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Bioaccumulation of Gold Nanomaterials by *Manduca sexta* through Dietary Uptake of Surface Contaminated Plant Tissue

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Supporting Information

ABSTRACT: We investigated the potential for bioaccumulation of engineered nanomaterials (ENMs) by tobacco hornworm (*Manduca sexta*) caterpillars resulting from the ingestion of plant tissue surface contaminated with ENMs. Caterpillars were fed tomato leaf tissue that had been surface contaminated with 12 nm tannate coated Au ENMs. After dosing was complete, bulk Au concentrations in individual caterpillars were measured after 0, 1, 4, and 7 days of elimination. Growth, mortality, and ingestion rate were monitored. This experiment revealed (1) no evidence that caterpillars were affected by ingestion of ENM contaminated plant tissue; (2) low bioaccumulation factors (BAF = 0.16) compared to a previous study where hornworm caterpillars were fed plants that had previously bioaccumulated Au ENMs (BAF = 6.2−11.6); (3) inefficient elimination of accumulated Au ENMs not associated with hornworm gut contents; and (4) regional differences in translocation of Au ENMs into tissues surrounding the hornworm gut, possibly the result of the interaction between ENM surface chemistry and regional differences in hornworm gut chemistry. These data, along with previous findings, indicate that although ENMs resuspended from soil onto plant surfaces by wind, water, biota, and/or mechanical disturbances are bioavailable to terrestrial consumers, bioaccumulation efficiency may be much lower via this pathway than through direct trophic exposure.

INTRODUCTION

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.[1] A consistent picture is emerging that suggests that during wastewater treatment, ENMs partition predominately to biosolids,[1−4] 60% of which are applied as fertilizer to agricultural land in the United States and in some parts of Europe.[1] 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[1] between 2008 and 2012.[1] 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,[5] biota,[6] mechanical disruption,[7] and raindrop splash.[8,9] 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 of the leaves of area understory vegetation.[10,11] 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 ^{137}Cs from soil to the surfaces of plants growing in Austrian soils contaminated by the Chernobyl disaster.[12,13] 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.[9,14]

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,[8,9,11] 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 of tobacco plants that had previously been exposed to Au ENMs under hydroponic growth conditions.[15] While there have been few studies that have demonstrated plant ENM uptake from soil, resuspension of soil

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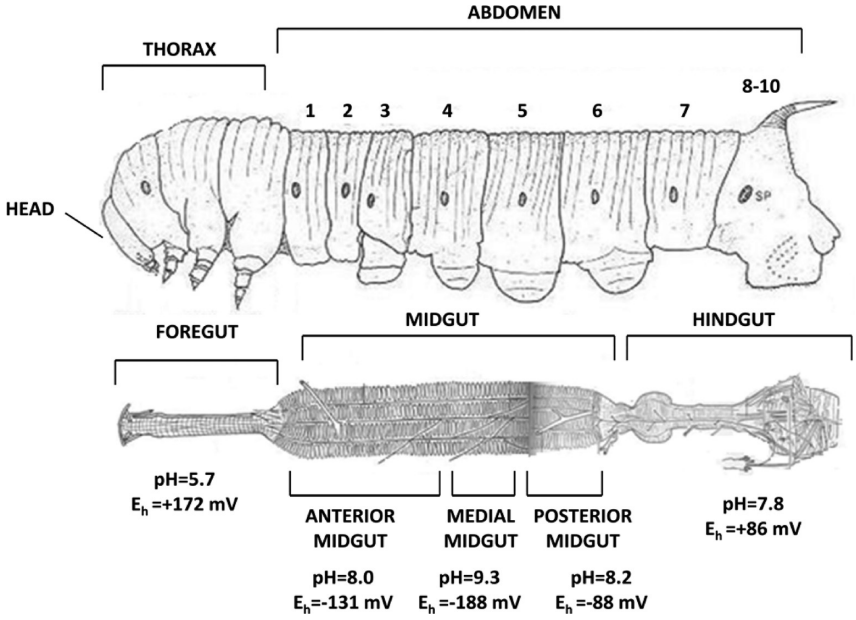

Figure 1. Standard anatomical regions of the tobacco hornworm. (top) Lateral view of 5th instar hornworm caterpillar with numbered abdominal segments (bottom) Lateral view of hornworm alimentary canal. Regional pH and E_h values are means reported in Appel et al. 1990.[24] Adapted and reproduced with permission from Eaton, J. Lepidopteran Anatomy, New York, Wiley Interscience. Copyright 1988, John Wiley & Sons, Inc.

