds were placed in Petri dishes and covered with 50 g of air dry sandy loam or clay loam wetted to 75% of water holding capacities using water or aqueous MNM suspensions. Ag MNMs inhibited seed germination at 10 mg L\(^{-1}\). There was no clear size dependence for effects as a result of exposure to the Ag MNMs. Inhibition in soil was more pronounced in sandy soil than
in clay soil. Again, no attempt was made to determine the extent of dissolution of the MNMs or control for effects of MNM dissolution once again calling into question the usefulness of the results.

Yin et al. (2011) exposed *Lolium multiflorum* to 6 and 25 nm Ag MNMs at 0-40 mg L\(^{-1}\). The plants were exposed to MNMs with or without the addition of cysteine, which is used to attempt to bind Ag\(^+\) and isolate particle specific effects.\(^{59}\) The efficacy of this technique is unclear, due to the potential for cysteine to sulfudize Ag MNMs.\(^{119}\) Plant growth was reduced by both MNM sizes, with the smaller MNMs reducing growth to a greater degree. Cysteine addition reduced but did not eliminate toxicity, suggesting that toxicity was the result of both dissolved Ag\(^+\) and MNM specific effects. Barrena et al. (2009) reported low or zero toxicity after exposing cucumber and lettuce to 2 nm Ag MNMs.\(^{120}\)

### 1.3.2.3 Carbon:
Carbon MNMs are the second most commonly employed MNM in consumer goods.\(^{115}\) Tan et al. (2009) found that 10-30 nm x 5-15 μm MWCNTs induced ROS generation at 20 and 80 mg L\(^{-1}\) in rice cells.\(^{121}\) Wild et al. (2009) reported that exposure of wheat seeds to 110-170 x 9μm MWCNT at 100 mg L\(^{-1}\) had no effect on root or shoot elongation.\(^{122}\) Canas et al. (2009) measured root elongation in cabbage (*Brassica oleracea*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), onion (*Allium cepa*), and tomato plants exposed to unmodified SWCNTs (CNT) and SWCNTs functionalized w/ poly-3-aminobenzenesulfonic acid (fCNT) at concentrations up to 1,750 mg L\(^{-1}\).\(^{123}\) Tomato root elongation was reduced compared to control in response to both CNT types. Lettuce root elongation was reduced in response to fCNT in lettuce. There was no effect on root elongation in cabbage and
carrot. Root elongation was increased in onion and cucumber in response to CNT. Lin et al. (2007) reported that MWCNTs did not affect seed germination in radish, rape, ryegrass, lettuce, corn, or cucumber. Larue et al (2012) reported no impact on plant development as a result of root exposure to MWCNT. Hawthorne et al. (2012) reported no effect of fullerenes on zucchini biomass or transpiration at concentrations of up to 750 mg L$^{-1}$.

1.3.2.4 CeO$_2$: In Europe, CeO$_2$ MNMs are used a diesel fuel additive to reduce NO$_x$ emissions. As a result, a potential exists for CeO$_2$ MNMs to accumulate to significant concentrations in soil near roadways. Fortunately, studies have consistently demonstrated that CeO$_2$ MNMs are non-phytotoxic and even in some cases beneficial to plants except at extremely high concentrations.

Ma et al. (2010) exposed radish, rape, tomato, lettuce, wheat, cabbage, and cucumber to 7.2 nm CeO$_2$ MNMs at 0-2000 mg L$^{-1}$. The CeO$_2$ were non-toxic at all concentrations to all species except at 2000 mg L$^{-1}$ to lettuce. L’opez-Moreno et al. (2010b) exposed alfalfa (M. sativa) tomato (L. esculentum), cucumber (C. sativus), corn (Z. mays) to 0-4000 mg L$^{-1}$ 7 nm cubic CeO$_2$ MNMs. CeO$_2$ MNMs increased root and stem elongation at all concentrations in cucumber. CeO$_2$ MNMs increased root elongation and decreased stem elongation at high concentration in corn. CeO$_2$ MNMs inhibited root elongation in alfalfa and tomato, but did not affect stem elongation in tomato and increased stem elongation at low concentration in alfalfa. Biomass decreased in corn, and decreased in alfalfa before increasing at 4000 mg L$^{-1}$. In a separate study, L’opez-Moreno et al. (2010a) exposed soybean (Glycine max) to 7 nm cubic CeO$_2$ MNMs at 0-4000 mg L$^{-1}$. CeO$_2$ MNMs had no effect on seed germination and
increased root elongation at all concentrations, although there was some evidence of genotoxicity. Similarly, Wang et al. (2012) reported that exposure to 0.1-10 mg L\(^{-1}\) cerium oxide MNMs with a mean diameter of 20 nm had either no effect or a slightly positive effect on tomato plants.\(^{126}\)

1.3.2.5 Phytotoxicity of Al, Fe, Au, Cu, Pd, Ni(OH)\(_2\), rare earth metals and other MNMs: In addition to the MNM species discussed above, the phytotoxicity of MNMs including La\(_2\)O\(_3\), Gd\(_2\)O\(_3\), Yb\(_2\)O\(_3\), Ni(OH)\(_2\), Fe\(_3\)O\(_4\), Au, Pd, CdSe/ZnS quantum dots, Cu, and Si has been evaluated. Although these studies have provided some important insights into MNM phytotoxicity, these materials are highly unlikely to ever accumulate in the environment in concentrations likely to induce toxicity in terrestrial biota based on current and expected trends in usage.

One early study investigating aluminum toxicity discovered a nano-sized aluminum\(_{13}\) (Al\(_{13}\)) polynuclear species that inhibited root growth in wheat and soybean more than the \(\text{Al}^{3+}\) cation.\(^{127,128}\) A follow up study using 6 different cultivars of wheat with different tolerances to aluminum toxicity was able to demonstrate that the Al\(_{13}\) nanocluster was more inhibitory to root growth in wheat and soybean than the hexaquo aluminum cation in every cultivar.\(^{129}\) Yang et al. (2005) demonstrated that exposure to uncoated 13 nm alumina MNMs inhibited root elongation.\(^{130}\) However, when the particles were loaded with phenanthrene, this inhibition was reduced, suggesting that either that the coating reduced dissolution and the release of ions, which were the likely source of toxicity, or that the coating altered the surface characteristics of the particle making them less available to react with the plant root surfaces. Lin et al. (2007) reported that MNMs with
an Al$^0$ core and either a carboxylate or an Al$_2$O$_3$ shell did not affect seed germination in radish, rape, ryegrass, lettuce, corn, or cucumber.$^{113}$

Ma et al. (2010) exposed radish, rape, tomato, lettuce, wheat, cabbage, and cucumber to 22 nm La$_2$O$_3$, 23 nm Gd$_2$O$_3$, and 12 nm Yb$_2$O$_3$ MNMs at 0-2000 mg L$^{-1}$. La$_2$O$_3$, Gd$_2$O$_3$, and Yb$_2$O$_3$ MNMs reduced root elongation in all plant species at 2000 mg L$^{-1}$. The IC$_{50}$ for rape was 40 mg L$^{-1}$, 20 mg L$^{-1}$ and 70 mg L$^{-1}$ for La$_2$O$_3$, Gd$_2$O$_3$ and Yb$_2$O$_3$, respectively. The concentration of dissolved ions liberated from each MNM treatment was measured. Plants were exposed to this concentration of each treatment and no significant effects were observed in any plant species. In another study researchers exposed cucumber (Cucumis sativis) to 22 nm La$_2$O$_3$ MNMs at 0-2000 mg L$^{-1}$. Acetic acid facilitated MNM dissolution, which led the author’s to speculate that exudation of organic acids by plants would enhance MNM dissolution in the rhizosphere. Both MNMs and an ionic control resulted in toxicity above 200 mg L$^{-1}$, suggesting that the observed toxicity is likely a result of dissolved ions. Formation of needle-like La$_2$O$_3$ nanostructures following exposure was also observed, providing evidence for plant mediated modification of the MNMs.

Shah et al. (2009) exposed lettuce to a variety of MNMs including Au (dodecanethiol functionalized), Cu, 3-aminopropyl Si, and Pd within an aluminum hydroxide matrix. Lettuce seeds were planted in garden top soil that was spiked with MNM treatments. Two experiments were conducted: seeds planted immediately after MNMs added to soil and seeds planted after MNMs had been in soil for 15 d. None of the MNM treatments affected assay endpoints in the day zero exposure. Pd and Au had a positive effect on endpoints in low concentration and Cu and Si in high concentrations in the exposure
Hawthorne et al. (2012) exposed zucchini plants to Au, Cu, and Si NMs and examined effects to biomass production and transpiration. Au MNMs did not adversely affect the plants. However, Cu and Si MNMs reduced biomass and transpiration relative to a bulk (micron sized) control. Toxicity was alleviated by addition of humic acid.

El-Temsah et al. (2010) reported that flax (Linum usitatissimum), ryegrass (Lolium perenne), and two-rowed barley (Hordeum vulgare) seed germination rates in aqueous suspension were strongly inhibited by Fe$^{0}$ MNMs. Inhibition was observed in ryegrass at 500 mg L$^{-1}$ and above 1000 mg L$^{-1}$ for flax and barley. Barrena et al. (2009) reported low or zero toxicity after exposing cucumber and lettuce to 10 nm Au and 7 nm Fe$_{3}$O$_{4}$ MNMs. Parsons et al. (2010) reported that exposure mesquite plants to Ni(OH)$_{2}$ MNMs had no effect on biomass production, shoot elongation or root elongation. Navarro et al. (2012) found that Arabidopsis plants produced reduced glutathione relative to oxidized glutathione after exposure to CdSe/ZnS quantum dots, indicating that the MNMs induced oxidative stress in the plants.

1.4 Uptake and toxicity of MNMs to soil bacteria: In addition to serving as important prey species and aiding plants defend against pathogens, soil bacteria participate in nutrient cycling, organic matter decomposition, and nitrogen fixation, among other important processes. As previously mentioned, many studies have demonstrated that MNMs can inhibit pathogenic bacteria and as the concentration of MNMs in biosolid amended soils increases, there are concerns that beneficial soil bacteria could be adversely affected. For example, one early study demonstrated that 12 nm Ag MNMs induced increases in membrane permeability that led to cytotoxicity in the
model gram negative bacteria *E. coli*. Bactericidal effects have been observed with other types of MNMs as well. Brayner *et al.* (2006) reported that 10-15 nm ZnO MNMs induced cytotoxicity as a result of membrane disorganization in *E. coli*. Stoimenov *et al.* (2002) demonstrated that 4 nm polyhedral and square MgO MNMs with a positive surface charge are toxic to both *E. coli* and the gram positive bacterium *Bacillus megaterium*. Auffan *et al.* (2008) found that magnetite (Fe$_3$O$_4$) and particularly zero-valent Fe MNMs induced oxidative stress in *E. coli*, whereas maghemite (Fe$_2$O$_3$) MNMs were non-toxic.

Recently, Xiu *et al.* (2012) exposed *E. coli* to 0-6.2 mg L$^{-1}$ Ag MNMs with a mean diameter of 35.4 nm coated with amorphous carbon. The authors reported that Ag MNMs were 20 times less toxic than Ag$^+$ and that toxicity in the MNM treatment was reduced under anaerobic conditions that would reduce MNM oxidation or reactive oxygen species generation. However, the authors also presented data indicating that under aerobic conditions, the presence of many common ligands (Cl$^-$, S$_2^-$, PO$_4^{3-}$, and cysteine) reduced the toxicity of silver nitrate, whereas the presence of Cl$^-$ did not affect the toxicity of Ag MNMs, implying that toxicity was not solely the result of free ion activity. The authors speculated that the persistent toxicity of Ag MNMs even in the presence of Cl$^-$ may have been related to intercellular Ag$^+$ delivered by internalized Ag MNMs.

Other studies have reported that intrinsic MNM characteristics may be important factors in predicting toxicity to bacteria via other mechanisms, such as membrane disruption. Morones *et al.* (2005) exposed *E. coli*, *V. cholera*, *P. aeruginosa* and *S. typhus* to a suspension of 1-100 nm carbon coated Ag MNMs. MNMs with a mean
diameter of 5 nm were observed associated with the bacterial membranes and within cells, whereas larger MNMs were not seen interacting with bacterial membranes or internalized within bacterial cells. The authors asserted that the observed toxicity was the result of membrane disruption, intracellular uptake, and subsequent chronic disruption of normal cell function. The authors also suggested that free ion activity resulting from experimentally observed partial MNM dissolution would likely also contribute to toxicity. Cells treated with a AgNO₃ control manifested low density regions enriched in agglomerated DNA, which differs from the damaged membranes and intracellular uptake observed for in cells treated with Ag MNMs. This difference in cellular response suggests a different mechanism of toxicity between the ionic and MNM phases.

Another recent study reported that exposure of *Cupriavidus necator* to either 1-3 nm ZnO MNMs suspended in an acetate solution or Zn (II) (zinc acetate) resulted in different protein expression profiles. Bacteria exposed to ZnO MNMs increased production of membrane bound proteins whereas bacteria exposed to Zn (II) increased proteins associated with metabolism. Also, differences in growth and acetate utilization in response to exposure to ZnO MNM and Zn (II) were observed. Growth was similarly reduced in both MNM and Zn (II) treatments above 25 µM. However, growth was increased in the Zn (II) treatment between 1000-25 nM, a pattern that was not observed in the MNM treatment. At 1 µM Zn (II) in the presence of no added nutrients, growth rate and acetate utilization are increased, likely the result of nutrition from low, non-toxic concentrations of zinc. However, no increase in growth or acetate utilization was observed in bacteria exposed to 1 µM ZnO MNMs, suggesting little dissolution of the
ZnO MNMs. These results indicate that the mechanisms of toxicity are likely not the same for both treatments, i.e., there are particle specific effects.

Different shapes of nanoparticles will have different facets and patterns of surface defects, both of which could affect the reactivity of the particle. Pal et al. (2007) demonstrated that nanotriangles were more inhibitory to bacteria than nanospheres. These researchers hypothesized that this effect could be due to the presence of more available facets with \{111\} planes on the nanotriangles that can interact with bacteria surfaces. Earlier research has demonstrated that the reactivity of silver is higher on high-atom-density facets such as \{111\}. Therefore, shapes that present more of these faces will be more reactive, and may have a higher potential for toxicity. Differences in reactivity would also be expected for shapes with more edges, corners, and surface defects, where atoms are likely to be active and easily dissolved.

How MNM accumulation in soils will affect soil microbial communities is unclear. Shah et al. (2009) reported that Au, Si, Pd and Cu MNMs had no effect on soil microbial populations. Doshi et al. (2008) found that MNMs with an \text{Al}^0 \text{ core and either a carboxylate or an Al}_2\text{O}_3 \text{ shell did not affect respiration or glucose production. Tong et al. (2007) reported that neither aqueous nor granular C}_60 \text{ fullerenes affected soil microbial communities or soil enzyme activities. However, Choi et al. (2008) demonstrated that 14 nm Ag MNMs inhibited respiration of autotrophic nitrifiers taken from an area wastewater treatment facility by 86\% compared to 42\% and 46\% for AgNO}_3 \text{ and 250 nm AgCl colloids, respectively. Hänsch and Emmerling (2010) reported concentration dependent reduction of soil microbial respiration, microbial biomass and enzyme production in a sandy loam in response to application of a commercially available Ag}
MNM containing plant growth promoting spray.\textsuperscript{146} The Ag MNM spray was not characterized and ionic silver controls were not used and considering that dissolved Ag has been shown to be detrimental to soil bacteria,\textsuperscript{147} it is unclear if toxicity is the result of dissolved Ag or Ag MNMs.\textsuperscript{146} Interestingly, Calder \textit{et al.} (2012) reported that soil components alleviated toxicity of 10 nm Ag MNMs at 1 and 3 mg L\textsuperscript{-1} to the free-living plant growth promoting rhizobacteria (PGPR) \textit{Pseudomonas chlororaphis}.\textsuperscript{148-149} When bacteria were exposed in soil, no toxicity was observed. However, when bacteria were exposed to the MNMs in sand, toxicity was observed. This toxicity was ameliorated though addition of either soil pore water or humic acid.

Ge \textit{et al.} (2011) reported that TiO\textsubscript{2} and ZnO MNMs reduced microbial biomass and microbial community composition and diversity in a pH 6 microcosm containing a Mollisol.\textsuperscript{150} Kumar \textit{et al.} (2011) investigated how 0.066\% 20 nm Cu, 20 nm Ag, and 15 nm SiO\textsubscript{2} affected soil microbial communities.\textsuperscript{151} The toxicity of Ag MNMs to cultured \textit{Bradyrhizobium canariense} was also evaluated. The Cu and SiO\textsubscript{2} MNMs did not affect the soil microbial community. However, the Ag MNMs were toxic both to the soil microbial community and to the isolated bacteria. Du \textit{et al.} (2011) found that two months after applying predominately 20 nm TiO\textsubscript{2} and platy, 40 nm ZnO MNMs to a pH 7.5 loamy clay, catalase, peroxidase and soil protease activity were inhibited compared to a control.\textsuperscript{80} No ionic controls were used in either Ge \textit{et al.} (2011), Kumar \textit{et al.} (2011) or Du \textit{et al.} (2011) and as a result, it is unclear if the effects reported from Ag and ZnO MNM applications are the result of free metal ions. The effects of MNMs on soil bacteria are reviewed in detail elsewhere.\textsuperscript{149,152}
1.5 Fungal bioaccumulation and mycotoxicity of MNMs: Terrestrial fungi are involved in providing many critical ecosystem services including driving carbon and nitrogen cycling\textsuperscript{153} and mineral weathering,\textsuperscript{154} as well as serving as plant growth promoting agents. Arbuscular mycorrhizae (AM), now wholly classified within the phylum \textit{Glomeromycota}, form obligate symbioses with greater than 80\% of vascular plants.\textsuperscript{155} Being endomycorrhizae, AM hyphae penetrate root cell walls and form dendritic structures called arbuscles within host plant root cells.\textsuperscript{155-157} The fungi take up inorganic ions, notably phosphate, from the soil using their hyphal network, or mycelium, and transfer them to the host plant in exchange for carbohydrates via the arbuscles.\textsuperscript{155-157} This symbiosis is also important in enhancing plant drought resistance,\textsuperscript{158} disease resistance\textsuperscript{159} and heavy metal tolerance.\textsuperscript{160} Given the intimate interaction between mycorrhizae and the soil, mycorrhizae may be particularly sensitive to MNM contamination. If the soil accumulation of MNMs due to land application of biosolids can result in toxicity to mycorrhizae, there is potential for secondary consequences to the terrestrial plants that depend on mycorrhizae as well as potential impacts to nutrient and carbon cycling.\textsuperscript{153}

Currently, very little information is available with which to assess possible toxicity of MNMs to beneficial terrestrial fungi. To our knowledge, only one study has been published examining the effect of MNMs on mycorrhizae. In this study, researchers exposed \textit{Helianthus annuus} grown in soil either inoculated with mycorrhizae or not to Ag MNM, either in the presence or absence of activated carbon. These researchers reported that Ag MNMs inhibited the rate of mycorrhizal colonization, Cs uptake, and biomass production. However, the researchers did not measure Ag dissolution and did not use
ionic Ag controls. As a result, MNM specific toxicity cannot be confirmed, as it is unclear whether the reduced Cs uptake is the result of competing Ag\(^+\) ions and whether the reduced mycorrhizal colonization and plant biomass production are the result of Ag\(^+\) toxicity. Another study by Whiteside et al. (2009) used quantum dots conjugated with amino groups and confocal microscopy to trace hyphal uptake of nitrogen from culture media by the saprotroph Penicillium solitum\(^{79}\). These researchers reported that the fungi readily bioaccumulated the quantum dots conjugated with amino groups, but not the bare quantum dots. No effects were measured and the potential that surface charge may have been an important variable in explaining their results was not considered.

