ECOTOXICOLOGY OF GOLD NANOMATERIALS: EFFECTS ON PERIPHYTON, *L. STAGNALIS,* AND *H. AZTECA* IN AN AQUATIC FOOD CHAIN STUDY

by

Michelle Lynn Sawyers Hudson

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Thesis Committee:
Professor Allen Burton, Chair
Professor Nil Basu

ABSTRACT

The growing use of nanomaterials poses serious concerns to aquatic environments. Research into the toxicity and potential trophic transfer of nanomaterials in aquatic ecosystems is needed to assess the risk of these novel pollutants. Our study investigates the movement of gold nanomaterials (AuNM) through an aquatic food chain. Field-collected (Huron River, Ann Arbor, Michigan) and lab cultured (*Oscillatoria*) periphyton (60 replicates per periphyton type) were exposed to AuNM in a closed recirculating flume system with three treatments: control (0 µg/L), low (100 µg/L), and high (500 µg/L), respectively. *Hyalella azteca* and *Lymnaea stagnalis* were then exposed to periphyton from the flumes. AuNM quickly aggregated and precipitated from the water column and gold was measured in periphyton. After feeding trials, gold was detected in *L. stagnalis* (high average 2.3 µg/L and low average 1.8 µg/L dryweight) but not in *H. azteca*. Although gold was detected in *L. stagnalis*, we observed no significant mortality or biomagnification in either *L. stagnalis* or *H. azteca.* These data suggest that trophic transfer of AuNM can occur, but the exposure is organism specific and does not have toxicological effects to exposed organisms. These results suggest that environmentally relevant concentrations of AuNM will not adversely affect aquatic ecosystems.

ACKNOWLEDGEDMENTS

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Contents

CHAPTER 1: Literature Review

INTRODUCTION

The field of nanotechnology is quickly expanding, and scientists are finding applications for nanomaterials in a wide variety of fields. Nanotechnology is defined as the "intentional and controlled generation or modification of materials at the nanometer scale" (Handy et. al. 2008) while the definition of a nanomaterial is a particle with at least one dimension less than 100 nanometers (Klaine et. al. 2008). Uses of nanomaterials (NM) include drug delivery systems, electronics, cosmetics, and clothing (National Research Council 2012). Proponents of nanotechnology cite reasons for its use including, "efficient energy consumption, a cleaner environment, and eradicating health problems" (Klaine et. al. 2012). On the other side are those more cautious about nanotechnology as much is unknown regarding nanomaterials function, particularly in the environment. For every nanomaterial application, there is both great benefit and risk involved (Klaine et. al. 2012). Risk assessment of ecotoxicology of nanomaterials in the environment is essential as their use and manufacturing are rapidly expanding.

In a 2012 report on namomaterials, the United States National Research Council stressed the need for increased research on environmental, health, and safety hazards of NM with an umbrella goal of mitigation (National Research Council 2012). Research gaps identified include occupational, consumer, and environmental exposure. Environmental exposure may occur through NM application for remediation or incidental exposure. As a result, NM modification in the environment, transport and fate, and bioaccumulation were identified as a high research priority (National Research Council 2012). Virtually nothing is known about NM behavior and its effects on the environment

since each case differs dramatically (Nowack et. al. 2012). Aquatic environments face potential risks from NM through wastewater treatment plant spills, rain, and runoff (Glenn et. al. 2012). Another concern for aquatic environments is bioavailability of nanomaterials which requires data collection concerning fate and behavior (Johnston et. al. 2010 and Klaine et. al. 2012). In particular, aquatic sediments will serve as a sink for nanomaterials, thus affecting benthic organisms (Kennedy et. al. 2008 and Velzeboer et. al. 2011).

The various types of nanomaterials and opportunities for application are astounding which presents the challenge of determining risk. For example, metal oxides, such as titanium dioxide (TiO_2), are becoming widely used in cosmetics and sunscreens and should be deemed safe for human use. Nanoparticulate silver is most often used as an antibacterial agent (Griffitt et. al. 2011). In particular, gold nanomaterials (AuNM) have many applications including industrial catalysis, chemical sensing, electronics, clothing, and pharmaceuticals (Ferry et. al. 2009, Diegoli et. al. 2008, and National Research Council 2012). Due to their unique size and structure, research on AuNM as building blocks for superlattices to program DNA is being carried out (Macfarlane et. al. 2011). AuNM have also been identified as possible mercury, lead, and copper ion detectors (Lin et. al. 2011).