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 nonmobile organic and inorganic contaminants.[10−14]

In this investigation, we evaluated the potential for bioaccumulation of Au ENMs from surface contaminated plant tissue to the terrestrial herbivore, *Manduca sexta* (tobacco hornworm). Although Au ENMs are being used in applications including medical imaging,[16] drug delivery,[17] and fuel cell catalysis,[18] 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 measurable concentrations. However, we have chosen Au ENMs for this study because, as demonstrated in our previous investigations,[15,19] they are resistant to oxidative dissolution and are also present in very low natural in background concentrations, properties which make Au ENMs ideal probes for investigating ENM bioaccumulation, translocation, and particle specific effects.[20] 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.[21,22]

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.[23]

■ EXPERIMENTAL SECTION

Nanoparticle 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) was purchased and characterized using transmission electron microscopy, electrophoretic mobility measurements, 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 7500cx ICP−MS (Agilent, Santa-Clara, CA). 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*g* for 2 h, removing Au particles smaller than about 1.2 nm. The Au concentration in the supernatant was analyzed using ICP-MS.

Hornworm Exposure Protocol. *Manduca sexta* eggs were purchased (Carolina Biological, Burlington, NC), hatched, and allowed to age 3 days prior to dosing. After 3 days, 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 days. 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 days. 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 days of elimination. At 1 and 7 days 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).[24−26] To investigate regional differences in Au ENM bioaccumulation, hornworm cross sections were keyed to standard anatomical regions (Figure 1).[27]

Synchotron X-ray Analysis. Scanning X-ray fluorescence microscopic measurements of Au were collected at the Au L-α_1 emission line (9713 eV) using beamline X-26A at the National Synchrotron Light Source at Brookhaven National Laboratory (Upton, NY). Analyses were conducted using either two Vortex single element silicone drift detectors or a four 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 sample was translated through the beam in fly scanning mode and data were integrated at 0.2 s intervals. The monochromator was calibrated to the absorption edge using a Au foil standard. To correct for interference from the Zn K-β_1 emission line (9572 eV), hornworm cross sections were mapped at energies above (12 110 eV) and below (11 850 eV) the Au L-α_{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.

Inductively Coupled Plasma-Mass Spectrometry Analysis. Tomato and hornworm samples were oven-dried for 7 days 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 μL of nitric acid. The following day, 300 μL of hydrochloric acid were added and the samples were heated for an additional 4 h, after which the digestate was brought to a 3 mL volume and analyzed by ICP-MS.[15] Samples weighing more than 10 mg were placed in 15 mL polypropylene centrifuge tubes, into which 0.75 mL of nitric acid was added. The samples were digested using a MARS microwave digestion system (CEM, Matthews, NC), 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% ± 8.6% (mean ± standard deviation) and the mean relative percent difference between duplicate dilutions was 0.7% ± 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% ± 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% ± 6.0%.

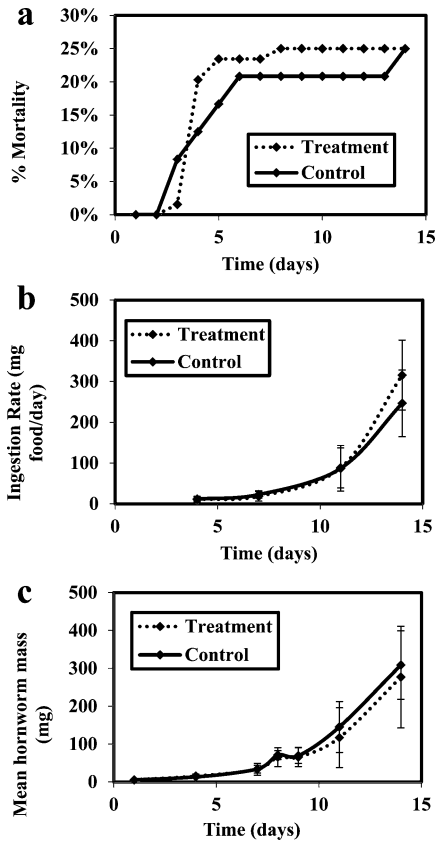

Figure 2. Effects of Au nanomaterials on tobacco hornworm caterpillars. (a) mortality; (b) ingestion rate; (c) growth. Mortality increased sharply in treatment population at beginning of dosing (day 3), but by the end of the 14 day exposure, treatment mortality and control mortality were both equal to 25%. Error bars represent standard deviation.