A handful of studies have evaluated the possibility of using Ag MNMs as a fungicide for pathogenic fungi. In one such study, researchers reported that Ag MNMs increased the zone of inhibition, or distance from a treated disk where fungal growth was inhibited, of fluconazole, a common fungicide, to several pathogenic fungi.\(^{161}\) The same researchers used flow cytometry, TEM, and measurements of membrane fluorescence anisotropy to demonstrate that the same 3 nm Ag-MNMs induced toxicity through the disruption of cellular membranes and interruption of the cell cycle in Candida albicans cells.\(^{162}\) Another study demonstrated significant antifungal activity of Ag MNMs to cells from several species of dermatophytes.\(^{163}\) Unfortunately, none of these studies adequately investigate the relative contribution to toxicity of the free metal ion, pristine MNMs, and a-MNMs, limiting the interpretation of their results. Another study that did use ionic controls reported that Ag MNMs inhibited Candida. spp. cell growth at lower concentrations (0.21 mg L\(^{-1}\)) than solutions of AgNO\(_3\).\(^{164}\) However, this study did not attempt to quantify the amount of Ag\(^+\) that had been liberated from the MNMs. To date,
there have been no studies published investigating how MNM properties such as surface charge, coating hydrophobicity, or particle size might affect MNM-fungal interactions.

Fungal cells are different from animal and bacteria cells in that they possess a chitinous cell wall and their membranes contain different sterols than bacterial, mammalian, or plant membranes. How these structural traits will affect mycotoxicity of MNMs is unknown. Research has demonstrated that there are variations in fungal spore surface chemistry between different fungal species\textsuperscript{165} and between fungal spore types.\textsuperscript{166-168} Douglas \textit{et al.} (1959) demonstrated that spores from different fungal species have different electrophoretic mobilities and speculated that differences in density of spore wall bound carboxylic groups might be responsible. Blastospores and conidia from \textit{Beauveria bassiana} have both been shown to carry a negative surface charge over the pH range 4.5-9.0, whereas aerial conidia have hydrophobic and blastospores have hydrophilic surfaces.\textsuperscript{167-168} Additionally, different spore types will have different cell-wall-surface carbohydrates, resulting in variations in protein binding properties and exposed functional groups.\textsuperscript{165,168} These variations affect how fungal spores bind to various substrates, as aerial conidia have been shown to adhere rapidly to both hydrophobic and hydrophilic surfaces, whereas blastospores bind poorly to hydrophobic surfaces and rapidly to hydrophilic surfaces.\textsuperscript{166} These differences in fungal cell surface chemistry will likely affect how MNMs having variable surface charge and hydrophobicity will interact with fungal cells and what role this variable surface chemistry may have in differential toxicity between fungal species. However, this has yet to be systematically investigated and very little information exists with which to predict possible effects of MNM and a-MNM exposure to terrestrial fungi. Considering the
demonstrated toxicity of many types of MNMs\textsuperscript{2,161-162} and the important ecosystem services performed by mycorrhizae,\textsuperscript{157} there is an urgent need to begin to investigate the risk to ecosystem health posed by potential mycotoxicity of soil accumulated MNMs.

**1.6 Uptake and toxicity of MNMs to soil invertebrates:** Soil invertebrates such as arthropods, nematodes and anelids participate in many important terrestrial ecosystem services including soil formation, nutrient cycling and improvement of soil physical properties in addition to being important prey species in numerous terrestrial food webs.\textsuperscript{37,169} As MNMs accumulate in the soil, a potential exists for soil invertebrates to be affected, influencing the ecosystem services and food webs that these organisms participate in.

**1.6.1 Arthropods:** Only a handful of studies have examined the bioavailability and toxicity of MNMs to arthropods (Table 1.1). One early study examined the effects of feeding *Porcellio scaber* individuals dried hazelnut leaves with 15 nm bare TiO\textsubscript{2} MNMs dried onto the leaf surfaces at 0.1-3,000 mg kg\textsuperscript{-1}.\textsuperscript{170} No effects were observed on isopod mortality, growth or reproduction after 3 d of exposure. However, enzyme activities were affected in a concentration independent manner. A follow up study, designed very similarly, examined the effects of <25 and <75 nm bare TiO\textsubscript{2} MNMs on *Porcellio scaber*.\textsuperscript{171} The isopods were exposed for either 3 d or 14 d. Toxicity was observed in individuals who were exposed for 14 d. Toxicity was not correlated with either particle size or concentration.

Other researchers have examined the effects of bare ZnO MNMs on *Folsomia candida* (springtail)\textsuperscript{172-173}. The results of these studies suggest that springtail will not be adversely affected by exposure to concentrations of ZnO MNMs that are likely to exist in
the environment. Manzo et al. (2011) examined soil avoidance and reproductive toxicity in *Folsomia candida* exposed to 103 nm bare ZnO MNMs at 286 mg kg\(^{-1}\) in an artificial soil\(^{172}\). The authors reported that there was little evidence that the springtail strongly avoided the spiked soil. Furthermore, the MNMs seemed to stimulate springtail reproduction. Kool et al. (2011) exposed the arthropods to < 200 nm in a pH 5.5 sandy loam at 100-6400 mg kg\(^{-1}\).\(^{173}\) Mortality was unaffected at even the highest concentration, whereas the EC\(_{50}\) for reproduction was 1954 mg kg\(^{-1}\).

**1.6.2 Nematodes: Caenorhabditis elegans**, a soil nematode, is often used as a model soil invertebrate due to its rapid life cycle, the ease in which it is cultured, and the fact that the entire organism genome has been sequenced. Additionally, useful transgenic and mutant strains of *C. elegans* have been developed and coupled with RNAi technology, allow for sophisticated gene expression analyses.\(^{174}\) As a result, many studies have been conducted examining the toxicity of MNMs to *C. elegans* (Table 1.2) One early study by Kim et al. (2008) reported that exposure to platinum MNMs behaved as a superoxide dismutase (SOD) catalase mimetic, alleviating oxidative stress and prolonging the lifespan of *C. elegans*.\(^{175}\) However, studies exposing *C. elegans* to environmentally relevant MNM compositions such as CeO\(_2\), Ag, TiO\(_2\) and ZnO have consistently demonstrated toxicity\(^{58,176-177}\).

**1.6.2.1 Metal MNMs: Ag, Au, Cu/CuO:** Roh et al. (2009) exposed *C. elegans* to < 100 nm Ag MNMs and AgNO\(_3\) controls at 0.05 to 0.5 mg L\(^{-1}\) and examined survival, growth, reproduction and changes in transcription profiles.\(^{176}\) Exposure to the Ag MNM treatment resulted in significant, concentration dependent reduction in reproduction and
increases of transcription of superoxide dismutase-3 (sod-3) and abnormal dauer formation protein (daf-12) compared to the ionic control.

Yang et al. (2012) exposed C. elegans to 7 nm citrate, 5 and 22 nm gum arabic, and 8 and 38 nm polyvinylpyrrolidone coated Ag MNMs in moderately hard water and in K⁺ media. EC₅₀ values in the K⁺ media ranged from 0.9-50 mg MNM L⁻¹ compared to 10 mg L⁻¹ for the control. EC₅₀ values in the moderately hard water ranged from 0.09-11.7 mg Ag L⁻¹ compared to 1 mg L⁻¹ for the control. The authors speculated that the amount of dissolved Ag liberated from the MNMs was directly related to toxicity, as measured by growth inhibition. However, MNM dissolution was not measured at the MNM EC₅₀. No significant differences in toxicity as a function of particle size were reported and exposing the nematodes to MNM treatments in a higher ionic strength medium reduced growth inhibition.

Meyer et al. (2010) investigated uptake and toxicity of 7 nm mean diameter citrate coated and 21 and 75 nm PVP coated Ag MNMs at 0.5-50 mg L⁻¹ to wild type and oxidative stress, metals, and genotoxin sensitive mutant strains of C. elegans in pH 6.5 K⁺ media. MNMs aggregated within the K⁺ media, with aggregates between 1-1.6 µm settling to the bottom of the exposure plates and aggregates smaller than 140 nm remaining in suspension. Considering the nematode and their food settled, the authors’ speculate that a local dose higher than the original MNM concentration existed at the bottom of the exposure plates. Uptake of all MNMs was detected via microscopic techniques, with qualitatively higher uptake of smaller MNMs over larger MNMs and of the citrate MNMs over the PVP MNMs was observed. Citrate coated MNMs inhibited growth at 5 mg L⁻¹, whereas the PVP coated MNM did not affect growth until 50 mg L⁻¹.
A metallothionein (mtl-2) deficient mutant strain was more susceptible to the MNMs than the wild type. Measurements of Ag dissolution from the MNMs and use of ionic controls revealed that toxicity was at least in part due to dissolved Ag.

Ellegaard-Jensen et al. (2012) investigated the toxicity of to PVP coated 28 nm Ag MNMs (at 0-3 mg L\(^{-1}\)) and 1 nm Ag MNMs (at 0-10 mg L\(^{-1}\)) to C. elegans in K-medium\(^{179}\). The authors reported lower LC\(_{50}\) and EC\(_{50}\) values for the 28 nm treatment (2.8 and 0.7 mg L\(^{-1}\)) compared to the smaller 1 nm treatment (13.9 and 2.1 mg L\(^{-1}\)), suggesting that the larger Ag MNMs were more toxic. MNM dissolution was measured but no ionic controls were used. As a result, it is unclear if the toxicity observed in this study is the result of dissolved Ag or MNMs. Pluskota et al. (2009) used fluorescence microscopy to demonstrate that Rhodamin B labeled amorphous 50 nm Si MNMs were taken up during feeding by C. elegans and translocated into the intestinal epithelial cells and secondary reproductive organs. Exposure concentrations ranged from 250 mg L\(^{-1}\) to 5000 mg L\(^{-1}\). Exposure to Si MNMs did not affect lifespan. However, reproductive senescence was observed. Pluskota et al. (2009) used fluorescence microscopy to demonstrate that Rhodamin B labeled amorphous 50 nm Si MNMs were taken up during feeding by C. elegans and translocated into the intestinal epithelial cells and secondary reproductive organs\(^{180}\). Exposure concentrations ranged from 250 mg L\(^{-1}\) to 5000 mg L\(^{-1}\). Exposure to Si MNMs did not affect lifespan. However, reproductive senescence was observed.

Tsyusko et al. (2012b) examined toxicogenomic effects induced in C. elegans by exposure to 4 nm citrate coated Au MNMs\(^{181}\). Global genome response using full genome microarrays demonstrated significant differential expression of 797 genes
between Au MNM exposed and control worms, observations that were validated for key genes with QRT-PCR, mutants, and RNA1. Upregulation of genes involved with the noncanonical unfolded protein response (UPR) pathway and molecular chaperones was detected and is likely symptomatic of endoplasmic reticulum stress. Upregulation of genes involved endocytosis including adaptin (apb-1), clathrin heavy chain (chc-1), and rab-11.1 was observed. Further investigation of the role of endocytosis through exposure of endocytosis mutants (dyn-1, chc-1, and rme-2) revealed that two of the three mutants had significantly different responses to the MNMs than the wild type nematodes. Taken together, these results strongly suggest that endocytosis was likely involved in the response of *C. elegans* to the Au MNMs.

1.6.2.2 Metal oxides: ZnO, TiO₂, Al₂O₃, etc.: Ma et al. (2009) exposed a metallothionein-2 green fluorescent protein (mtl-2::GFP) transgenic strain of *C. elegans* to either 1.5 nm ZnO MNMs suspended in an acetic acid-acetate buffered K-medium (0.032 M KCl, 0.051 M NaCl, 0.14 M acetic acid; pH 6) or ZnCl₂ controls in either the buffered K-medium or an unbuffered K-medium at concentrations between 325 and 1,625 mg Zn L⁻¹. Mortality, reproduction, movement behavior and mtl-2 expression, as measured by GFP fluorescence, were reported. Mortality was observed in all three treatments, with LC₅₀ equal to 780, 884, and 348 mg L⁻¹ for the MNM, ZnCl₂ in buffered K-medium, and ZnCl₂ in unbuffered K-medium treatments, respectively. There were no significant differences in reproduction between nematodes exposed to the MNM treatment (EC₅₀=46 mg L⁻¹) and those exposed to the ZnCl₂ in buffered K medium treatment (EC₅₀=59 mg L⁻¹), with no data for this test were reported for the unbuffered K-medium treatment. Movement was affected at similar concentrations in the MNM.
treatment (EC$_{50}$=636 mg L$^{-1}$) compared to the buffered K-medium treatment (EC$_{50}$=546 mg L$^{-1}$) but movement was only affected at a significantly higher concentration for the unbuffered K-medium treatment (EC$_{50}$=906 mg L$^{-1}$). No significant differences in *mil-2* expression, induced at 33 mg L$^{-1}$ in all three treatments, between the MNM and control treatments between 0-150 mg L$^{-1}$ were reported.

Wang *et al.* (2009) investigated the toxicity of 20 nm ZnO (0.4-8.1 mg L$^{-1}$), 60 nm Al$_2$O$_3$ (10.2-407.8 mg L$^{-1}$), and 50 nm TiO$_2$ MNMs (24-239.6 mg L$^{-1}$) compared to bulk and ionic controls to *C. elegans*. The ZnO MNMs carried an approximately neutral surface charge, whereas the Al$_2$O$_3$ MNMs had a positive surface charge and the TiO$_2$ MNMs had a negative surface charge. The degree of dissolution in the ZnO and Al$_2$O$_3$ treatments was measured and control treatments were formulated based upon the degree of dissolution. Mortality, growth, egg production and reproduction were assessed. The 24h LC$_{50}$ of the ZnO MNMs was not different from the bulk control. However, the 24 h LC$_{50}$ for the Al$_2$O$_3$ (153 mg L$^{-1}$) and TiO$_2$ MNMs (80 mg L$^{-1}$) were significantly different than the bulk material treatments. Toxicity was reported in all treatments and could only partially be explained by MNM dissolution.

Roh *et al.* (2010) examined the effects of 15 and 45 nm CeO$_2$ and 7 and 20 nm TiO$_2$ MNMs at 1 mg L$^{-1}$ on gene expression, survival, growth and fertility of *C. elegans*. The 15 and 45 nm CeO$_2$ and the 7 nm TiO$_2$ MNMs reduced survival and fertility. Toxicity appeared to be size dependent. Exposure to the ceria MNMs and the 7 nm titania MNM induced significantly higher transcription of the stress response gene cytochrome P450 (*cyp35a2*).
1.6.3 Annelids: Segmented worms such as *Eisenia fetida* are also often used as model soil invertebrates and standard toxicity testing protocols have been developed for use with earthworms by the USEPA and the Organization for Economic Cooperation and Development (OECD). Unlike *C. elegans*, these organisms can easily be exposed within soil, allowing for more environmentally realistic exposures.

1.6.3.1 Metal MNMs: Ag, Au, Cu/CuO: Several studies have now demonstrated bioaccumulation and toxicity of a variety of metal MNMs to earthworms (Table 1.3). A consistent theme that has emerged from these studies is that earthworms can bioaccumulate metal MNMs from soil, that these materials can be distributed through tissues, often far from the port of entry with little evidence for elimination. Considering the role of earthworms as a prey species for many terrestrial organisms, these findings have important implications for the mobility of MNMs within terrestrial food webs.

Shoultz-Wilson *et al.* (2011b) exposed *Eisenia fetida* to AgNO₃ and two sizes of polyvinylpyrrolidone (PVP) coated Ag MNMs in two different soils: a naturally occurring sandy loam and a standardized artificial soil. In the artificial soil, significant reproductive toxicity was only observed in organisms exposed to the Ag MNMs at concentrations approximately eight times higher than those at which the effects from ionic Ag were observed. *Eisenia fetida* exposed in the sandy loam accumulated significantly higher concentrations of both Ag MNMs and ionic Ag than those exposed in the artificial soil. Earthworms exposed to AgNO₃ also accumulated significantly higher concentrations of Ag than those exposed to Ag MNMs. No differences in toxicity were observed between based on particle size. Extended x-ray absorption fine structure
spectroscopy analysis of the soils indicated that Ag ions may be responsible for effects on growth and reproduction caused by exposure to Ag MNMs. These results also suggest that soil type is a more important determinant of Ag bioaccumulation from Ag MNMs than particle size.

This finding is consistent with earlier research that examined the role of particle size on the bioavailability and toxicity of copper (Cu) and Au MNMs to *Eisenia fetida*. Unrine *et al.* (2010) exposed *Eisenia fetida* to 5, 20 and 50 mg kg\(^{-1}\) 20 or 55 nm citrate coated Au MNMs\(^{183}\). Upregulation of *mtl* was observed in a HAuCl\(_4\) control population but was not observed as a response to either Au MNM treatment. The authors reported no evidence that the Au MNMs affected growth or mortality, although they did report limited evidence that exposure to the MNMs may affect reproduction at 20 and 50 mg kg\(^{-1}\) for the 20 and 55 nm MNMs, respectively. Bioaccumulation was clearly observed via µXRF mapping, with the Au MNMs demonstrated to be present throughout earthworm tissues. There were no significant differences in toxicity or bioaccumulation as a function of particle size.