CHALLENGES WORKING WITH NANOMATERIALS

One of the most commonly cited issues with nanomaterials is their dynamic properties. Nanomaterial behavior and characteristics, including size, shape, and surface charge, make it extremely difficult to detect and quantify NM at low concentrations, particularly at environmentally relevant ones (Klaine et. al. 2012). Creation of artifacts

while working with nanomaterials often occurs due to changing of physiochemical properties in a colloidal system. Addition of nanomaterials at low concentrations in an already complex matrix calls for an advance of analytical techniques which includes standard reference and testing materials coupled with methodology for NM suspension (von der Kammer et. al. 2012 and Klaine et. al. 2008). Maintaining test concentrations of NM is another concern for researchers. Moreover, characterization of media is essential during experimentation, yet there is no analytical method available presently (Bouwmeester et. al. 2011; Handy et. al. 2012; Klaine et. al. 2012). In most cases, it is difficult to keep nanomaterials in solution, so special techniques such as sonication, stirring, mixing, synthetic dispersing agents, or natural dispersants must be employed. Although each of these methods encourages dispersion, they may also have negative effects (Handy et. al. 2012 and Kennedy et. al. 2008).

In particular, bioaccumulation studies involving nanomaterials present unique challenges. Traditionally, organisms are exposed to a chemical until an equilibrium state is reached and bioconcentration factors (BCF) can be calculated. However, because of nanomaterials' dynamic state it is unsuitable to apply this typical calculation. To include colloid behavior and uptake, researchers suggest development of new tests specific to nanomaterials (Handy et. al. 2012 and Klaine et. al. 2012).

BEHAVIOR

Nanomaterial behavior varies greatly depending on its environment and stabilization mechanisms. Due to existence in a colloidal system, aggregation of nanomaterials significantly impacts their behavior as well (Keller et. al. 2010). Natural organic matter (NOM) is ubiquitous throughout the environment and may significantly impact NM aggregation. Three different metal oxide nanoparticles were examined in

seawater media and researchers found electrophoretic mobility of the NM were heavily impacted by presence of NOM and ionic strength (Keller et. al. 2010). Nanomaterials will inevitably make their way into terrestrial ecosystems through the soil compartment which may affect physical and chemical processes due to NM and soil characteristics. For example, NM agglomeration, aggregation, and dissolution are possible behavior changes as well as bioavailability to organisms (Tourinho et. al. 2012). Fullerenes, or C_{60}, were shown to decrease bacteria growth in soil which may have negative ecotoxicology and genetic diversity implications alike (Johansen et. al. 2008). Studies with single-walled carbon nanotubes indicate size-dependent toxicity of the materials on a marine copepod (Templeton et. al. 2006). Surface functionalization may slow settling of multi-walled carbon nanotubes (MWNTs) especially in the presence of NOM (Kennedy et. al. 2008).

Specifically, in aquatic ecosystems, NOM has the ability to bind metal ions and minerals thus stabilizing said materials. Citrate-stabilized gold nanomaterial effects were examined in the presence of different types of NOM isolates. In terms of aggregation, researchers found each strain of NOM isolate increased stabilization in the AuNPs because of NOM's adsorption to particle surfaces (Nason et. al. 2012). Similarly, another study found NOM increased stabilization of carbon nanotubes (Hyung et. al. 2006). Research focused on silver nanoparticle (AgNP) effects on bacterial activity in a natural aquatic system indicated that low concentrations of AgNP in nanogram per liter range will most likely not negatively affect aquatic biogeochemical cycles. However, bacterial exposure to AgNP in the microgram per liter range may have negative effects (Das et. al. 2012).

Exposure of nanomaterials to aquatic ecosystems is inevitable and coagulation will greatly affect nanomaterial fate (Holbrook et. al. 2010). In an effort to limit human exposure to NM from aquatic ecosystems a study addressed multi-walled carbon nanotube (MWCNT) and their relationship to source water quality in water treatment facilities. Coagulant type and dosage most significantly affected MWCNT removal and further illustrate aquatic environmental factors dictate NM behavior (Holbrook et. al. 2010). Currently, silver, titanium dioxide, and zinc oxide nanomaterials may present the highest risk to aquatic organisms due to their use in sewage treatment effluents (Gottschalk et. al. 2009).

TROPHIC TRANSFER

With an increasingly wide use of nanomaterials, concerns have arisen about exposure to both low level and, subsequently, higher level organisms. Studies have shown that various types of nanomaterials, including silver and cerium dioxide, in both terrestrial and aquatic ecosystems alike may cause adverse effects (Gaiser et. al. 2010; Griffitt et. al. 2011; Navarro et. al. 2008; Scown et. al. 2010). Biomagnification in both terrestrial and microbial food chains has been illustrated (Judy et. al. 2011 and Werlin et. al. 2011). The National Institute of Standards and Technology (NIST) identified nanomaterials as a cause for potential environmental risks while illustrating quantum dot trophic transfer in rotifers (Holbrook et. al. 2008). Similarly, it has been shown AuNM can be taken up by cells in culture as well as in saltwater clams (Ferry et. al. 2009 and Hull et. al. 2011). In addition, plant uptake may play an important role in aquatic exposure routes to AuNM (Glenn et. al. 2012).