Data Analyses. In our previous trophic exposure, bioaccumulation factors (BAFs) were reported after 0 days elimination.[15] To compare the results of this direct exposure with our previous study, BAF at 0 days elimination for each individual hornworm was estimated using eq 1.

$$\mathrm{BAF} = \frac{\text{Au concentration in hornworm}(\mu\text{g g}^{-1})}{\left(\frac{(\text{dose administered}(\mu\text{g}) - \text{dose recovered on leaves}(\mu\text{g}))}{\text{mass tomato tissue consumed during dosing phase}(\text{g})}\right)} \quad (1)$$

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

$$\mathrm{AE} = \frac{\text{mass Au accumulated in hornworms}(\mu\text{g})}{(\text{dose administered}(\mu\text{g}) - \text{dose recovered on leaves}(\mu\text{g}))} \quad (2)$$

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 homoscedascity using Barlett's test. Significant differences were tested using ANOVA and Tukey's means comparisons at α = 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 pairwise Mann−Whitney

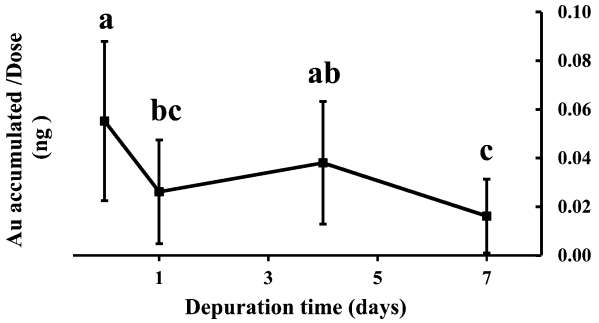

Figure 3. Results of inductively coupled plasma mass spectrometry (ICP-MS) analysis of oven-dried hornworm tissue and subsequent calculations of assimilation efficiency (AE). Means with any like superscripts do not differ at $\alpha = 0.05$ as per pairwise Mann−Whitney U-tests. Number of observations, n, is equal to 10 for each elimination time. Error bars represent standard deviation.

U-tests at $\alpha = 0.05$. Differences in mortality between the treatment and control populations were evaluated using survival time analysis (SAS Lifetest procedure).

RESULTS AND DISCUSSION

Electron micrographs revealed that the Au ENMs had a mean diameter of 14.6 ± 3.1 (mean ± standard deviation, $n = 104$ from 10 separate micrographs, see Supporting Information (SI) Figures S1 and S2) 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 2a). However, mortality in the control population was also high during this period and by the end of the 14 day 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 days (Figure 2b,c). The hornworms that received the maximum dose had significantly higher growth rates after 11 days (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