These researchers conducted another study examining the bioavailability and toxicity of 5, 20, and 50 mg kg\(^{-1}\) 20-40 or <100 nm bare Cu MNMs to *Eisenia fetida* \(^{37}\). Neither the Cu MNMs nor a CuSO\(_4\) control affected growth, mortality, or reproduction. Significant upregulation of *mtl* was observed in response to both 20 and 50 mg kg\(^{-1}\) Cu MNMs and CuSO\(_4\), suggesting oxidative dissolution of Cu. However, XANES and XRF imaging provided strong evidence that particles were taken up and distributed in tissues. As with the Au MNM study, there were no significant differences in bioaccumulation or toxicity as a function of particle size.
In a follow up study, Shoults-Wilson et al. (2011c) studied the effect of surface coating on the toxicity of Ag MNMs in soil to Eisenia fetida.\textsuperscript{185} Earthworms were exposed to AgNO\textsubscript{3} and Ag MNMs with similar size ranges coated with either PVP (hydrophilic) or oleic acid (amphiphilic). No significant effects on growth or mortality were observed in any treatment. Reproduction was affected only at very high MNM concentrations (727.6 mg kg\textsuperscript{-1} for oleic acid coating and 773.3 mg kg\textsuperscript{-1} for polyvinylpyrrolidone). No significant differences were observed in Ag accumulation or toxicity between earthworms exposed to Ag MNMs with PVP or oleic acid coatings.

Shoults-Wilson et al (2011a) also reported that Eisenia fetida consistently avoid soils containing Ag MNMs and AgNO\textsubscript{3} at concentrations as low as 6.92 mg kg\textsuperscript{-1}.\textsuperscript{60} However, avoidance of Ag MNMs occurred over 48 h, while avoidance of AgNO\textsubscript{3} was immediate. Based on XAFS data, it was determined that avoidance of Ag MNMs could not be explained by release of silver ions since that maximum oxidative dissolution would have yielded Ag\textsuperscript{+} concentrations below where effects were observed in the Ag ion controls or changes in microbial communities caused by the introduction of Ag.

Recently, Tsyusko et al. (2012) investigated stress response gene expression and oxidative protein damage in Eisenia fetida in response to exposure to soils spiked with either 10 or 30-50 nm PVP coated Ag MNMs as a function of time\textsuperscript{186}. Overall, gene expression patterns were similar between MNM and ionic treatments, though there were some exceptions, and protein oxidation occurred as a result of exposure to both forms of Ag. However, this study suggests that the mechanisms of delivery of MNMs compared to ionic Ag may result in different uptake and internalization rates, with internalized Ag MNMs emitting Ag\textsuperscript{+} within the organism cells as a result of \textit{in vivo} dissolution.
1.6.3.2 Metal oxides: ZnO, TiO₂, Al₂O₃, etc.: In a study examining the toxicity of a wide range of both metal and metal oxide MNMs to earthworms, Henkmann et al. (2011) reported increased mortality and reduced reproduction in *Eisenia fetida* as a result of exposure to 30-50 nm Ag, 80 nm Cu MNMs, and 21 nm TiO₂ MNMs, but observed no effects in worms exposed to Ni, Al₂O₃, SiO₂, TiO₂ and ZrO₂ MNMs. Coleman et al. (2010) investigated toxicity of Al₂O₃ MNMs to *Eisenia fetida*. The MNMs affected reproduction above 5000 mg kg⁻¹ and the worms avoided spiked soil above 2500 mg kg⁻¹. Similarly, McShane et al. (2012) reported that *Eisenia fetida* and *Eisenia andrei* avoided soil contaminated with 21 nm bare TiO₂ MNMs between 1000 and 5000 mg kg⁻¹, despite no evidence of overt toxicity.

Hu et al. (2010) exposed *Eisenia fetida* to 10-20 nm TiO₂ and 10-20 nm ZnO MNMs in pH 6 artificial soil at 100-5000 mg kg⁻¹. Bioaccumulation of both MNM types was observed. Both MNM types damaged gut cell mitochondria at 5000 mg kg⁻¹ and inhibited antioxidant responses above 1000 mg kg⁻¹. Hooper et al. (2011) exposed *Eisenia veneta* to <100 nm bare ZnO MNMs at 250 and 750 mg kg⁻¹. Toxicity in the worm population exposed to MNMs was lower than in the population exposed to ionic controls.

Canas et al. (2011) investigated the effects of 32 nm TiO₂ and 40-100 nm ZnO MNMs on *Eisenia fetida*. Fourteen day acute toxicity tests were conducted on filter paper and in sand at 0.1-10,000 mg MNMs L⁻¹. In the filter paper contact test, ZnO MNMs were found to be acutely toxic at even 0.1 mg L⁻¹, whereas TiO₂ were not acutely toxic even at 10,000 mg L⁻¹. Neither MNM treatment was acutely toxic in sand. Four week reproduction assays were performed in the sand and a pH 6-7 artificial soil media. Both MNM treatments reduced reproduction in 4 week toxic to the earthworms during a
4 week exposure in artificial soil. However, no attempts were made to quantify ZnO MNM dissolution in any of the three media and no Zn\textsuperscript{+} controls were used and as a result, it is unclear how much of the observed toxicity is the result of the presence dissolved Zn.

In another study, the toxicity of bare 30 nm ZnO MNMs was evaluated in agar plates spiked with MNMs and made using either reconstituted water (RW) or deionized water (DI).\textsuperscript{193} Toxicity was greater on the agar plates made with DI (96 h LC\textsubscript{50}=232 mg kg\textsuperscript{-1}) than on the plates made with RW (96 h LC\textsubscript{50}=374 mg kg\textsuperscript{-1}), which the authors speculated was due to the high ionic strength of the RW inducing MNM aggregation and reducing bioavailability. A separate filter paper contact exposure investigated the effect of background humic acid concentration on toxicity. Humic acid both reduced toxicity as well as increased dissolution. Significant mortality was observed at a lower concentration (50 mg kg\textsuperscript{-1}) in this assay than was observed for the agar plate exposure, suggesting that the exposure environment was an important variable.

Henkmann \textit{et al.} (2011) investigated the toxicity of Ag, Cu, Ni, Al\textsubscript{2}O\textsubscript{3}, SiO\textsubscript{2}, TiO\textsubscript{2} and ZrO\textsubscript{2} MNMs to \textit{Eisenia fetida} at 1000 mg kg\textsuperscript{-1}.\textsuperscript{187} Toxicity was observed as a result of exposure to Ag, Cu, and TiO\textsubscript{2} MNMs. However, the contribution of dissolved material for the Ag and Cu MNMs was unclear.

1.6.3.3 Carbon: A series of studies conducted by researchers at the National Institute of Standards and Technology (NIST) that investigated bioaccumulation of single-walled carbon nanotubes (\textit{SWCNTs}) and multi-walled carbon nanotubes (\textit{MWCNTs}) by earthworms reported little bioaccumulation or toxicity. In one such study, little bioaccumulation of radiolabeled SWCNTs and MWCNTs was observed in the earthworm \textit{Eisenia fetida}.\textsuperscript{194} Similar results were reported when exposing \textit{Lumbriculus vaeriegatus}
to the same CNT treatments\textsuperscript{195} or when exposing \textit{Eisenia fetida} to polyethylenimine (PEI) modified SWCNTs and MWCNTs.\textsuperscript{196}

\textbf{1.7 Dietary uptake and trophic transfer of MNMs in terrestrial ecosystems:} In the past, dietary exposure and trophic transfer have proven to be important terrestrial exposure pathways to polychlorinated biphenyls (PCBs), methylmercury, and dichlorodiphenyltrichloroethane (DDT).\textsuperscript{21} As terrestrial food webs are a possible pathway for human exposure,\textsuperscript{20} there is an urgent need to examine the trophic transfer and fate of MNMs in terrestrial ecosystems under environmentally relevant scenarios.

Some early studies examined uptake through the ingestion of MNMs and reported that although bioaccumulation occurred, dietary uptake was not an efficient exposure route by which nanoparticles could be absorbed. In one example, 98\% of water solubilized C\textsubscript{60} fullerenes administered to rats were excreted within 2 days.\textsuperscript{197} Another study reported minimal bioaccumulation as well as size dependency for uptake via the rat GI tract, with 6.6\% of administered 50nm, 5.8\% of 100nm, 0.8\% 1 \textmu m, and 0\% of 3 \textmu m particles taken up via Peyer’s patches.\textsuperscript{198}

However, these investigations were confined to mammals. Many of the more recent studies discussed in section 8 have reported bioaccumulation in soil invertebrates through ingestion of MNM contaminated soil or food, a simple form of trophic uptake. Recently, a handful of studies have examined the potential for trophic transfer from one terrestrial organism to another in simulated simplified food chains. We demonstrated the biomagnification of 5, 10 and 15 nm tannic acid coated Au MNMs from \textit{Nicotiana tabaccum} to \textit{Manduca sexta}. Tobacco plants were hydroponically exposed to MNM suspensions and subsequently fed to hornworm caterpillars. Tobacco plants
bioaccumulated significant concentrations of all three MNM treatments. The hornworms accumulated and biomagnified the ingested plant-accumulated Au MNMs by mean factors of 6.2, 11.6, and 9.6 for the 5, 10, and 15 nm treatments, respectively.\textsuperscript{87} A follow up study was conducted examining bioaccumulation in hornworms via consumption of surface contaminated plant and comparing the efficiency of bioaccumulation by this pathway with the earlier study.\textsuperscript{199} Bioaccumulation efficiency (bioaccumulation factor=0.16) was much lower for dietary consumption of surface contaminated plant material than for consumption of plant material within which MNMs had been accumulated via root uptake.

Similarly, Unrine \textit{et al.} (2012) investigated trophic transfer of tannic acid Au MNMs from earthworms to bullfrogs and compared transfer efficiency of this pathway with direct gut gavage exposure in the presence of control worms.\textsuperscript{200} As was the case with the hornworms, bioaccumulation efficiency in the bullfrogs was higher via ingestion of the earthworms than by gut gavage. The authors speculated that this observation may have been the result of MNMs remaining in the bullfrog gut longer when incorporated into the earthworm tissues, surface modification of the MNMs during bioaccumulation by the earthworms, or trophic filtering.\textsuperscript{200-201}

\textbf{1.8 Conclusion and future direction:} The nanotechnology industry continues to expand rapidly, with research predicting that $2.6$ trillion in products employing nanotechnology will be sold in 2014.\textsuperscript{202} Commensurate with the growth in the number of products containing MNMs is the expected increase in the release of MNMs from these products to the environment. While many of the benefits of nanotechnology are transformative, there remain questions regarding the potential longer-term risks of
MNMs to the environment and human health. Furthermore, there is a general consensus that there currently is a lack of data on the hazards of many of the MNMs currently in use and concern over the ability to generate data to evaluate hazards of the large number of products anticipated to reach the market. What data are available appear to be contradictory in many cases. Although the reasons for these contradictions are unclear, they are possibly the result of inadequate MNM characterization, experimental artifacts, or variations in experiment methods, such as different exposure times, MNM exposure media, and analysis detection limits, which limit the identification of unifying principles.

What is now apparent is that bioaccumulation of MNMs is possible for many terrestrial organisms and particle specific toxicity is possible under certain circumstances. What is less clear is whether or not this will be the case under environmentally realistic scenarios, although the demonstration of MNM uptake by earthworms from soil and the distribution in tissues remote from the portal of entry suggests that this looms as a very real possibility for a wider range of biota. As our understanding has evolved and techniques used in examining nanotoxicology have advanced, concerns have been raised about the relevance of data collected under exposure scenarios using traditional exposure media such as K-medium or Hoagland’s nutrient solutions. The high ionic strengths of these media heavily favor aggregation and may not adequately represent environmental conditions. Furthermore, many studies have reported data examining the toxicity of pristine MNMs to a variety of test organisms without considering the likely transformations that these MNMs will undergo prior to accumulation in soil. Thus, many of these data likely have limited environmental relevance and there is an urgent
need to move more swiftly to environmentally relevant exposure scenarios to advance the assessment of realistic ecological risks.

As described above, several studies have examined genomic and proteomic responses of organisms to metal and metal oxide MNMs and aged or transformed MNMs and compared these to the comparable concentrations of the metal free ion. Some of these studies have demonstrated particle and dissolved ion specific responses or common responses that could not be explained by the total free ion concentrations alone, providing unequivocal evidence for particle specific effects (Meyer 2010, Neal 2012, Tsyusko 2012a, Tsyusko 2012b). As this research area continues to emerge we believe that unique particle responses in gene and protein regulation can be potentially exploited as a biosensor for detecting MNMs and teasing out MNM vs. dissolved metal ion effects in complex media. Thus, organismal response manifested in differential gene and protein expression potentially represent a signal related to what the organism is experiencing in complex systems at environmentally relevant concentrations, where particle characterization is otherwise extremely challenging, impractical, or impossible. Exploring this possibility presents an interesting and important area for future research.

There is a critical need to conduct further research to inform the development of a framework for predicting the hazards and risks of MNMs and a-MNMs supported by advances in fundamental science and engineering. Variations in intrinsic MNM properties such as size, surface charge and coating molecule may be critical variables controlling uptake for some terrestrial organisms and should be systematically investigated under environmentally realistic conditions using a broad variety of ecoreceptors. Sequential aging experiments evaluating the ultimate speciation of MNMs
in different depositional environments should be conducted and these a-MNMss should be tested for toxicity to relevant terrestrial organisms. Studies examining the toxicity of a-MNMss to important terrestrial organisms are necessary. Experiments such as these will clarify the risks associated with MNMs in environmentally relevant conditions and as nanotechnology continues to expand, will be critical in the development of any predictive or regulatory framework.
## Tables

### Table 1.1 Summary of studies investigating bioaccumulation and/or toxicity of MNMs to arthropods

<table>
<thead>
<tr>
<th>MNM Type</th>
<th>Organism</th>
<th>Exposure media</th>
<th>Concentrations tested</th>
<th>Analyzed endpoints</th>
<th>Particle Size, nm</th>
<th>Key Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO$_2$, bare</td>
<td><em>Porcellio scaber</em></td>
<td>Surface contaminated hazelnut leaves (dried)</td>
<td>0.5–3000 mg kg$^{-1}$</td>
<td>Mortality, growth, enzyme activity, feeding and defecation rates</td>
<td>15</td>
<td>Delivered dose unclear; Enzyme activity affected in concentration independent manner; No effect on other endpoints</td>
<td>Jemec <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>TiO$_2$, bare</td>
<td><em>Porcellio scaber</em></td>
<td>Surface contaminated hazelnut leaves (dried)</td>
<td>10–1000 mg TiO$_2$ kg$^{-1}$ dry food (1.35–1025 mg)</td>
<td>Mortality, growth, enzyme activity, feeding and defecation rates</td>
<td>&lt;25, &lt;75</td>
<td>Toxicity observed after 14 d; Toxicity more closely linked to exposure duration than concentration or particle size</td>
<td>Drobne <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>ZnO, bare</td>
<td><em>Folsomia candida</em></td>
<td>Artificial soil</td>
<td>286 mg kg$^{-1}$</td>
<td>Reproduction and avoidance</td>
<td>103</td>
<td>MNMs produced stimulatory effect; Little avoidance (16%) of spiked soil observed</td>
<td>Manzo <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>ZnO, bare</td>
<td><em>Folsomia candida</em></td>
<td>Sandy loam (pH 5.5)</td>
<td>100–6400 mg kg$^{-1}$</td>
<td>Mortality, reproduction</td>
<td>&lt;200</td>
<td>Mortality unaffected up to 6400 mg kg$^{-1}$; MNM EC$_{50}$ for reproduction at 1964 mg kg$^{-1}$; Toxic effects result of dissolution</td>
<td>Kool <em>et al.</em> (2011)</td>
</tr>
<tr>
<td>Au, tannic acid coated</td>
<td><em>Manduca sexta</em></td>
<td>Surface contaminated tomato leaves (undried)</td>
<td>100 mg kg$^{-1}$ suspension applied to leaves; doses ranged from 0-3 mg MNMs</td>
<td>Bioaccumulation</td>
<td>12</td>
<td>Bioaccumulation observed via X-ray fluorescence and bulk analysis but efficiency low</td>
<td>Judy <em>et al.</em> (2012)</td>
</tr>
</tbody>
</table>
### Table 1.2 Summary of studies investigating bioaccumulation and/or toxicity of MNMs to the model nematode *C. elegans*

<table>
<thead>
<tr>
<th>MNM Type</th>
<th>Exposure media</th>
<th>Concentrations tested</th>
<th>Analyzed endpoints</th>
<th>Particle Size, nm</th>
<th>Key Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt</td>
<td>Liquid S-medium with OP 50</td>
<td>0.1-1 mM</td>
<td>Oxidative stress generation</td>
<td>2.4</td>
<td>Oxidative stress was alleviated and lifespan was prolonged</td>
<td>Kim <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>Si</td>
<td>OP 50 treated with either MNMs or control</td>
<td>250-5000 mg L(^{-1})</td>
<td>Bioaccumulation and translocation, mortality, reproduction</td>
<td>50</td>
<td>No effect on mortality or lifespan; reproductive senescence observed</td>
<td>Pluskota <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>ZnO</td>
<td>Buffered K(^{+}) medium</td>
<td>325-1,625 mg L(^{-1})</td>
<td>Mortality, reproduction, movement behavior and gene expression</td>
<td>1.5</td>
<td>No significant differences between MNM treatment and ZnCl(_2) control</td>
<td>Ma <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>ZnO(_2), Al(_2)O(_3), and TiO(_2)</td>
<td>Ultrapure water</td>
<td>0.4-8.1 mg L(^{-1})</td>
<td>Mortality, growth, fertility and reproduction</td>
<td>ZnO=20, Al(_2)O(_3)=60, TiO(_2)=50</td>
<td>All MNM treatments toxic compared to bulk and ionic controls; Toxicity only partially explained by MNM dissolution</td>
<td>Wang <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Ag</td>
<td>K(^{+}) media, no food</td>
<td>0.05 to 0.5 mg L(^{-1})</td>
<td>Mortality, growth, reproduction, gene expression</td>
<td>&lt; 100</td>
<td>Incubated without food for 20 h prior to MNM exposure; Concentration dependant reduction in reproduction and upregulation of superoxide dismutase compared to ionic control</td>
<td>Roh <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>CeO(_2) and TiO(_2)</td>
<td>K(^{+}) media, no food</td>
<td>1 mg L(^{-1})</td>
<td>Mortality, growth, fertility, reproduction, gene expression</td>
<td>CeO(_2)=15, TiO(_2)=7, 20</td>
<td>Incubated without food for 20 h prior to MNM exposure; Both sizes of CeO(_2) MNM toxic and 7 nm TiO(_2) reduced survival and fertility and increased stress response gene expression; toxicity size dependant</td>
<td>Roh <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>Ag; citrate and PVP coated</td>
<td>pH 6.5 K(^{+}) media</td>
<td>0.5-50 mg L(^{-1})</td>
<td>Bioaccumulation, mortality, growth, gene expression</td>
<td>Citrate=7, PVP=21, 75</td>
<td>MNMs aggregated in K-media; MNMs inhibited growth; Toxicity at least partly due to dissolution</td>
<td>Meyer <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>Ag; PVP coated and other (coating not reported on second treatment; likely bare)</td>
<td>K(^{+}) media plus cholesterol; with and without food</td>
<td>Bare=0, 0.5, 1, 3, 5, 7, 10 mg L(^{-1}); PVP=0, 0.6, 1, 1.5, 2, 2.5, 3 mg L(^{-1})</td>
<td>Mortality, adverse effect condition</td>
<td>PVP=28, Bare=1</td>
<td>Both MNMs toxic; PVP MNMs more toxic; MNMs more toxic when food is present; MNM dissolution was measured but no ionic controls were used; Contribution by Ag(^{+}) to toxicity unclear</td>
<td>Elleegaard-Jensen <em>et al.</em> (2012)</td>
</tr>
</tbody>
</table>
Table 1.2 Continued