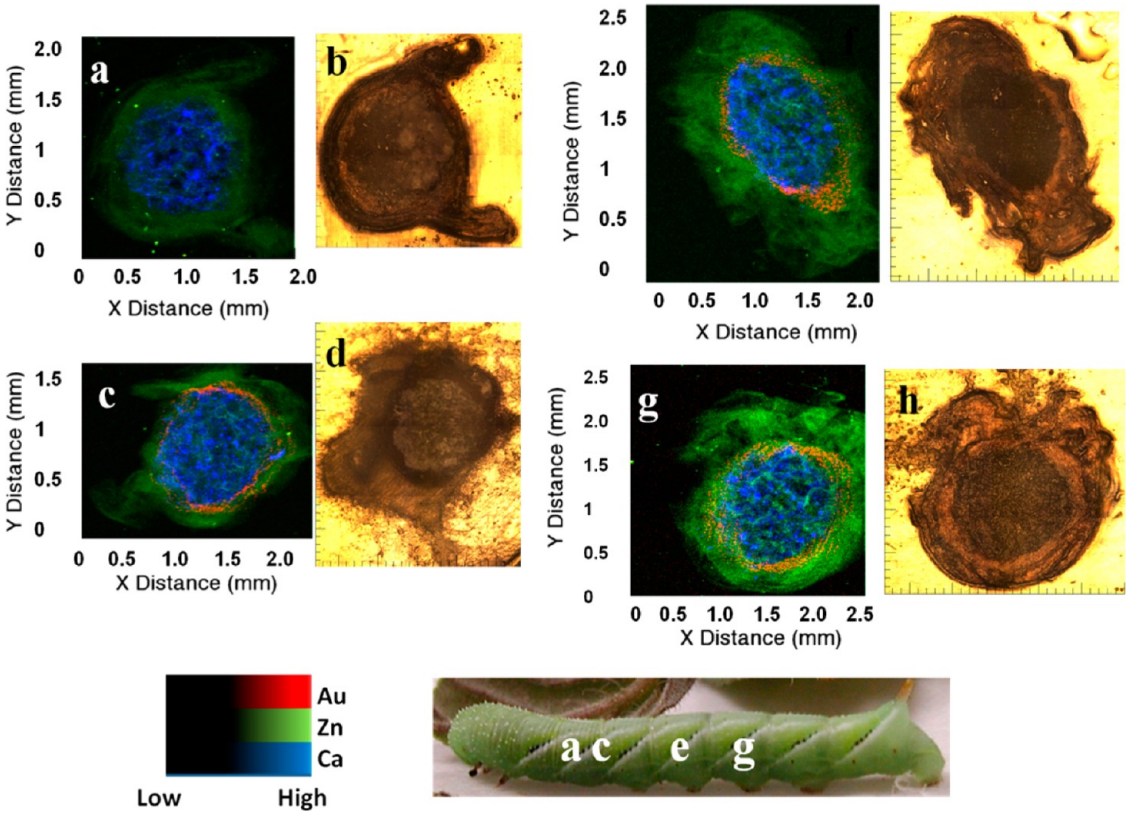

Figure 4. Synchrotron X-ray fluorescence microprobe (μXRF) maps and light micrographs of cross sections of a fourth instar tobacco hornworm larva taken from (a,-b) abdominal segment 1, anterior midgut, (c,d) abdominal segment 2, posterior anterior midgut, (e,f) abdominal segment 4, medial midgut, and (g,h) abdominal segment 5, posterior midgut. Hornworm received a 3 μg dose and was depurated for 1 day. 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.

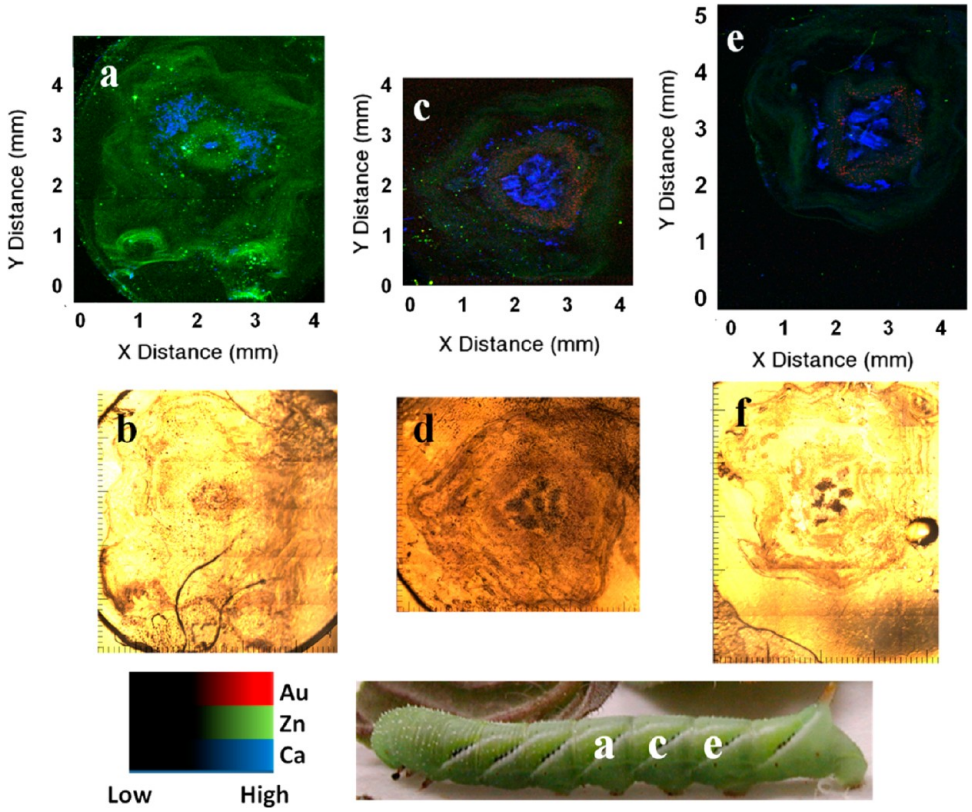

Figure 5. Synchrotron X-ray fluorescence microprobe (μXRF) maps and light micrographs of cross sections of a fifth instar tobacco hornworm larva taken from (a-b) abdominal segment 4, anterior midgut, (c-d) abdominal segment 5, medial midgut, and (e-f) abdominal segment 6, posterior midgut. Hornworm received 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.

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 days elimination. Pairwise means comparisons indicate AE of Au ENMs at 0 days elimination is significantly higher than at 1 day elimination, which is consistent with the hornworms voiding their gut contents over this period (Figure 3). No significant differences exist in AE between 1 and 7 days 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 days 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 days of elimination in *Eisenia fetida* exposed to Au MNMs via indirect exposure.[28]

After 1 day of elimination, 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}).[27] Au ENMs are not present in detectable concentrations in the anterior midgut (abdominal segment 1, Figure 4a) but are evident in the posterior anterior midgut (abdominal segment 2, Figure 4c), medial midgut (abdominal segment 4, Figure 4e) and posterior midgut (abdominal segment 5, Figure 4g). Au ENMs are also detected within caterpillars that experienced 7 days of elimination and had received only 1 μg Au ENMs (Figure 5). Au ENMs are detected in cross sections from the hornworm medial and posterior midguts (abdominal segments 5−6, Figure 5c-e) but not in the anterior midgut (abdominal segment 4, Figure 5a). We detected no Au in cross sections from the posterior midgut in control caterpillars (see SI Figure S3).

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.[25] This high pH is possibly an adaptation to tannin-rich diets and is most likely the result of ion transport.[25] Considering the negative surface charge on the Au ENMs used in this exposure, the high pH near the hornworm medial midgut may have provided a large degree of stabilization, reducing aggregation and facilitating translocation into tissues near the 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 activity, 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.[25] 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 days

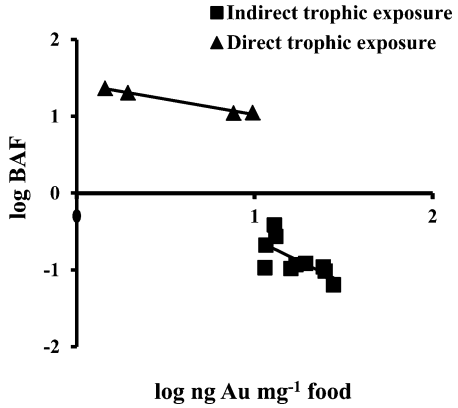

Figure 6. Relationship between log bioaccumulation factor (BAF) and log plant tissue gold (Au) engineered nanomaterial (ENM) concentration in both our previous exposure during which caterpillars were fed plants that had been exposed to Au ENMs via their roots (direct exposure) and the current study where caterpillars were fed tomato tissue surface contaminated with ENMs similar to what were used in the earlier study (indirect exposure).

elimination ranged from 6.2 to 11.6 for 5−15 nm tannate coated Au ENMs compared to the 0.16 observed in this indirect exposure.[15] 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 investigating direct trophic exposure is striking.[15] 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.[29] 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.[28] 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.[16,28,30] 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.

We have demonstrated that a potential exists for secondary terrestrial consumers to bioaccumulate ENMs via indirect trophic exposure through soil resuspension and that *Manduca sexta* bioaccumulate dramatically smaller masses of ENMs through dietary consumption of surface contaminated plant tissues than through direct trophic consumption of plants that have taken up ENMs and incorporated them into their tissues. 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 ENM surface functionalization and gut chemistry. These results suggest that dietary uptake of ENMs by terrestrial herbivores exposed via surface contaminated plant tissue could be an important pathway by which ENMs might enter terrestrial food webs. They further indicate that resuspension of ENM 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

TEM micrographs of nanoparticle suspensions, nanoparticle characterization data, μXRF scans of cross sections from control hornworm. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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