<table>
<thead>
<tr>
<th>Ag; citrate, gum arabic and PVP coated</th>
<th>Moderately hard water and in K⁺ media</th>
<th>0.1-50 mg L⁻¹</th>
<th>Mortality, adverse effect condition</th>
<th>Citrate=7 Gum arabic=22 PVP=8, 38</th>
<th>EC₅₀s in the moderately hard water as low as 0.09 mg L⁻¹ compared to 1 mg L⁻¹ for the control; Toxicity related to Ag dissolution; No significant differences based on particle size</th>
<th>Yang et al. (2012)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au; citrate coated</td>
<td>50% K⁺ media</td>
<td>0, 2.5, 5.5, 7, 15, and 30 mg L⁻¹</td>
<td>Mortality, growth, reproduction, gene expression</td>
<td>4</td>
<td>797 genes affected; MNMs likely induced endoplasmic reticulum stress; endocytosis implicated in uptake</td>
<td>Tsyusko et al. (2012)</td>
</tr>
<tr>
<td>MNM Type</td>
<td>Organism</td>
<td>Exposure media</td>
<td>Concentrations tested</td>
<td>Analyzed endpoints</td>
<td>Particle Size, nm</td>
<td>Key Details</td>
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<tr>
<td>Carbon; Radiolabeled single walled (SWCNT) and multi walled (MWCNT) carbon nanotubes</td>
<td><em>Eisenia fetida</em></td>
<td>2 natural Michigan soils</td>
<td>SWNTs = 0.03 mg/g, MWNTs = 0.3 or 0.03 mg/g</td>
<td>Bioaccumulation</td>
<td>SWCNT=1-2 MWCNT=30-70 (diameters)</td>
<td>No significant bioaccumulation observed</td>
</tr>
<tr>
<td>Carbon; Radiolabeled SWCNTs and MWCNTs</td>
<td><em>Lumbriculus variegatus</em></td>
<td>Artificial soil</td>
<td>SWNTs = 0.03 or 0.003 mg/g, MWNTs = 0.37 or 0.037 mg/g</td>
<td>Bioaccumulation</td>
<td>SWCNT=1-2 MWCNT=30-70 (diameters)</td>
<td>No significant bioaccumulation observed</td>
</tr>
<tr>
<td>Au, citrate coated</td>
<td><em>Eisenia fetida</em></td>
<td>Artificial soil</td>
<td>5, 20, 50 mg kg⁻¹</td>
<td>Growth, mortality, reproduction, gene expression, bioaccumulation</td>
<td>20, 55</td>
<td>Bioaccumulation observed but low; No differences in toxicity or bioaccumulation based on particle size</td>
</tr>
<tr>
<td>Cu, bare</td>
<td><em>Eisenia fetida</em></td>
<td>Artificial soil</td>
<td>5, 20, 50 mg kg⁻¹</td>
<td>Growth, mortality, reproduction, gene expression, bioaccumulation</td>
<td>20-40,&lt;100</td>
<td>Bioaccumulation observed but low; No differences in toxicity or bioaccumulation based on particle size</td>
</tr>
<tr>
<td>TiO₂, ZnO</td>
<td><em>Eisenia fetida</em></td>
<td>pH 7 artificial soil</td>
<td>100-5000 mg kg⁻¹</td>
<td>Bioaccumulation, antioxidant responses</td>
<td>TiO₂= 10-20 ZnO=10-20</td>
<td>Bioaccumulation of both MNM type observed; Toxicity observed only at very high concentrations</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td><em>Eisenia fetida</em></td>
<td>Natural soil</td>
<td>100-10,000 mg kg⁻¹ for toxicity, 625-10,000 mg kg⁻¹ for avoidance</td>
<td>Growth, mortality, reproduction, avoidance</td>
<td>11</td>
<td>Reproduction affected at &gt;3000 mg kg⁻¹; Avoidance unlikely below 2500 mg kg⁻¹</td>
</tr>
<tr>
<td>Ag, Polyvinylpyrrolidone (PVP) coated</td>
<td><em>Eisenia fetida</em></td>
<td>Sandy loam, artificial soil</td>
<td>10, 100, 1000 mg kg⁻¹</td>
<td>Growth, mortality, reproduction, bioaccumulation</td>
<td>10 and 30-50</td>
<td>No differences in toxicity based on particle size; Observed toxicity though to be result of dissolved Ag⁺. Exposure media affected toxicity</td>
</tr>
<tr>
<td>Test Substance</td>
<td>Organism</td>
<td>Test Media</td>
<td>Treatment</td>
<td>End Points</td>
<td>Comments</td>
<td></td>
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<tr>
<td>Ag, PVP and oleic acid (OA) coated</td>
<td><em>Eisenia fetida</em></td>
<td>Artificial soil</td>
<td>10, 100, 1000 mg kg⁻¹</td>
<td>Growth, mortality, reproduction, bioaccumulation</td>
<td>PVP= 10 OA=30-50</td>
<td>No differences in toxicity or bioaccumulation as a function of coating molecule</td>
</tr>
<tr>
<td>Ag, PVP and OA coated</td>
<td><em>Eisenia fetida</em></td>
<td>Sandy loam, low and high Ca artificial soils</td>
<td>0.3-54 mg kg⁻¹</td>
<td>Avoidance</td>
<td>PVP= 10 and 30-50, OA=30-50</td>
<td>MNM avoidance observed at concentrations as low as 6.92 mg kg⁻¹ after 48 h</td>
</tr>
<tr>
<td>TiO₂, ZnO</td>
<td><em>Eisenia fetida</em></td>
<td>On filter paper and in sand</td>
<td>0.1-10,000 mg L⁻¹</td>
<td>14 d acute toxicity</td>
<td>TiO₂= 32 ZnO=40-100</td>
<td>TiO₂ non toxic even at 10,000 mg L⁻¹. ZnO toxic even at 0.1 mg L⁻¹, although no attempt to determine Zn dissolution. No Zn²⁺ controls.</td>
</tr>
<tr>
<td>Carbon; polyethylenimine coated radiolabeled MWCNTs</td>
<td><em>Eisenia fetida</em></td>
<td>2 natural Michigan soils</td>
<td>0.5 mg/g</td>
<td>Bioaccumulation</td>
<td>30-70 nm in diameter, mean length = 407 nm</td>
<td>No significant bioaccumulation observed</td>
</tr>
<tr>
<td>Ag, Cu, Ni, Al₂O₃, SiO₂, TiO₂ and ZrO₂</td>
<td><em>Eisenia fetida</em></td>
<td>Sandy loam</td>
<td>1000 mg kg⁻¹</td>
<td>Growth, mortality, reproduction</td>
<td>Ag=30-50, Cu=80, Ni=20, Al₂O₃=12-14, SiO₂=5-15, TiO₂=21 and ZrO₂=20-30</td>
<td>Toxicity observed in Ag, Cu and TiO₂; Contribution of free ions for Ag and Cu MNMs unclear</td>
</tr>
<tr>
<td>ZnO, bare</td>
<td><em>Eisenia veneta</em></td>
<td>Artificial soil</td>
<td>250 and 750 mg kg⁻¹</td>
<td>Bioaccumulation, growth, mortality, reproduction, immune activity</td>
<td>&lt;100</td>
<td>Bioaccumulation observed with SEM; toxicity greater in ionic controls than in MNM treatments</td>
</tr>
<tr>
<td>ZnO, bare</td>
<td><em>Eisenia fetida</em></td>
<td>Deionized water and reconstituted water agar plates; filter paper</td>
<td>0, 50, 100, 200, 500, and 1000 mg kg⁻¹</td>
<td>Mortality, enzyme activity</td>
<td>30</td>
<td>Toxicity observed in both media unexplained by dissolution; Toxicity occurred at lower conc. (50 mg kg⁻¹) on filter paper; Presence of salts and humic acid reduced toxicity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>Species</th>
<th>Soil Type</th>
<th>Concentration</th>
<th>Endpoint(s)</th>
<th>Study Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂, bare</td>
<td><em>Eisenia fetida</em>, <em>Eisenia andrei</em></td>
<td>Sandy loam and artificial soil</td>
<td>200-20,200 mg kg⁻¹</td>
<td>Growth, mortality, reproduction, avoidance</td>
<td>No toxic effects observed; Worms avoided spiked soil between 1000 and 5000 mg kg⁻¹ and differentiated between bulk and nano TiO₂</td>
<td>McShane <em>et al.</em> (2012)</td>
</tr>
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</tr>
<tr>
<td>Cu</td>
<td><em>Enchytraeus albidus</em></td>
<td>LUFA 2.2 natural standard soil, natural soil</td>
<td>100 and 200 mg Cu kg⁻¹ added to soil</td>
<td>Allocation of energy reserves to lipids, proteins, or carbohydrates</td>
<td>No differences in effects observed between Cu MNMs and ionic Cu control</td>
<td>Amorim <em>et al.</em> (2012)</td>
</tr>
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</tr>
<tr>
<td>Ag, PVP coated</td>
<td><em>Eisenia fetida</em></td>
<td>Sandy loam</td>
<td>100, 500 mg kg⁻¹</td>
<td>Gene expression (stress response), oxidative damage to proteins</td>
<td>Gene expression response to ionic control similar to response from MNM; both sizes induced oxidative stress; toxicity likely result of dissolved Ag⁺</td>
<td>Tsyusko <em>et al.</em> (2012)</td>
</tr>
</tbody>
</table>
Chapter 2: Introduction


http://pubs.acs.org/articlesonrequest/AOR-DXBvBMH93897XUnmHTS9

Manufactured nanomaterials (MNMs) are being discharged into waste streams from the rapidly increasing number of consumer products that employ nanotechnology.\textsuperscript{16,19,45} Studies have consistently demonstrated that MNMs concentrate in the sludge during wastewater treatment,\textsuperscript{16-19} 60\% of which is applied to agricultural land as biosolids in the U.S. and the majority of Europe.\textsuperscript{19} As a result, the MNM concentrations in sludge-treated soil in the U.S. are expected to rise rapidly.\textsuperscript{19} A recent model conservatively predicted increases from 0.1 to 0.5 mg kg\(^{-1}\) for TiO\(_2\) MNMs, from 6.8 to 22.3 \(\mu\)g kg\(^{-1}\) for ZnO MNMs, and from 2.3 to 7.4 \(\mu\)g kg\(^{-1}\) for Ag MNMs between 2008 and 2012.\textsuperscript{19} Despite this, little is known about the toxicity and bioavailability of sludge-accumulated MNMs to plants and other terrestrial organisms following their introduction into the soil.\textsuperscript{21}

In the past few years, many studies have investigated plant uptake of a wide variety of MNMs in many different plant species. For example, early studies demonstrated the uptake of uncoated 20 nm Fe\(_3\)O\(_4\) MNMs by pumpkin plants,\textsuperscript{83} uncoated 50 nm Cu MNMs by wheat and mungbean,\textsuperscript{203} and natural organic matter (NOM) coated 1.19 nm C\(_{70}\) fullerenes by rice.\textsuperscript{100} More recent research has demonstrated the uptake of alizarin red and sucrose coated 2.8 nm TiO\(_2\) MNMs by Arabidopsis thaliana,\textsuperscript{77} 15 nm tannate coated and 3.5 nm citrate coated Au MNMs by Nicotiana tabaccum,\textsuperscript{86-87} and of 6 nm gum arabic
coated silver MNMs by *Lolium multiflorum*.\(^{59}\) Conversely, many studies investigating plant uptake of MNMs have reported no uptake. For example, researchers reported no uptake of uncoated 19 nm ZnO MNMs by ryegrass,\(^{72}\) no uptake of uncoated 37 nm CeO\(_2\) MNMs by maize,\(^{81}\) and no uptake of uncoated 20 nm TiO\(_2\) or uncoated 40 nm ZnO by wheat.\(^{80}\) Many of these studies have been published without adequate MNM characterization or investigation of the localization of MNMs within cells or tissues. Without measurements to establish MNM localization, it can be difficult to eliminate the possibility that reported bioaccumulation is simply reflective of MNMs or dissolved species on the exterior surfaces of the plants.\(^{21}\)

The majority of these studies exposed a single plant species to one MNM treatment or to several different types of MNMs. There have been few studies that have attempted to systematically evaluate the relative importance of particle characteristics or plant species on plant bioaccumulation of MNMs. There is a fundamental difference in the nature of root exudates between monocots and dicots, as these groups have different strategies for obtaining metal nutrients from the soil.\(^{84}\) Therefore, it is reasonable to hypothesize that differences in type and amount of root exudates between plant species might also affect uptake, either by facilitating uptake or by inducing MNM aggregation. Furthermore, each MNM has intrinsic properties that may affect mobility, bioavailability, or toxicity, as well as the likely transformations it will undergo in the environment. These properties include composition, crystal structure, size, shape and surface chemistry. Nanomaterial size is likely to be important to MNM bioaccumulation in plants as plant cell wall pores have been shown to be size selective to macromolecules.\(^{67}\) Nanomaterial surface
chemistry is also likely to be an important factor in plant uptake, as plant cell surfaces will present barriers of varying hydrophobicity and surface charge.\textsuperscript{84}

Considering the fact that plants comprise the base of many terrestrial food webs, there is an urgent need to systematically characterize the factors that control the bioavailability of MNMs to plants. To begin addressing this need, we used the model organisms \textit{Nicotiana tabacum} L. cv \textit{Xanthi} and \textit{Triticum aestivum} to investigate plant uptake of 10, 30, and 50 nm diameter tannate (T-MNMs) or citrate (C-MNMs) coated Au MNMs.\textsuperscript{12} The objectives of the study were: to systematically investigate the importance of MNM size between the range of 10 and 50 nm in plant bioaccumulation of MNMs, to collect data elucidating the importance of MNM surface chemistry on plant bioaccumulation of MNMs, and to collect data clarifying the importance of plant species to plant bioaccumulation of MNMs.

\textbf{2.1 Experimental section}

\textbf{2.1.1 Nanomaterial characterization.} Stable suspensions of 10, 30, and 50 nm diameter primary particle size Au MNMs surface modified with either tannate (Nanocomposix San Diego, CA, USA) or citrate (Ted Pella, Redding, CA, USA) were purchased and the stock suspensions were characterized using transmission electron microscopy (Figure 2.1, Table 2.1).

Au MNMs are being used in applications including medical imaging,\textsuperscript{5} drug delivery,\textsuperscript{6} and fuel cell catalysis.\textsuperscript{7} We selected Au MNMs for this study due to their resistance to oxidative dissolution and low natural background concentrations. These properties make Au MNMs an idea probe for investigating MNM bioaccumulation and translocation.\textsuperscript{204} Tannate is a high molecular weight polyphenol with a log $K_{ow}$ of -0.19$^{205}$ with $pK_{a1}$=
Citrate is low molecular organic acid with log $K_{ow}$ of -1.74 with $pK_a1=3.1$, $pK_a2=4.7$, and $pK_a3=5.4$. Tannate and citrate coated MNMs were selected for this study because we consider these two molecules to be reasonable analogous for common low molecular weight organic acids found in soil and high molecular weight NOM complexes, respectively, both of which we envision could adsorb to MNMs in the soil as is often observed for fine-grained soil mineral surfaces. The suspension concentrations were verified through aqua regia digestion and elemental analysis via inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500cx ICP–MS (Agilent, Santa-Clara, CA, USA). To determine the concentration of dissolved Au in the stock suspensions, samples of the stock suspensions were filtered through a 3 kDa regenerated cellulose membrane (Amicon Ultra, Millipore, Billerica, MA, USA), after which the resulting filtrate was analyzed for Au using ICP-MS. Measurement of the amount of dissolved Au present in suspension filtrates reveals very low concentrations that are mostly below detection (detection limit = 1.37 ng Au mL$^{-1}$). A solution of 10 µg L$^{-1}$ HauCl$_4$ was filtered through one of the membrane filters used to estimate dissolved Au and a recovery of 82.4% was determined, which we have found to be a typical recovery at low concentrations, presumably due to interactions with trace functional groups associated with the membranes.

TEM size analysis of the T-MNMs was provided by the manufacturer (Nanocomposix San Diego, CA, USA) using a Jeol 1010 TEM. The size of the C-MNMs was derived from TEM images performed using a Jeol 2010 TEM. Mean MNM diameter and size ranges were quantified based on measurements of at least 100 individual particles using ImageJ software.
2.1.2 Nanomaterial treatment preparation. Prior to dilution to 30 mg L\(^{-1}\), MNMs were treated to purify the MNM suspensions, buffer the solution to prevent destabilization of the coating, and to attempt to homogenize the pH and electrophoretic mobilities of each MNM treatment. The C-MNMs were washed with a pH 7 sodium citrate-citric acid buffer of a concentration normalized to the surface area of each treatment. For the 10, 30 and 50 nm MNMs, a 1 mM, 0.4 mM and 0.33 mM buffer was used, respectively. All three T-MNM treatments were diluted to 30 mg L\(^{-1}\) with 0.1 mM tannic acid and adjusted to pH 7 with dilute NaOH. T-MNMs were not stable at higher concentrations of tannic acid, possibly due to the relatively high molecular weight of tannic acid, so it was not possible to use the same approach as was used with the C-MNMs. The T-MNMs had been washed 10 times with 18 M\(\Omega\) cm\(^{-1}\) deionized water (DI) by the manufacturer prior to purchase. Mean intensity weighted hydrodynamic diameters and electrophoretic mobilities of the exposure suspensions were measured with a Nano-ZS zetasizer (Malvern, Worcestershire, UK) using 173° backscatter analysis method. Hydrodynamic diameter distributions were converted to a volume basis using a refractive index of 0.2 and absorption of 3.32 (see supporting information, Table S2). All MNM treatment suspensions were highly negatively charged with zeta potentials >-50 mV (Table 2.2). Electrophoretic mobilities were converted to zeta potentials using the Hückel model.

2.1.3 Plant exposure protocol. *Nicotiana tabacum* L. cv Xanthi was selected as a model primary producer for this study due to its demonstrated ability to bioconcentrate metals\(^{213}\) whereas *Triticum aestivum* was selected as a model organism for this experiment because it is an important food crop.
Nicotiana tabacum L. cv Xanthi seeds were sterilized by shaking with 6% sodium hypochlorite for 10 minutes and washing three times with 18 MΩ deionized water (DI)\textsuperscript{214}. Seeds were then placed in plates of gellan gum slanted at 70° (Phytagel, Sigma-Aldrich, St. Louis, MO USA) containing a half strength nutrient mixture (Phytotechnology Laboratories, Shawnee Mission, KS USA) and grown in a growth chamber at a mean temperature of 24 °C and 25% mean relative humidity with a 12 hour light cycle.

Triticum aestivum cv. Cumberland seeds were imbibed in aerated 3 mM CaCl\textsubscript{2} overnight \textsuperscript{215}. Afterwards, the seeds were placed in the dark between moistened paper towels. After three days, the seedlings were moved into a full strength nutrient solution (Phytotechnology Laboratories, Shawnee Mission, KS, USA) in a growth chamber at a mean temperature of 32 °C and 27% mean relative humidity with a 12 hour light cycle.

At 30 days post germination for tobacco and 7 days post germination for wheat, plants were randomly divided into treatment populations and placed in 1.5 mL microcentrifuge tubes. We elected to perform a hydroponic exposure over a soil exposure because we believe that it would be virtually impossible to separate the importance of intrinsic particle properties to plant uptake from the importance of extrinsic properties imparted by soil components to uptake. Fifteen plants were exposed to each of the six treatment combinations. Controls consisted of 5 plants each in DI, 1 mM pH 7 sodium citrate-citric acid buffer and pH 7 0.1 mM tannic acid. Treatment solutions were periodically adjusted to the initial volume with the appropriate buffer. Since wheat does not tolerate nutrient stress as well as tobacco due to differences in its growth and
development, tobacco plants were exposed for 7 days whereas wheat plants could only be exposed for 3 days. Plant growth over the exposure period appeared negligible.

2.1.4 Sample collection and preparation. At the end of each exposure, plant roots were cut from each plant above the level of the MNM suspensions to remove tissue that might have been surface contaminated with MNMs. The aerial portion of each plant was carefully washed with deionized water (DI), citranox, 0.5% HCl/0.5% HNO₃ and then again with DI prior to being dried for bulk analysis by ICP-MS. Other leaf samples were mounted on metal free polyimide film for spatially resolved analysis using laser ablation inductively coupled mass spectrometry (LA-ICP-MS) and scanning x-ray fluorescence microscopy (µXRF). In the wheat exposure, roots were fixed in 10% formalin acetate and subsequently placed in optimal cutting temperature embedding medium (Sankura Finetek, Torrance, CA, USA), frozen using dry ice and stored at -80° C for later cryo-sectioning to 15 µm thickness and µXRF analysis.

2.1.5 Post exposure treatment suspension characterization. The degree to which the MNMs were aggregated during the wheat and tobacco exposures was characterized through post exposure sedimentation analysis of the treatment suspensions. One mL of each suspension was vortex mixed thoroughly and then centrifuged at 1100 x g for 1 minute to sediment aggregates larger than approximately 840 nm according to Stoke’s law calculations. After collecting a 10 µL sample from the supernatant, the treatment suspensions were vortex mixed again and then centrifuged at 11000 x g for 1 minute to sediment aggregates larger than approximately 80 nm after which another 10 µL was removed. As wheat plants have been demonstrated to strongly alkalinize their rhizosphere, the post exposure pH of each treatment suspension from both plants was
recorded and Ca and Mg, as well as Au, concentrations were measured using ICP-MS. These data will be used to clarify the role of root exudates on any observed treatment aggregation.

2.1.6 Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS). LA-ICP-MS depth profiles were collected using a series of controlled laser pulses from a LSX-213 laser ablation system (CETAC, Omaha, NE, USA) that removed 400 x 400 µm² craters. The laser energy and burst duration were calibrated so that these craters were 15 µm in depth. The elemental composition of the material removed during each laser pulse was measured using ICP–MS. Calibration standards for analysis consisted of pellets created by spiking dried and finely ground tobacco to a range of concentrations. A calibration curve was created by simple linear regression of the summed counts from the laser bursts within the depth profile for each standard. Semi-quantitative sample concentrations were calculated by fitting the summed counts from each sample depth profile to this calibration curve.

2.1.7 Synchrotron X-ray analysis. Scanning x-ray fluorescence microscopic measurements of Au were collected at the Au L-α₁ emission line (9,713 eV) employing beamline X-26A at the National Synchrotron Light Source at Brookhaven National Laboratory (Upton, NY, USA). To correct for interference from the Zn K-β₁ emission line (9,572 eV), leaves and root cross-sections were mapped at energies above (12,110 eV) and below (11,850 eV) the Au L-α₃Ⅲ absorption edge (11,919 eV). The below edge signal was subtracted from the above edge signal and the difference reported as the Au signal. Analysis conducted using either a 9 element Ge array detector (Canberra, Merridian, CT) or a 4 element silicone drift Vortex ME4. The incident x-ray beam was
monochromatized at 13 keV. The beam had a spot size of 9 µm horizontally and 5 µm vertically. The beam was translated through the sample in 3 µm steps, and spectra were collected at each step for 0.5 seconds. The monochromator was calibrated to the absorption edge using a Au foil standard.

2.1.8 Inductively coupled plasma-mass spectrometry (ICP-MS) analysis of bulk tissue. Plant samples were oven dried for 48 h at 60° C, weighed and placed in microcentrifuge tubes. The samples were digested overnight at 60° C in a mixture of 50 µL of hydrogen peroxide and 150 µL of nitric acid. Then, 300 µL of hydrochloric acid were added and the samples were heated for an additional 4 hours, after which the digestate was brought to a 3 mL volume12 and analyzed by ICP-MS. Analytical runs contained calibration verification samples, duplicate dilutions, and spike recovery samples. As there is no widely available standard reference material containing Au in plants, a laboratory control sample was prepared using finely ground dried tobacco leaves spiked with Au standard to a concentration of 10 mg kg⁻¹. The measured concentrations were 111.7% of the nominal concentration with a standard deviation of 11.5%. Spike recovery averaged 99.2%, and the mean relative percent difference between duplicate dilutions was 5.5%.

2.1.9 Statistical analyses. All data were tested for normality and homoscedascity using Shapiro-Wilk’s test and Barlett’s test, respectively. Data were log transformed if found not to be normally distributed and retested. Significant differences between ICP-MS plant bioaccumulation data, LA-ICP-MS bioaccumulation data and Ca concentrations in the treatment suspensions post exposure were tested using ANOVA and Student-Newman-Keuls (SNK) means comparisons at α=0.05 when data were normal and
homogenously distributed. Significance of non-normal or data with non-homogenous variance were analyzed using a Kruskal-Wallis and Mann-Whitney U-tests at $\alpha=0.05$. In rare cases, outliers were removed using Grubb’s test.

2.2 Results and discussion

Bulk analysis of the oven dried aerial tobacco biomass reveals mean Au concentrations between 2.2 and 53.5 mg kg$^{-1}$ (Figure 2.3). Significant uptake occurred in every treatment combination. The mean Au concentration in plants exposed to the 50 nm T-MNMs is significantly lower than three of the other treatments but is not significantly different from the 10 nm C-MNM and 30 nm T-MNM treatments. Bulk analysis of the oven dried aerial wheat biomass reveals no significant uptake in any treatment combination.

The Au concentration in the majority of the tobacco leaf samples determined by scanning x-ray fluorescence is below the estimated detection limit of $\sim$1 mg kg$^{-1}$ for Au at beamline X-26A. However, one image demonstrates the presence of Au in detectable concentrations in the leaf mid rib of a plant exposed to 30 nm C-MNMs (Figure 2.4). Images of wheat leaf tissues reveal no evidence of accumulation of Au MNMs in the aerial portions of the plants (Figure 2.5). In subtraction maps of root cross-sections from the wheat roots, Au MNMs are detected adsorbed to the surface of the roots, but there is no evidence that Au MNMs penetrated the root surface in any treatment (Figure 2.6).

Au is detected by LA-ICP-MS throughout cross-sections of tobacco leaves from each treatment (Figure 2.7). Semi-quantitative Au concentrations determined by LA-ICP-MS are $< 1$ mg kg$^{-1}$, consistent with the Au in these samples being below the detection limits of $\mu$XRF imaging at beamline X-26A (Figure 2.8). Each calibration curve $r^2$ is $>$
0.999 and there are no significant differences between mean concentrations as a function of MNM treatment or in concentration as a function of cross-section depth in any treatment.

Characterization of the treatment suspensions after exposures demonstrates that, in general, the wheat plants basified their exposure suspensions more than the tobacco plants, although the mean pH for the 30 nm and 50 nm citrate were similar for the tobacco and wheat samples (Tables 2.3 and 2.4). The results of the sedimentation studies indicate that the MNMs in the wheat exposure suspension aggregated to a greater degree than the MNMs in the tobacco exposures. For example, in the 10 nm tobacco treatments, 61.4% and 67.9% of the T-MNMs and C-MNMs, respectively, were found to be within aggregates larger than 80 nm compared to 88.3% and 93.3% for the wheat treatments.

Post-exposure analyses of Mg in the treatment suspensions show concentrations mostly below the detection limit (~1.1 mg Mg L⁻¹ supernatant). Detectable levels of Ca were measured in each treatment for both plant exposures. However, there are no significant differences in mean Ca concentrations based on MNM size, surface coating or plant species at α=0.05 (Tables 2.3 and 2.4).

This study provides little evidence that primary particle size between 10 and 50 nm is an important factor in plant bioavailability of Au MNMs. We found that bioaccumulation of 50 nm T-MNMs is significantly lower than bioaccumulation of 10 and 30 nm T-MNMs in tobacco, but this trend was not evident for the C-MNMs and the tobacco did accumulate a significant concentration of 50 nm T-MNMs compared to the control. However, it is possible that the large variability in the data set could be masking
trends. Although the reasons for this variability are unclear, we speculate that it is in part the result of the large genetic variability between individual tobacco plants. Regardless, this result contradicts the commonly repeated hypothesis that the MNMs must passively pass through cell wall pores to be taken up by plants and that the cell wall will exclude most MNMs larger than 20 nm. The diameter of most cell wall pores have been estimated to be between 5-20 nm, although recent gas adsorption measurements of cell wall porosity suggest than some cell wall pores may be as large of 50 nm. The mechanism by which MNMs might bypass the plant cell wall is not well understood. Studies on fungal cells have demonstrated that Ag MNMs are able to induce plasma membrane depolarization and protoplast leakage suggesting that MNMs can induce pore formation in cell walls in certain cases. Other studies have provided evidence using confocal microscopy and TEM that clearly demonstrate penetration of the plant cell wall by carbon MNMs. Another possibility is that minor cuts and other physical damage to the root during the exposure could lead to uptake. Alternatively, Liu et al. (2009) demonstrated cellular uptake of single-walled carbon nanotubes by intact tobacco bright yellow (BY-2) cells and reported evidence that endocytosis was the mechanisms of uptake. The results presented here seemingly contradict our earlier study that used μXRF mapping to demonstrate that tobacco plants would bioaccumulate 3.5 nm Au MNMs to a greater extent than 18 nm Au MNMs. In the earlier study we presented spatial data confirming uptake, but did not provide bulk, volumetrically averaged quantitative analysis, thus the data were only semi-quantitative. The present study is not the first to report plant bioaccumulation of larger MNMs. For example, evidence of uptake of magnetite MNMs with a hydrodynamic diameter of approximately 40 nm in
pumpkin plants was presented by Zhu et al., 2008, although they provided no spatially resolved data. Lee et al. (2008) demonstrated uptake of Cu MNMs with a diameter of approximately 50 nm in mungbean and wheat.

These data do not conclusively demonstrate differences in plant bioaccumulation between the two MNM surface coatings. Although the 50 nm C-MNMs were taken up to a significantly ($\alpha=0.05$) lesser degree than the 50 nm T-MNMs, this trend did not exist for the 10 and 30 nm MNMs. Considering that tannate and citrate could be considered similar to the NOM coatings that could adsorb to MNM surfaces after introduction into natural ecosystems, this result suggests that MNM coating might be of minor importance in many environmentally relevant scenarios.

The large difference in uptake between the wheat and tobacco suggests that MNMs might be more bioavailable to some plant species than to others. This is consistent with the number of studies presenting both volumetrically averaged and spatially resolved MNM concentrations reporting greater uptake in dicots than in monocots. However, there has been little systematic examination of variation in MNM bioaccumulation based on plant species and there have been reports of positive and negative results in bioaccumulation studies exposing both monocots and dicots. It is possible that the difference in uptake between tobacco and wheat is the result of the longer exposure time for the tobacco. However, one recent study did not see any leaf translocation of MNMs in wheat after several months of exposure, albeit in soil, and our observation of almost complete aggregation of the MNMs in the wheat treatments at the end of 3 d make it unlikely that additional exposure time would have resulted in fundamentally different results. Additionally, Nedoskin et al. (2011) recently used in
plant flow cytometry to demonstrate that uptake of CNT-quantum dot conjugates by
tomato plants occurs within minutes.\textsuperscript{78} We speculate that the differential
bioaccumulation between the two plant species is likely the result of the differences in
MNM aggregation induced in the treatment suspensions during the exposures and that
these differences in aggregation are the result of root exudation of different compounds
between the two plants. Wheat plants were observed to alkalize their treatment
suspensions to a greater degree than the tobacco plants in most treatments, although Ca
and Mg exuded into the treatment suspensions by the two plant species was not
significantly different. Considering that increased alkalinization should have further
stabilized the negatively charged MNMs, we cannot explain the increased aggregation
observed in the wheat treatments.

In addition to potentially modifying the pH and concentrations of divalent cations in
the rhizosphere, plant roots also exude many other solutes including mucilage, enzymes,
sugars, phenolics, and amino acids that were not measured in this study, any of which
could potentially affect MNM aggregation and bioavailability.\textsuperscript{84} As previously
mentioned, in some cases the differences between the exudates of monocots and dicots is
dramatic. For example, monocots like wheat exude amino acids such as mugineic acid in
response to iron deficiency whereas dicots like tobacco exude phenolic and reducing
compounds\textsuperscript{84}. Differences in the amount and nature of the exudation between species
such as this could play a major role in inducing aggregation and affecting bioavailability
of MNMs and further investigation into this possibility represents an interesting area for
future investigations.
Tables

Table 2.1 Summary of characterization data for nanomaterial stock suspensions. Includes TEM measurements of particle size and measurements of the amount of dissolved Au in stock suspension filtrate. TEM data for tannate coated MNMs provided by manufacturer (Nanocomposix, San Diego, CA USA).

<table>
<thead>
<tr>
<th>Nominal Diameter (nm)</th>
<th>Coating</th>
<th>TEM Diameter (mean ± SD, nm)</th>
<th>TEM min/max diameter (nm)</th>
<th>Dissolved Au in filtrate (% wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Tannate</td>
<td>11.0 ± 0.9</td>
<td>9.1/14.2</td>
<td>0.046</td>
</tr>
<tr>
<td>30</td>
<td>Tannate</td>
<td>28.8 ± 3.3</td>
<td>20.1/35.2</td>
<td>BDL</td>
</tr>
<tr>
<td>50</td>
<td>Tannate</td>
<td>47.7 ± 4.7</td>
<td>37.7/59.2</td>
<td>BDL</td>
</tr>
<tr>
<td>10</td>
<td>Citrate</td>
<td>9.7 ± 0.8</td>
<td>8.1/11.6</td>
<td>BDL</td>
</tr>
<tr>
<td>30</td>
<td>Citrate</td>
<td>35.4 ± 3.5</td>
<td>25.8/44.0</td>
<td>BDL</td>
</tr>
<tr>
<td>50</td>
<td>Citrate</td>
<td>66.5 ± 5.7</td>
<td>51.2/84.0</td>
<td>BDL</td>
</tr>
</tbody>
</table>

Table 2.2 Summary of characterization data for nanomaterial treatment suspensions. Includes DLS (dynamic light scattering, electrophoretic mobility, and pH. PDI= polydispersivity index

<table>
<thead>
<tr>
<th>Nominal Diameter (nm)</th>
<th>Coating</th>
<th>Hydrodynamic Diameter (by volume, nm ± SD)</th>
<th>Z-Average Diameter (nm ± SD)</th>
<th>PDI</th>
<th>Zeta potential (mV ± SD)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Tannate</td>
<td>14.5 ± 0.3</td>
<td>22.9 ± 1.82</td>
<td>0.34</td>
<td>-57.5 ± 0.5</td>
<td>6.2</td>
</tr>
<tr>
<td>30</td>
<td>Tannate</td>
<td>26.0 ± 5.2</td>
<td>33.3 ± 0.58</td>
<td>0.31</td>
<td>-60.8 ± 1.3</td>
<td>7.0</td>
</tr>
<tr>
<td>50</td>
<td>Tannate</td>
<td>46.0 ± 1.2</td>
<td>55.5 ± 0.25</td>
<td>0.07</td>
<td>-65.5 ± 1.3</td>
<td>6.7</td>
</tr>
<tr>
<td>10</td>
<td>Citrate</td>
<td>9.6 ± 0.8</td>
<td>24.4 ± 4.22</td>
<td>0.43</td>
<td>-50.4 ± 0.3</td>
<td>6.3</td>
</tr>
<tr>
<td>30</td>
<td>Citrate</td>
<td>27.6 ± 1.2</td>
<td>47.9 ± 0.52</td>
<td>0.35</td>
<td>-73.6 ± 0.4</td>
<td>6.4</td>
</tr>
<tr>
<td>50</td>
<td>Citrate</td>
<td>47.7 ± 0.4</td>
<td>63.5± 0.13</td>
<td>0.16</td>
<td>-81.8 ± 0.9</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Table 2.3 Summary of characterization data for tobacco post-treatment suspensions including Au, Ca, and pH measurements. Au concentrations presented at a percentage of the original treatment concentration.

<table>
<thead>
<tr>
<th>Nominal Diameter (nm)</th>
<th>Coating</th>
<th>Au in Supernatant 1100 x g (mean % ± STD %)</th>
<th>Au in Supernatant 11000 x g (mean % ± STD %)</th>
<th>Ca (mg L⁻¹, mean ± STD)</th>
<th>pH (mean ± STD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Tannate</td>
<td>51.4 ± 11.2</td>
<td>38.6 ± 10.4</td>
<td>12.3 ± 1.0</td>
<td>6.39 ± 0.10</td>
</tr>
<tr>
<td>30</td>
<td>Tannate</td>
<td>18.5 ± 5.6</td>
<td>3.9 ± 2.1</td>
<td>12.4 ± 2.9</td>
<td>6.54 ± 0.20</td>
</tr>
<tr>
<td>50</td>
<td>Tannate</td>
<td>13.3 ± 10.0</td>
<td>3.8 ± 2.0</td>
<td>22.2 ± 11.12</td>
<td>6.17 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>Citrate</td>
<td>48.4 ± 8.9</td>
<td>32.1 ± 4.3</td>
<td>65.73 ± 68.9</td>
<td>7.51 ± 0.23</td>
</tr>
<tr>
<td>30</td>
<td>Citrate</td>
<td>21.9 ± 7.5</td>
<td>9.5 ± 2.6</td>
<td>21.1 ± 5.5</td>
<td>8.10 ± 1.26</td>
</tr>
<tr>
<td>50</td>
<td>Citrate</td>
<td>20.5 ± 10.0</td>
<td>2.1 ± 0.9</td>
<td>18.6 ± 4.3</td>
<td>8.09 ± 1.45</td>
</tr>
</tbody>
</table>

Table 2.4 Summary of characterization data for wheat post-treatment suspensions including Au, Ca, and pH measurements. Au concentrations presented at a percentage of the original treatment concentration.

<table>
<thead>
<tr>
<th>Nominal Diameter (nm)</th>
<th>Coating</th>
<th>Au in Supernatant 1100 x g (mean % ± STD %)</th>
<th>Au in Supernatant 11000 x g (mean % ± STD %)</th>
<th>Ca (mg L⁻¹, mean ± STD)</th>
<th>pH (mean ± STD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Tannate</td>
<td>13.9 ± 8.1</td>
<td>11.7 ± 8.8</td>
<td>27.6 ± 7.4</td>
<td>7.27 ± 0.22</td>
</tr>
<tr>
<td>30</td>
<td>Tannate</td>
<td>16.9 ± 7.0</td>
<td>5.9 ± 4.1</td>
<td>24.4 ± 11.9</td>
<td>7.31 ± 0.20</td>
</tr>
<tr>
<td>50</td>
<td>Tannate</td>
<td>20.8 ± 12.5</td>
<td>2.7 ± 1.9</td>
<td>23.4 ± 0.3</td>
<td>7.25 ± 0.67</td>
</tr>
<tr>
<td>10</td>
<td>Citrate</td>
<td>10.1 ± 3.4</td>
<td>6.7 ± 4.4</td>
<td>23.32 ± 5.2</td>
<td>8.59 ± 0.05</td>
</tr>
<tr>
<td>30</td>
<td>Citrate</td>
<td>8.9 ± 2.3</td>
<td>4.3 ± 0.5</td>
<td>24.2 ± 2.3</td>
<td>7.98 ± 0.35</td>
</tr>
<tr>
<td>50</td>
<td>Citrate</td>
<td>4.7 ± 2.2</td>
<td>1.6 ± 0</td>
<td>22.5 ± 5.4</td>
<td>7.66 ± 0.36</td>
</tr>
</tbody>
</table>
Figures

**Figure 2.1** TEM micrographs of nanomaterials. a, 10 nm, b, 30 nm, c, 50 nm tannate coated Au nanomaterials (T-MNMs), and d, 10 nm, e, 30 nm, f, and 50 citrate coated Au MNMs (C-MNMS). Scale bars for d, e and f equal to 20 nm. TEM data for d, e and f provided by manufacturer (Nanocomposix, San Diego, CA USA).
Figure 2.2 Bulk inductively coupled plasma-mass spectrometry (ICP-MS) analysis of tobacco leaf tissue. Error bars represent SD. Treatments with the same letter are not significantly different. T-MNMs= tannate coated manufactured nanomaterials (MNMs). C-MNMs=citrate coated MNMs.
Figure 2.3 Synchrotron X-ray fluorescence microprobe map of leaf from a tobacco plant treated with 30 nm citrate coated Au manufactured nanomaterials. Fluorescence from the L-α1 edge of Au, depicted in red, and K-α1 edge of K, depicted in green. MNMs detected within leaf mid rib near petiole.

Figure 2.4 Synchrotron X-ray fluorescence microprobe map of wheat tissue. The Au L-α edge of gold is depicted in red and potassium is depicted in green. Left) Tip of wheat grass blade exposed to 50 nm citrate coated MNMs. Right) Tip of wheat grass blade exposed to 50 nm tannate coated MNMs. No gold was detected within the wheat leaves.
Figure 2.5 Synchrotron X-ray fluorescence microprobe maps of wheat root cross-sections. Fluorescence from the L-α1 edge of Au is depicted in red, fluorescence from the Zn K-β1 edge is depicted in green, and fluorescence from the K-α1 edge of Fe is depicted in blue. **a**, 10 nm, **b**, 30 nm, **c**, 50 nm citrate coated manufactured Au nanomaterials (C-MNMS), and **d**, 10 nm, **e**, 30 nm, **f**, 50 nm tannate coated Au MNMs (T-MNMs). No evidence was found indicated that the MNMs penetrated the plant root surface.
Figure 2.6 Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) depth profiles from mesophyll of tobacco leaves. a, 10 nm, b, 30 nm, c, 50 nm citrate coated manufactured Au nanomaterials (C-MNMS), and d, 10 nm, e, 30 nm, f, and 50 nm tannate coated Au MNMs (T-MNMs). The presence of Au within leaf tissue removed during each laser burst demonstrates the presence of Au throughout the leaf. Au concentration reported as counts per second (CPS) of m/z 197 (Au) normalized by CPS for m/z 66 (Zn) to account for the mass of tissue removed from each laser burst.
Figure 2.7 Bulk laser ablation inductively coupled plasma-mass spectrometry analysis of tobacco leaf tissue. Error bars represent SD. Treatments means were not significantly different from one another. T-MNMs= tannate coated manufactured nanomaterials (MNMs). C-MNMs=citrate coated MNMs.
Chapter 3: Introduction


http://pubs.acs.org/articlesonrequest/AOR-zebwe2VkJmZu3U5BaSMx

The rapidly increasing number of consumer products containing engineered nanomaterials (ENMs) is predicted to result in exponentially increasing quantities of ENMs being discharged into waste streams.19 A consistent picture is emerging that suggests that during wastewater treatment, ENMs partition predominately to biosolids,16-19 60% of which are applied as fertilizer to agricultural land in the United States and in some parts of Europe.19 Given the rapid increase in the mass of ENMs used in consumer products, a rapid rise in ENM concentrations in biosolid-amended soil is anticipated. For example, a recent calculation conservatively estimates increases in soil concentrations of ENMs from 0.1 to 0.5 mg kg\(^{-1}\) for TiO\(_2\), from 6.8 to 22.3 \(\mu g\) kg\(^{-1}\) for ZnO ENMs, and from 2.3 to 7.4 \(\mu g\) kg\(^{-1}\) for Ag ENMs\(^{19}\) between 2008 and 2012.\(^{19}\) This model also estimates the current total concentration of ENMs in biosolids may be as high as 7.5 mg kg\(^{-1}\), suggesting that localized regions of biosolid-amended soil may contain much higher ENM concentrations. Unfortunately, the bioavailability, toxicity, and fate of ENMs introduced into terrestrial ecosystems in this manner are not well understood.

Soil particles can be transferred from soil to plant surfaces by wind erosion\(^{221}\), biota\(^{222}\), mechanical disruption\(^{223}\) and raindrop splash.\(^{224-225}\) Mass loading of soil
particles onto plant surfaces is a pathway by which metals and radionuclides that are associated with these particles could potentially enter terrestrial food webs. For example, Punshon et al. (2003, 2004) reported that high concentrations of U and Ni in soil resulted in high levels of mass loading of these metals onto the leaves of area understory vegetation.\textsuperscript{101,226} Other studies have reported mass loading of soil Pu onto the surfaces of plants grown near a nuclear fuel chemical separations facility in the United States and resuspension of \textsuperscript{137}Cs from soil to the surfaces of plants growing in Austrian soils contaminated by the Chernobyl disaster.\textsuperscript{227-228} Researchers have also raised concerns that resuspension of soil particles might result in deposition of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) onto plant surfaces and ultimately in the bioaccumulation of these contaminants in higher trophic level consumers.\textsuperscript{225,229}

As the concentration of ENMs in soil increases, it is likely that ENMs will be transferred onto vegetative surfaces via soil resuspension. Since the length of time that particles will remain on leaf surfaces increases as particle sizes decreases,\textsuperscript{224-226} a potential exists for significant concentrations of ENMs to irreversibly accumulate on plant surfaces as a result of resuspension of biosolid-amended soil and present a pathway for trophic exposure of consumers to ENMs. We recently demonstrated biomagnification of Au ENMs in tobacco hornworm caterpillars exposed through dietary uptake tobacco plants that had previously been exposed to Au ENMs under hydroponic growth conditions.\textsuperscript{87} While there have been few studies that have demonstrated plant ENM uptake from soil, resuspension of soil particles containing ENMs to plant tissues could be an important indirect trophic exposure pathway for a variety of terrestrial herbivores and
humans, as demonstrated for other relatively non-mobile organic and inorganic contaminants.\textsuperscript{101,226-229}

In this investigation, we evaluated the potential for bioaccumulation of Au ENMs from surface contaminated plant tissue to the terrestrial herbivore, \textit{Manduca sexta} (tobacco hornworm). Although Au ENMs are being used in applications including medical imaging,\textsuperscript{5} drug delivery,\textsuperscript{6} and fuel cell catalysis,\textsuperscript{7} these applications do not result in large masses of Au ENMs being discharged into waste streams and as a result, Au ENMs are unlikely to accumulate in the environment in high concentrations. However, we have chosen Au ENMs for this study because, as demonstrated in our previous investigations,\textsuperscript{85,87} they are resistant to oxidative dissolution and are also very low natural in background concentrations, properties which make Au ENMs ideal probes for investigating ENM bioaccumulation, translocation, and particle specific effects.\textsuperscript{204} Tannate, a intermediate molecular weight polyphenol, was selected as the ENM coating molecule for this study because we consider tannate to be comparable to intermediate molecular weight NOM complexes, which may adsorb to ENMs in the soil as is often observed for soil mineral colloids.\textsuperscript{211-212}

The objectives of the study were: to investigate the bioaccumulation and elimination of ENMs, as well as the potential for toxicity, as a result of dietary uptake of surface contaminated plant tissue. We were also interested in comparing bioaccumulation by indirect trophic exposure as the result of consumption of surface contaminated plant tissue with bioaccumulation via direct trophic exposure, or consumption of plants that have previously bioaccumulated ENMs through root exposure. This comparison will allow us to test the hypothesis that bioaccumulation will be enhanced via direct trophic
exposure resulting from the anticipated surface modification of Au ENMs with biocompatible molecules.25

3.1 Experimental section

3.1.1 Nanomaterial characterization. A suspension of Au ENMs having a nominal primary particle size of 12 nm diameter and surface modified with tannate (Nanocomposix San Diego, CA, USA) was purchased and characterized using transmission electron microscopy, electrophoretic mobility, and dynamic light scattering. TEM size analyses were derived from TEM images collected using a Jeol 2010 TEM. Mean ENM diameter and size ranges were quantified using ImageJ software. Electrophoretic mobilities were converted to zeta potentials using the Hückel approximation.

The suspension concentration was verified through digestion in a 2:1 mixture of concentrated hydrochloric and nitric acid and subsequent elemental analysis via inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500ex ICP–MS (Agilent, Santa-Clara, CA, USA). To determine the concentration of dissolved Au in the stock suspensions, samples of the stock suspensions were diluted to 10 mg L\(^{-1}\) and centrifuged at 239,311 \(\times\) g for 2h, removing Au particles smaller than about 1.2 nm. The Au concentration in the supernatant was analyzed using ICP-MS.

3.1.2 Hornworm exposure protocol. Manduca sexta eggs were purchased (Carolina Biological, Burlington, NC, USA), hatched, and allowed to age 3 d prior to dosing. After 3 d, hornworm caterpillars that had entered their second instar were selected and randomly allocated to control or treatment populations. Mean hornworm mass on d=1 was 5.6 ± 1.0 mg. Twenty-five hornworms were allocated to the control
population and 65 were allocated to the treatment population. Each hornworm was placed within a Petri dish lined with moistened filter paper and sealed with parafilm. The dosing period lasted 7 d. On d=1, each treatment hornworm was given tomato tissue with two 5 µL droplets of Au ENMs diluted to 100 mg L⁻¹ dried onto the surface, resulting in a dose of 1 µg of Au ENMs. After a hornworm had consumed its dose, another leaf with 1 µg of Au ENMs dried onto the surface was provided. Any unconsumed plant tissue was collected for bulk analysis and the mass of Au ENMs remaining on the tissue was subtracted from the individual hornworm’s dose. Hornworms received a maximum dose of 3 µg of Au ENMs.

After completion of the dosing phase of the exposure, hornworms were allowed to eliminate Au for up to 7 d. Hornworms were fed tomato tissue with no ENMs dried on the surface during this time. Hornworms were collected for bulk analysis at 0, 1, 4 and 7 d of elimination. At 1 and 7 d depuration, hornworms were collected for analysis via scanning x-ray fluorescence microscopy (µXRF). These samples were fixed in 10% formalin and subsequently embedded in hydrophilic glycol methacrylate resin. Once embedded, approximately 1 mm thick cross-sections were cut perpendicularly to the caterpillar body axis using a diamond wafering blade and mounted onto metal free polyimide film (Kapton; Dupont, Wilmington, DE) for analysis. Hornworms have unusual gut chemistry with regional differences which include variations in pH, redox potential, and enzyme activity (Figure 1).²³⁰⁻²³² To investigate regional differences in Au ENM bioaccumulation, hornworm cross-sections were keyed to standard anatomical regions (Figure 1).²³³
3.1.3 Synchrotron X-ray analysis. Scanning X-ray fluorescence microscopic measurements of Au were collected at the Au L-\(\alpha_1\) emission line (9,713 eV) using beamline X-26A at the National Synchrotron Light Source at Brookhaven National Laboratory (Upton, NY, USA). Analyses were conducted using either two Vortex single element silicone drift detectors or a 4 element silicone drift Vortex ME4. The incident X-ray beam was monochromatized at 13 keV. The beam had a spot size of 9 \(\mu\)m horizontally and 5 \(\mu\)m vertically. The sample was translated through the beam in fly scanning mode and data were integrated at 0.2 seconds intervals. The monochromator was calibrated to the absorption edge using a Au foil standard. To correct for interference from the Zn K-\(\beta_1\) emission line (9,572 eV), hornworm cross sections were mapped at energies above (12,110 eV) and below (11,850 eV) the Au L-\(\alpha_{III}\) absorption edge (11,919 eV). The below edge signal was subtracted from the above edge signal and the difference reported as the Au signal.

3.1.4 Inductively coupled plasma-mass spectrometry analysis. Tomato and hornworm samples were oven dried for 7 d at 60 °C. To reduce the detection limit for low mass samples, samples weighing less than 10 mg were placed in microcentrifuge tubes. These samples were digested overnight at 60 °C in 150 \(\mu\)L of nitric acid. The following day, 300 \(\mu\)L of hydrochloric acid were added and the samples were heated for an additional 4 hours, after which the digestate was brought to a 3 mL volume and analyzed by ICP-MS.\(^87\) Samples weighing more than 10 mg were placed in 15 mL polypropylene centrifuge tubes, into which 0.75 mL of concentrated trace metal grade nitric acid was added. The samples were digested using a MARS microwave digestion system (CEM, Matthews, NC, USA), allowed to cool, and digested a second time after
adding 1.5 mL of hydrochloric acid. Concentrated trace metal grade acid was used for all
digestions. Analytical runs contained calibration verification samples, duplicate
dilutions, and spike recovery samples. Spike recovery averaged 113.2% \pm 8.6% (mean \pm
standard deviation) and the mean relative percent difference between duplicate dilutions
was 0.7% \pm 3.8%. As there is no widely available standard reference material containing
Au in plants, a laboratory fortified matrix (LFM) sample was prepared using finely
ground dried tobacco leaves spiked with Au standard to a concentration of 10 mg kg\(^{-1}\)
and digested. The measured concentrations of the LFM averaged 110.6% \pm 5.6%.
Additional laboratory control samples (LCS) consisting of spiked digestion blanks were
analyzed as well. The measured concentrations in the LCS averaged 100.0% \pm 6.0%.

3.1.5 Data analyses. In our previous trophic exposure, bioaccumulation factors
(BAFs) were reported after 0 d elimination.\(^{87}\) To compare the results of this direct
exposure with our previous study, BAF at 0 d elimination for each individual hornworm
was estimated using equation 1.

\[
BAF = \frac{\text{Au concentration in hornworm (\(\mu g \ g^{-1}\))}}{\left(\text{Dose administered (\(\mu g\))} - \text{Dose recovered on leaves (\(\mu g\))}\right) / \text{Mass tomato tissue consumed during dosing phase (g)}}
\]

(1)

Assimilation efficiency (AE) of Au ENMs for each individual caterpillar was
calculated using equation 2.

\[
AE = \frac{\text{Mass Au accumulated in hornworms (\(\mu g\))}}{\left(\text{Dose administered (\(\mu g\))} - \text{Dose recovered on leaves (\(\mu g\))}\right)}
\]

(2)

Undried tissue masses were used to calculate BAFs. Growth, ingestion rate, AE, and
mass Au ENMs accumulated data were tested for normality using Shapiro-Wilk’s test
and for homoscedasticity using Barlett’s test. Significant differences were tested using
ANOVA and Tukey’s means comparisons at \(\alpha=0.05\) when data were normal and
homogenously varied. When data were not normally or homogenously distributed, they were analyzed using a Kruskal-Wallis test and pair-wise Mann-Whitney U-tests at $\alpha=0.05$. Differences in mortality between the treatment and control populations were evaluated using survival time analysis (SAS Lifetest procedure).

### 3.2 Results and discussion

Electron micrographs revealed that the Au NPs had a mean diameter of 14.6 ± 3.1 (mean ± standard deviation, $n=104$ from 10 separate micrographs, Figure 3.2) and ranged in diameter from 10.7 to 28.8 nm. After dilution to 100 mg L$^{-1}$, the Z-average hydrodynamic diameter of the Au ENMs was 29.4 ± 1.13 (mean ± standard deviation, $n=3$) and the polydispersity index, a measure of the width of the particle size distribution, was 0.44 ± 0.014. Electrophoretic mobility measurements indicate that the Au ENMs are highly negatively charged with a mean zeta potential of -58.5 ± 4.18 mV at pH 5.2. Analysis of the Au concentrations in the treatment suspension supernatant revealed a mean dissolved ion fraction of 0.088 ± 0.077 (mean wt/wt % ± standard deviation, $n=3$).

Hornworm mortality was high during the dosing phase of the exposure in both control and treatment populations. In the treatment population, mortality increased dramatically once dosing commenced (Figure 3.3a). However, mortality in the control population was also high during this period and by the end of the 14 d exposure period, the control mortality and treatment mortality rates were both 25%. Survival time analysis indicates no significant differences in survival time between the two populations. There are no significant differences in growth or ingestion rate between the overall treatment population and the control population at 4, 7, 11 or 14 d (Figure 3.3b-c). The hornworms that received the maximum dose had significantly higher growth rates after
11 d (data not shown). However, we speculate that this difference is likely the result of the fact that hornworms that were healthier as a result of genetic variation and more successful ecdysis were more likely to consume their whole dose rather than a stimulatory effect. Regardless, these data provide little evidence that consumption of plant tissue surface contaminated with ENMs would result in particle specific toxicity to hornworms, even at the very high concentrations tested here.

Based on the bulk chemical analysis, the BAF was 0.16 ± 0.1 (mean ± standard deviation, n=10) after 0 d elimination. Pairwise means comparisons indicate AE of Au ENMs at 0 d elimination is significantly higher than at 1 d elimination, which is consistent with the hornworms voiding their gut contents over this period (Figure 3.4). No significant differences exist between mass of Au ENMs accumulated or AE between 1 d and 7 d elimination, suggesting that little elimination occurred after the material in the gut lumen was voided. The retention of Au ENMs in the hornworms after 7 d elimination indicates that the hornworms were not able to eliminate bioaccumulated Au ENMs efficiently. This finding is consistent with a recent study by Unrine et al. (2012) that reported near zero elimination of accumulated Au MNMs after 60 d of elimination in *Eisenia fetida* exposed to Au MNMs via indirect exposure.200

After 1 d of depuration, caterpillars that had received the maximum dose retained Au ENMs in the tissues surrounding the medial and posterior midgut in concentrations detectable by synchrotron µXRF scans (Figure 4, detection limit ~ 1 mg kg⁻¹).233 Au ENMs are not present in detectable concentrations in the anterior midgut (abdominal segment 1, Figure 3.5a) but are evident in the posterior anterior midgut (abdominal segment 2, Figure 3.5c), medial midgut (abdominal segment 4, Figure 3.6e) and posterior
midgut (abdominal segment 5, Figure 3.5g). Au ENMs are also detected within caterpillars that experienced 7 d of depuration and had received only 1 µg Au ENMs (Figure 3.6). Au ENMs are detected in cross-sections from the hornworm medial and posterior midguts (abdominal segments 5-6, Figure 3.6c-e) but not in the anterior midgut (abdominal segment 4, Figure 3.6a). We detected no Au in cross-sections from the posterior midgut in control caterpillars (Figure 3.7).

The observed bioaccumulation of higher concentrations of Au ENMs in the posterior regions of the caterpillar midgut compared to the anterior regions of the midgut may be related to the hornworm’s unique gut chemistry. Hornworm caterpillars have alkaline gut fluids, the pH of which can approach 12 in the medial midgut. This high pH is possibly an adaptation to tannin-rich diets and is most likely the result of ion transport. Considering the negative surface charge on the Au ENMs used in this exposure the high pH in the near the hornworm medial midgut may have provided a large degree of stabilization, reducing aggregation and facilitating translocation into tissues near medial midgut and in regions of the gut downstream of the medial midgut. This observation could have important implications for predicting how ENM surface charge and gut chemistry interact to affect bioaccumulation of ENMs and warrants additional study. However, there are other differences between the anterior, medial and posterior sections of the hornworm gut including degree of gut folding, redox potential, enzyme activities, and potentially varying residence time of ingested material which may play a role in the differences in uptake observed in the different regions of the caterpillar gut. Additionally, it is possible that the bioaccumulation patterns we observed are linked to
the exposure parameters used in this study and that different exposure times, ENM drying
times, and ENM doses may have resulted in differences in bioaccumulation.

In our earlier direct trophic exposure study examining bioaccumulation by tobacco
hornworms, mean BAFs at 0 d elimination ranged from 6.2-11.6 for 5-15 nm tannate
coated Au ENMs compared to the 0.16 observed in this indirect exposure. The
relationship between log BAF and log Au ENM tissue concentration for the hornworms
in this study and hornworms exposed to a similar size tannate coated Au ENM in our
earlier study are compared in Figure 6. Although a quantitative evaluation of the
differences between the two studies may have limitations, the difference between the
mean BAF observed in this study where surface contaminated foliage was ingested and
that observed in our previous study with direct trophic exposure is striking. A portion
of the difference in BAF between the two studies is likely the result of the relatively high
Au ENM tomato tissue concentrations in this study, as BAF and plant tissue
concentration are inversely related. However, the observed higher bioavailability by
direct trophic exposure is consistent with our recent study examining bioaccumulation of
ENMs in bull frogs by either direct trophic exposure or indirect exposure via oral
gavage. Unrine et al. (2012) demonstrated that bull frogs accumulated ENMs more
efficiently through dietary uptake of exposed earthworms (Eisenia fetida) than through
indirect exposure. The reasons for this trend are unclear. Unrine et al. (2012) provided
several possible explanations including ingested ENMs remaining in the gut longer when
incorporated in the tissues of a prey species, modification of ENM surfaces by the
adsorption of biocompatible macromolecules during uptake into the initial trophic level,
and trophic filtering. Another possible explanation is that the process of drying
ENMs onto the surface of the plant tissue in this study resulted in aggregation that reduced ENM bioavailability.

**Figures**

**Figure 3.1** Standard anatomical regions of the tobacco hornworm. (top) Lateral view of 5th instar hornworm caterpillar with numbered abdominal segments (bottom) Lateral view of hornworm alimentary canal. Regional pH and Eₕ values are means reported in Appel *et al.* 1990. Adapted and reproduced with permission from Eaton, J. Lepidopteran Anatomy, New York, Wiley Interscience. Copyright 1988, John Wiley & Sons, Inc.
Figure 3.2 TEM micrograph of 12 nm tannate coated gold nanomaterials
Figure 3.3 Effects of Au nanomaterials on tobacco hornworm caterpillars. (a) mortality; (b) ingestion rate; (c) growth. Mortality increased sharply in treatment population at beginning of dosing (day 3), but by the end of the 14 d exposure, treatment mortality and control mortality were both equal to 25%. Error bars represent standard deviation.
Figure 3.4  Results of inductively coupled plasma mass spectrometry analysis of oven dried hornworm tissue and subsequent calculations of assimilation efficiency (AE). Means with any like superscripts do not differ at $\alpha=0.05$ as per pairwise Mann-Whitney U-tests. Number of observations, $n$, is equal to 10 for each elimination time. Error bars represent standard deviation.
Figure 3.5 Synchrotron X-ray fluorescence microprobe maps and light micrographs of cross-sections from a fourth instar tobacco hornworm larva. Cross-sections taken from (a-b) abdominal segment 1, anterior midgut, (c-d) abdominal segment 2, posterior anterior midgut, (e-f) abdominal segment 4, medial midgut, and (g-h) abdominal segment 5, posterior midgut. Hornworm received a 3 µg dose and was depurated for 1d. Fluorescence from the L-α1 edge of Au, depicted in red, K-β1 edge of Zn, depicted in green, and from the L-α1 edge of Ca, depicted in blue. ENMs detected within hornworm tissues of posterior anterior midgut, medial midgut, and posterior midgut.
Figure 3.6 Synchrotron X-ray fluorescence microprobe maps and light micrographs of cross-sections from a fifth instar tobacco hornworm larva. Cross-sections taken from (a-b) abdominal segment 4, anterior midgut, (c-d) abdominal segment 5, medial midgut, and (e-f) abdominal segment 6, posterior midgut. Hornworm received a 1 µg dose and was depurated for 7d. Fluorescence from the L-α1 edge of Au, depicted in red, K-β1 edge of Zn, depicted in green, and from the L-α1 edge of Ca, depicted in blue. ENMs detected within hornworm tissues of medial and posterior midgut.
Figure 3.7 Synchrotron X-ray fluorescence microprobe map and light micrograph of a cross-section taken from the posterior midgut of a control caterpillar. Fluorescence from the L-α1 edge of Au, depicted in red, and K-α1 edge of Zn, depicted in green. No Au is present.

Figure 3.8 Relationship between log bioaccumulation factor (BAF) and log plant tissue gold nanomaterial concentration. Results compared between our previous exposure during which caterpillars were fed plants that had been exposed to Au ENMs via their roots (direct exposure) and the current study where caterpillars were fed tomato tissue
surface contaminated with ENMs similar to what were used in the earlier study (indirect exposure).

Chapter 4: Introduction


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Advances in nanotechnology have led to the development of many new consumer products containing nanomaterials and large increases in the mass and volume of several nanoparticles (MNMs) produced. Silver (Ag) MNMs have been studied extensively, due largely to their demonstrated antimicrobial effects. This property gives Ag MNMs a wide variety of commercial applications, and their production and resultant environmental loading has increased to reflect this. Titanium dioxide (TiO₂) and zinc oxide (ZnO) MNMs are being used in products such as sunscreens, pharmaceuticals, and UV protective coatings, and as these uses become more widespread, they will inevitably be discharged into the environment in increasing concentrations.

Between 2005 and 2010, the amount of listed products employing nanotechnology has increased from 54 to 1015. As a result, the amount of nanomaterials entering waste streams is increasing steadily. During wastewater treatment, nanoparticles have been shown to concentrate in the sludge, 60% of which is applied to agricultural land as biosolids in the U.S. and the majority of Europe. Probabilistic material flow analysis models have predicted that between 2008 and 2012, the nanomaterials concentrations in
sludge-treated soil in the U.S. will increase from 0.1 to 0.5 mg kg\(^{-1}\) for TiO\(_2\) MNMs, from 6.8 to 22.3 \(\mu\)g kg\(^{-1}\) for ZnO MNMs, and from 2.3 to 7.4 \(\mu\)g kg\(^{-1}\) for Ag MNMs\(^{19}\).

Plant uptake\(^{100}\), gastrointestinal absorption\(^{235}\), and trans-membrane transport of nanomaterials\(^{25}\) have already been demonstrated. In the past, dietary exposure and trophic transfer have proven to be important terrestrial exposure pathways to chemicals such as polychlorinated biphenyls (PCBs), methylmercury, and dichlorodiphenyltrichloroethane (DDT)\(^{21}\). Despite these facts, there has been little research published investigating the trophic transfer of nanoparticles, and even less addressing the transfer from plants to animals. In one such study, trophic transfer was reported when freshwater algae (Pseudokirchneriella subcapitata) exposed to 10-25 nm CdSe quantum dots were fed to Ceriadaphnia dubia\(^{236}\). Another study reported trophic transfer of 6 by 12 nm ellipsoid carboxylated and biotinated CdSe quantum dots from ciliated protozoans to rotifers\(^{237}\). A recent publication presented evidence for the transfer of 21 nm TiO\(_2\) MNMs from Daphnia magna to Danio rerio (zebrafish)\(^{238}\). We have demonstrated the accumulation of Au MNMs up to 55 nm in diameter\(^{21}\) and copper (Cu) MNMs up to 100 nm in diameter in earthworms from exposure to MNM spiked soil\(^{21,37}\). There are also some studies that provide indirect evidence of trophic transfer. In one such study, researchers presented indirect evidence that gold (Au) MNMs transferred from the water column to the marine food web within estuarine mesocosms\(^{239}\). Another study exposed Caenorhabditis elegans to ZnO, Al\(_2\)O\(_3\) and TiO\(_2\) MNMs in the presence of Escherichia coli (E. coli), which were provided as food\(^{240}\). It is possible that some of the MNMs were internalized by the E. coli and transferred to the nematodes through dietary uptake.
These initial results suggest that MNMs could be available for dietary uptake at lower trophic levels and subsequently transferred through terrestrial food webs. As terrestrial ecosystems are a possible pathway for human exposure\(^\text{20}\), there is an urgent need to examine the transport and fate of MNMs in terrestrial ecosystems under environmentally relevant scenarios. To begin addressing this knowledge gap, we used the model organisms \textit{Nicotiana tabacum} L. cv \textit{Xanthi} and \textit{Manduca sexta} (tobacco hornworm) to investigate plant uptake and the potential for trophic transfer of 5, 10, and 15 nm diameter Au MNMs. Au MNMs are being used in a variety of applications including the detection and imaging of cancer cells\(^5\), pharmaceuticals designed to combat HIV\(^6\), and catalysis in fuel cells\(^7\). We selected Au for this initial study due to its resistance to oxidative dissolution and release of dissolved Au ions, as well as because of its low natural background concentrations\(^204\). These properties make Au an ideal model to probe particle specific uptake in complex systems.

Based on previously published results\(^{21,37,100,235-236,238}\), we hypothesized that 5-15 nm Au MNMs may be transferred through dietary exposure from a primary producer to a primary consumer. We also hypothesize that due to the presence of size selective physiological barriers such as the as the plant cell wall, size will be an important factor in plant uptake and potential trophic transfer of Au nanoparticles. Finally, we hypothesize that Au nanomaterials would accumulate in the primary consumer from ingestion of the primary producer, or bioaccumulate\(^{241}\), but that the Au MNMS would not occur in a higher concentration in the primary consumer than seen in the tobacco, or biomagnify\(^{242}\), as metal ions, other than lipophilic complexes, i.e. methyl mercury, do not biomagnify\(^{243}\)
4.1 Experimental section

4.1.1 Nanomaterial characterization. Stable aqueous suspensions of 5, 10, and 15 nm diameter primary particle size gold (Au) nanoparticles surface modified with tannic acid and carrying a negative surface charge were purchased (Nanocomposix San Diego, CA USA) and characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS, Table 4.1, Figure 4.1).

Tannic acid coated nanoparticles were selected due to the fact that a suspension of bare Au nanoparticles would be unstable under these exposure conditions and would aggregate, making it very difficult to investigate differences in uptake based on size. Additionally, tannic acid is one of only a few environmentally relevant coatings available in our desired size range and surface charge with low polydispersivity. We also consider tannic acid to be a reasonable analog for a particle coated with natural organic matter, which we believe will sorb to nanoparticle surfaces once introduced into soil, similar to soil minerals\textsuperscript{211}.

The stock suspension was diluted to create the treatment suspensions. Mean hydrodynamic diameters and electrophoretic mobilities of the nanoparticle treatment suspensions were measured using a Nano-ZS zetasizer, and were conducted at a suspension concentration of 100 mg Au L\textsuperscript{-1} (Malvern, Worcestershire, UK). The 100 mg L\textsuperscript{-1} concentrations were verified through ICP-MS measurement and averaged 100.9 ± 4.61 mg L\textsuperscript{-1}. TEM size analysis was provided by the manufacturer (Nanocomposix San Diego, CA USA). The manufacturer fixed the particles on Formvar/carbon-coated copper transmission electron microscope (TEM) grids 200 mesh size (Ted Pella, Redding, CA). Imaging was performed on a Jeol 1010 TEM and the diameter of 103
randomly selected individual particles were quantified with Image J software (Figure 4.1). Particle size distribution for each size class is based on the relative percent of the total particle diameters measured.

4.1.2 Exposure of the primary producer. *Nicotiana tabacum* L. cv *Xanthi* was selected as a model primary producer for this study due to its ability to bioconcentrate heavy metals$^{213}$, as well as its similarity to *Lycopersicon esculentum* (tomato), a USEPA recommended test plant for uptake and translocation$^{214}$ and a member of the Solanaceae family of plants$^{244}$. *Nicotiana tabacum* L. cv *Xanthi* seeds were sterilized by shaking with 6% sodium hypochlorite for 10 minutes and washing three times with 18 MΩ deionized water (DI)$^{214}$. Seeds were then placed in plates of gellan gum (Phytagel, Sigma-Aldritch, St. Louis, MO USA) containing a half strength nutrient mixture (Phytotechnology Laboratories, Shawnee Mission, KS USA) and grown in a greenhouse at a mean temperature of 25.4 ±1.7 °C, with a mean relative humidity of 81.6 ± 9.8 % and a 12 hour light cycle. After four weeks, the plants were randomly divided into treatment populations, placed in microcentrifuge tubes, and grown for one week in either 1.5 mL of DI or 5, 10, or 15 nm nanoparticles suspended in DI at 100 mg Au L$^{-1}$. Treatment solutions were periodically adjusted to the initial volume with DI.

4.1.3 Exposure of the primary consumer. We selected *Manduca sexta* as a model consumer for this study due to its ability to digest tobacco, which is a result of its unique gut chemistry$^{230,232}$. *Manduca sexta* larvae generate a pH gradient in their gut that ranges between 5 and 12$^{232}$, and an Eh gradient that that varies between -188 to 172 mV$^{230}$, either of which could play a role in the dietary uptake of MNMs. This high pH is possibly adaptation to tannin-rich diets, and is most likely the result of ion transport$^{230,232}$. 

100
Manduca sexta eggs were purchased from Great Lakes Hornworm (Grand Rapids, MI, USA). The hornworms were randomly divided into their individual treatment enclosures at the beginning of the second larval instar, during which the hornworms were 9-18 mm long\textsuperscript{233,245}. Five hornworms were provided living plants growing in DI, and 10 hornworms each were provided living plants growing in DI that were previously exposed to the 5, 10, and 15 nm Au treatment suspensions as described above. The hornworm enclosures consisted of a 48.1 mL vial containing a 3 mL vial filled with DI, which was placed inside and glued to the bottom (Figure 4.2). A 2-3 mm diameter hole was punched into the inner vial cap through which a tobacco roots were threaded, with the leaves of the living plant resting on the top of the inner vial cap. This exposure setting was designed to disallow the hornworms access to the plant roots. Plants were rinsed with DI using a wash bottle and then submerged in water twice before transfer to the hornworm enclosures. The hornworms were then added to the enclosures, which were covered with nylon mesh. Enclosures were cleaned daily to reduce the incidence of disease.

When the hornworms had consumed the majority of the leaf material of their initial plants, a new plant was placed into their enclosure. The feeding cycle continued for one week. Each plant was weighed before and after placing it in the enclosures, and the final mass of tobacco consumed by each hornworm was recorded. To maximize the mass of tobacco tissue available to feed to each hornworm, tobacco tissue samples for the tobacco bulk analysis consisted of small triangles of tissue cut using a razor blade from a leaf tip of each individual plant that was fed to each hornworm. For each hornworm, leaf tip samples from each plant that was fed to the hornworm were pooled for bulk analysis.
Entire hornworms were embedded in hydrophilic glycol methacrylate resin and sectioned to approximately 1 mm using a diamond wafering blade. Several hornworm sections were cut perpendicularly to the body axis for each sample and were each keyed to standard anatomical divisions of the *Manduca sexta* larvae. Hornworm sections from the midgut and whole tobacco leaves were mounted on metal free polyimide film (Kapton; Dupont, Wilmington, DE). Hornworm sections were analyzed using x-ray absorption near edge spectroscopy (µXANES) and synchrotron x-ray fluorescence microprobe (µXRF) scans. Whole tobacco leaves were analyzed using µXRF and laser ablation inductively coupled mass spectrometry (LA-ICP-MS).

4.1.4 Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS). LA-ICP-MS depth profiles were collected using sequential controlled laser bursts from a LSX-213 laser ablation system (Cetac, Omaha, NE, USA) that removed 400 x 400 μm² craters, the depth of which ranged from 8-10 μm as measured using a Nikon Eclipse 90i light microscope (Nikon, Tokyo, Japan). The material removed during each laser pulse was measured using an Elan DRC Plus ICP–MS (PerkinElmer-Sciex, Waltham, MA). Six depth profiles from 3 samples were collected for each treatment. For each sample, one depth profile was collected from the leaf mesophyll and one was collected from the leaf midrib.

4.1.5 Synchrotron X-ray analysis. µXRF measurements of Au were based on the fluorescence from the L-α edge (9,713 eV), and were conducted at beamline X-26A at the National Synchrotron Light Source at Brookhaven National Laboratory (Upton, NY, USA) using a nine element Ge array detector (Canberra, Merridian, CT). The x-ray beam was monochromatized at 13 keV. The beam had a spot size of 9 μm horizontally
and 5 µm vertically. The beam was translated through the sample in 20 µm steps, and spectra were collected at each step for one second. Au L absorption edge (11,921 eV) µXANES was performed in fluorescence mode by scanning the monochrometer from 11850 to 12038 eV with 2 eV steps. µXANES spectra were analyzed using the Athena software package. The beam was calibrated to the absorption edge using a Au foil standard. µXANES spectra from the Au foil standard as well as from a Au chloride (HAuCl₄) standard were collected for comparison to µXANES spectra in the samples.

4.1.6 Inductively coupled plasma-mass spectrometry analysis of bulk tissue. Wet tobacco samples were weighed and placed in microcentrifuge tubes. The samples were digested in 50 µL of hydrogen peroxide and 150 µL of nitric acid. The tubes were heated overnight at 60° C in an Isotemp 2001 FS hot block (Fisher Scientific, Pittsburgh, PA). In the morning, 300 µL of hydrochloric acid was added and the samples were heated in the hot block for an additional 4 hours, after which the digestate was collected and brought to 3 mL volume. Each digestate was analyzed by ICP-MS using an Elan DRC Plus ICP–MS (PerkinElmer-Sciex, Waltham, MA). Moisture content of the tobacco leaves was measured to average 91.1% +/- 1.9% and the results of the tobacco bulk analysis were adjusted to dry weight using this estimate. Surviving hornworms were dried for 48 hours at 60° C and subsequently digested using the same protocol. Analytical runs contained calibration verification samples, duplicate dilutions, and spike recovery samples. As there is no widely available standard reference material containing Au, a standard reference material was synthesized using finely ground dried tobacco leaves spiked with Au standard to a concentration of 10 mg kg⁻¹. Mean standard recovery
using this material was 103.6%. Spike recovery averaged 94.2%, and the mean relative percent difference between duplicate dilutions was 7.4%.

4.2 Results and discussion

Concentrations of Au in whole, undried tobacco leaves were below the detection limits as determined by synchrotron µXRF. However, ICP-MS analysis of dried tobacco tissue revealed mean Au concentrations of 40.3, 95.8, and 61.7 mg Au kg⁻¹ dry tobacco in the 5, 10, and 15 nm treatments respectively. All of the LA-ICP-MS depth profiles of tobacco leaves that were collected provided similar results, and verified that Au was present throughout the plant tissue cross section, rather than just on the leaf surface (Figure 4.3). These results differ from the results from one early study where barley (*Hordeum vulgare* L.) was exposed to bare 4.6 nm diameter and 5.8 nm polyvinylpyrrolidone (PVP) coated Au MNMs for were used as opaque electron tracers with no uptake or translocation observed using electron microscopy over exposure periods of 41 h or 22 h, respectively. The differences in the exposure durations or detection methodology between this early study and the work presented here could be responsible for the differences in the results reported. It is also possible that the tannic acid coating stabilizes Au particles in a natural system more than PVP. However, how surface coating affects plant uptake and trophic transfer is currently unclear. Regardless, plant uptake of 15 nm diameter MNMs is consistent with recently published research on plant uptake of nanoparticles, which has reported uptake of 30-50 nm copper particles and 20 nm iron oxide particles.

*Manduca sexta* which consumed tobacco exposed to all three sizes of MNMs accumulated Au in the tissue surrounding the gut lumen in concentrations detectable by
synchrotron µXRF (Figure 4.4). Although Au is not expected to be oxidized under the conditions of this experiment, we validated this by collection of µXANES at Au hotspots elucidated by the µXRF scans of hornworm cross sections from each treatment. These spectra were compared to Au foil and HAuCl₄ standards, which clearly indicate that only Au⁰ was which confirmed that the amount of dissolved Au in the dosing material was negligible, providing additional evidence that the Au transferred to the hornworm via plant consumption was via Au MNMs rather than as dissolved Au ions (Table 4.2).

The mass of Au transferred was quantified by comparing the bulk analyses of the tobacco and hornworm tissues, which demonstrated that Au concentrations in the hornworm tissue exceeded that of the tobacco tissue by mean factors of 6.2, 11.6, and 9.6 for the 5, 10, and 15 nm treatments, respectively (Figure 4.6a). The bulk analysis data were log transformed and analyzed using ANOVA and post-hoc multiple pairwise comparisons of the means of the Au concentration in the hornworms for each treatment at α=0.05 using the Student-Newman-Keuls procedure. The SNK procedure grouped the control population alone, the 5 nm population alone, and the 10 and 15 nm populations together (Table 4.2). Assumptions of normality and homoscedasticity were tested using Shapiro-Wilk’s test and Barlett’s test. The mass of Au transferred was also converted to number of particles transferred (Figure 4.6b), which indicated that there was no significant difference between the number of particles transferred between treatments.

The concentration of Au measured in the hornworm tissues, though consistent with our first hypothesis, was unexpectedly high and implies that under certain conditions, stable MNMs as large as 15 nm may be available for transfer to higher trophic levels with the potential for biomagnification, contrary to our third hypothesis. The bulk
concentration data also demonstrate that the mass of Au in the hornworm tissues for the 10 nm and 15 nm treatments was significantly higher than the 5 nm treatment (Figure 4.6), inconsistent with our second hypothesis. The observed trend is similar to that demonstrated in previous work on the uptake of 10-100 nm Au MNMs into mammalian cells\textsuperscript{25}, and could be due to smaller particles being more tightly bound to membrane receptors\textsuperscript{25}. This trend could also be due to differences in zeta potential and pH between the treatment solutions rather than the size of the MNMs, as the 10 and 15 nm treatment suspensions had virtually identical zeta potentials (Table 4.1). Additionally, the 10 and 15 nm MNMs had similar hydrodynamic diameters (Table 4.1). The similarity in uptake between these two treatments may reflect the greater importance of hydrodynamic diameter over primary particle size in MNM uptake. The differences in uptake between the treatments could also be an effect of particle number, as the concentrations of Au in the hornworms based on particle number were not significantly different between treatments (Figure 4.6b). However, it is problematic to analyze trends based on particle number, as the dosages by particle number would be different for each treatment.

Of the previous studies that have been conducted investigating the trophic transfer of nanomaterials, none have considered terrestrial environments, and many have neglected to conduct crucial analyses. For example, spatial analysis of exposed organisms is required to verify that MNM uptake and transfer has occurred, as opposed to the MNMs being adsorbed to the external surfaces of the organisms, a very common occurrence with aquatic plants. Additionally, in many cases metal ions can be solubilized from MNMs during the course of an experiment and bulk analysis cannot discriminate metals in MNM
or ionic forms, nor whether the nanomaterials are adsorbed to the surface of an organism or have been incorporated into the organism’s tissues\textsuperscript{249}.

Tables

Table 4.1 Summary of characterization data for nanomaterial treatment suspensions. Characterization data for treatment suspensions of 5, 10, and 15 nm 100 mg L\textsuperscript{-1} gold nanoparticles surface modified with tannic acid. Transmission electron microscopy (TEM) diameter, hydrodynamic diameter, and zeta potential given as mean +/- one standard deviation. TEM data provided by manufacturer (Nanocomposix, San Diego, CA USA). The hydrodynamic diameter distributions are weighted by volume.

<table>
<thead>
<tr>
<th>Nominal Diameter (nm)</th>
<th>TEM Diameter (nm ± SD)</th>
<th>TEM Min/Max Diameter (nm ± SD)</th>
<th>Hydrodynamic Diameter (nm ± SD)</th>
<th>Zeta potential (mV ± SD)</th>
<th>pH</th>
<th>Dissolved Au (% wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.4 ± 0.6</td>
<td>3.2/6.7</td>
<td>9.05 ± 0.099</td>
<td>-48.6 ±1.63</td>
<td>4.66</td>
<td>4.7 x 10\textsuperscript{-6}</td>
</tr>
<tr>
<td>10</td>
<td>11.0 ± 0.9</td>
<td>9.1/14.2</td>
<td>14.74 ± 0.32</td>
<td>-77.7 ±3.00</td>
<td>5.28</td>
<td>4.6 x 10\textsuperscript{-5}</td>
</tr>
<tr>
<td>15</td>
<td>14.3 ± 1.3</td>
<td>12/18.6</td>
<td>14.69 ± 0.55</td>
<td>-77.0 ±0.76</td>
<td>6.08</td>
<td>8.0 x 10\textsuperscript{-8}</td>
</tr>
</tbody>
</table>
**Table 4.2** Bulk analysis of hornworm tissue. Results from bulk gold (Au) analysis, as well as results from post-hoc multiple pair wise comparisons of the log transformed means of the results from bulk gold (Au) analysis at $\alpha=0.05$ using the Student-Newman-Keuls procedure; the control population and the 5 nm population were each grouped alone, and the 10 and 15 nm populations were grouped together.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Observations, $n$</th>
<th>Mean Hornworm Au Conc. (mg L$^{-1}$)</th>
<th>Standard Error (SE)</th>
<th>Mean Au Conc. (log mg L$^{-1}$)</th>
<th>Standard Error (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>1.15</td>
<td>0.66</td>
<td>0.2433$^c$</td>
<td>0.17</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>241.02</td>
<td>60.59</td>
<td>2.37$^b$</td>
<td>0.11</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>929.27</td>
<td>257.36</td>
<td>2.9175$^a$</td>
<td>0.12</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>938.88</td>
<td>256.02</td>
<td>2.92$^a$</td>
<td>0.12</td>
</tr>
</tbody>
</table>

$abc$ Means with any like superscripts do not differ at 5% level as per SNK procedure.
Figures

Figure 4.1 TEM micrographs of nanomaterials. A) 5 nm, b) 10 nm, C) 15 nm; Scale bars in all three images are equal to 20 nm; TEM images provided by manufacturer Nanocomposix San Diego, CA USA).
Figure 4.2 Exposure setting. Tobacco plant roots were threaded through a hole in the cap of the inner vial, which was filled with deionized water. Once the tobacco plant was in place, a tobacco hornworm was placed onto the leaves of the tobacco plant. Nylon mesh and a rubber band were used to keep the hornworm from escaping the enclosure.
Figure 4.3 Laser ablation inductively coupled plasma-mass spectrometry depth profiles from mesophyll of tobacco leaves. Profiles from plants exposed to a, 5 nm, b, 10 nm, and c, 15 nm gold nanoparticles. The presence of gold within leaf tissue removed during each laser burst demonstrates the presence of gold throughout the leaf. Gold concentration reported as log counts per second (CPS) of m/z 197 (Au) normalized by CPS for m/z 66 (Zn) to account for the mass of tissue removed from each laser burst.
Figure 4.4 Synchrotron X-ray fluorescence microprobe maps and light micrographs of hornworm cross-sections. From the Au L-α edge of gold (Au), depicted in red, and zinc (Zn), depicted in blue, as well as light micrographs of cross-sections of *Manduca sexta* specimens that were fed plants exposed to a, 5 nm nanoparticles b, 10 nm nanoparticles and c, 15 nm nanoparticles. Au fluorescence, reported in counts per second (CPS), was detected throughout the hornworm tissues surrounding the gut lumen. *Manduca sexta* sections are from the midgut of each hornworm (30). Scale bars in the light micrographs are each equal to 0.5 mm. Lines crosscutting the light micrographs for sections a. and c. are from collecting LA-ICP-MS transects across these sections.
Figure 4.5 X-ray absorption near edge spectroscopy (XANES) spectra. Spectra shown for gold foil, Au$^{3+}$ as gold chloride (HAgCl$_4$), gold hotspots within cross sections of hornworm fed tobacco treated with each of the three nanoparticle sizes (5, 10, and 15 nm hornworm), and samples of the original dosing material (5, 10, 15 nm Au). The absence of the peak diagnostic of HAgCl$_4$ in the XANES spectra indicates that only Au$^0$ was present within hornworm tissues, and that there were no Au ions in the dosing material.
Figure 4.6 Bulk analysis of hornworm and tobacco tissue. Gold concentration reported as mean ± one standard error for tobacco and hornworms based on: a.) mass and b.) particle number. Groups with the same letter are not significantly different from each other.

Chapter 5. Conclusion and Future Direction

A series of experiments were conducted to clarify the bioavailability of MNMs to terrestrial organisms. In the first such experiment, tobacco and wheat plants were exposed hydroponically to Au MNMs of a range of diameter coated with one of two different organic ligands (Chapter 2). The results of these exposures produced some of the first data systematically examining the importance of particle size and MNM surface chemistry on the bioavailability of MNMs. Our data suggest that MNMs with a wide range of particle size and different surface coatings are bioavailable to plants in hydroponics and that MNMs do not need to passively move through cell wall pores to be taken up. Further study of the mechanisms, particularly endocytosis, by which MNMs could enter plant cells is critical to advancing our understanding plant uptake of MNMs. Extrinsic properties imparted by soil components, which were not investigated in this study, will likely influence uptake, possibly even affecting how MNM intrinsic properties
affect uptake. Therefore, investigating the importance of MNM intrinsic properties to plant uptake in soil exposures is a necessary area of future research. Furthermore, we observed large species dependent differences in MNM bioaccumulation that we speculate are the ultimate result of differences in the nature of chemical root exudation between plant species. Clarifying the role of differential root exudation on the bioavailability of MNMs to plants is another interesting area for future research.

Tobacco hornworms were exposed to surface contaminated tomato tissue to examine the possibility of bioaccumulation of MNMs from dietary uptake of surface contaminated plants (Chapter 3). The evidence resulting from this experiment demonstrates that a potential exists for secondary terrestrial consumers to bioaccumulate MNMs via indirect trophic exposure through soil resuspension. We have also revealed that bioaccumulation does not take place in the most anterior regions of the hornworm midgut, a result that may suggest important interactions between MNM surface functionalization and gut chemistry and warrants further study. These results suggest that dietary uptake of MNMs by terrestrial herbivores exposed via surface contaminated plant tissue could be an important pathway by which MNMs might enter terrestrial food webs. They further indicate that resuspension of MNM containing soil particles, such as might be expected in biosolid amended terrestrial agroecosystems represents an exposure pathway that should be adequately considered in risk assessments of nanotechnology.

The research presented in Chapter 4 represents the first evidence of trophic transfer of MNMs from a terrestrial primary producer to a primary consumer, as well as the first evidence of biomagnification of MNMs within a terrestrial food web. Past experience with chemicals such as methylmercury, DDT, and PCBs have revealed dietary uptake at
lower trophic levels and accumulation up the food chain to be an important route of contaminant exposure, resulting in chronic or even acute toxicity to a variety of ecoreceptors as well as humans. Comparing the results of this study to the results from Chapter 3, we find that *Manduca sexta* bioaccumulate dramatically larger concentrations of MNMs through direct trophic consumption of plants that have taken up MNMs and incorporated them into their tissues than through dietary consumption of surface contaminated plant tissues.

Furthermore, our observation that MNMs can biomagnify highlights the importance of considering dietary uptake as a pathway for MNM exposure, and raises questions about potential ecoreceptor and human exposure to MNMs from long-term land application of biosolids containing MNMs.

Given the lack of emphasis on studies focused on MNMs in terrestrial ecosystems to date, generating data such as the results from the experiments described here is critical for developing an understanding of the mechanisms and factors that control the bioavailability of MNMs in terrestrial ecosystems and will be required to help inform risk-based policy decisions on the regulation of nanomaterials.
REFERENCES


[27] Li, Q., Xie, Bin, Hwang, Yu, and Xu, Yuankai Kinetics of C\textsubscript{60} Fullerene Dispersion in water enhance by natural organic matter and sunlight. *Environmental Science and Technology*. **2009**, *43* (3574-3579), 3574.


[61] Yamakoshi, Y. Active oxygen species generated from photoexcited fullerene (C\(_{60}\)) as potential medicines: O\(_2\) versus \(^1\)O\(_2\). *Journal of the American Chemical Society*. **2003**, 125 (12803-12809).


[199] Judy, J. D., Unrine, J. M., Rao, W., Bertsch, P. M. Bioaccumulation of gold nanomaterials by *Manduca sexta* through dietary uptake of surface contaminated plant


[222] Sumerling, T. J., Dodd, N. J., Green, N. The transfer of $^{90}$Sr and $^{137}$Cs to milk in a dairy herd grazing near a major nuclear installation. *Sci. Total Environ*. **1984**, 34 (57-72)


[244] USDA, A. Germplasm Resources Information Network (GRIN), National Germplasm Resources Laboratory: Beltsville, Maryland, 2010.


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