ALGAE AND MACROINVERTEBRATE EXPOSURE

Carbon nanotubes have been shown to significantly impact survival or growth to several species of freshwater aquatic invertebrates including *Hyallela azteca, Chironomus dilutes, Lumbriculus variegates, Villosa iris,* and *Daphnia magna* (Mwangi et. al. 2012 and Petersen et. al. 2009). The aforementioned species are environmentally sensitive and commonly used in aquatic toxicity tests to assess potentially adverse effects to aquatic organisms. Another study addressed MWCNT contaminated sediments on benthic macroinvertebrate communities. Although there was a significant increase of individuals with higher MWCNT concentrations, biodiversity was not affected. In keeping, researchers determined the MWCNT dose was not statistically significant and therefore, negative community effects should not be seen in the future at environmentally relevant MWCNT concentrations (Velzeboer et. al. 2011). When compared to a known environmental contaminant, polycyclic aromatic hydrocarbon (PAH), exposed organisms did not readily absorb purified carbon nanotubes in their tissues (Petersen et. al. 2008). Similarly, no significant effects were recorded of SWNTs on a species of lungworm (Galloway et. al. 2010). Although bioavailable to several marine benthic organisms SWNT were not shown bioaccumulate or cause toxicity (Parks et. al. In Press).

Effects of amine-coated AuNM on a species of algae and benthic bivalve gene expression were investigated. After 24 hour direct exposure of the green algae *Scenedesmus subspicatus* to amine-coated AuNM, there was significant algal cell mortality. Based on TEM images, cell walls had adsorbed AuMP which led to intracellular and cell wall detrimental effects. The bivalve in question, *Corbicula fluminea,* experienced bioaccumulation of AuNM in the gills and digestive epithelia as well as oxidative stress (Renault et. al. 2008).

The most utilized nanomaterial in commercial products has been identified as silver nanoparticles (AgNP) (Bowman et. al. 2012). Fittingly, countless studies have investigated the effects of AgNP. Zebrafish (*Danio rerio*) exhibited adverse effects of the heart when exposed to AgNP (Bowman et. al. 2012).

Within metallic nanomaterials, different elements can be combined to enhance nanomaterial use. One study investigated toxicity of silver, gold, and silver-gold bimetallic nanoparticles effect on *Daphnia magna* mortality. The LC_{50} of AuNM ranged from 65-75 mg/L while the LC_{50} of AgNM ranged from 3-4µg/L for three different diameter sizes. In the case of AgNM, toxicity was not determined to be a function of nanoparticle diameter. Rather, toxicity was attributed to AgNM aggregation while suspended in freshwater. Silver-gold bimetallic nanoparticle LC_{50} fell between Au and Ag LC_{50}, but closer to that of AgNM suggesting that pairing Au with Ag nanoparticles decreases Ag bioavailability, thus reducing environmental impacts (Ting et. al. 2010). Ionic activity, which is associated with particle suspension, may significantly impact nanosilver behavior. Dissolved silver may be released when induced by centrifugal ultrafiltration and atomic absorption spectroscopy (Liu and Hurt 2010). From this study, authors were able to propose an empirical kinetic law which "reproduces the observed effects of dissolution time, pH, humic/fulvic acid content, and temperature . . . in the low range of nanosilver concentration most relevant for the environment" (Liu and Hurt 2010).

PERIPHYTON

Studies involving periphyton are of utmost importance as toxins, dissolved nutrients, and natural organic matter (NOM) encounter periphyton initially in natural waters (Sabater et. al. 2007). Periphyton, also known as biofilms, is an ideal candidate to

study toxin effects due to rapid growth, small size, and community makeup. Supplying food and nutrients to higher trophic levels is another function of periphyton, thus making it an integral part in trophic transfer experiments (Hill et. al. 2010). As a result, periphyton may serve as "early warning systems" regarding environmental issues (Sabater et. al. 2007).

CONCLUSION

In order to safely recommend said AuNM use, extensive research must be conducted. AuNM were chosen as the ENM in this case due to their relative ease of synthesis and stable suspension. The purpose of this research was to characterize the movement of nanomaterials through an aquatic food chain. Effects of AuNM addition to water column, uptake by periphyton, and transfer to grazing macroinvertebrates were investigated. A secondary objective was to compare the effects of AuNM in a mixed periphyton community to that of a single species of algae, *Oscillatoria prolifera.* In turn, the exposed algae were fed to two commonly used aquatic macroinvertebrates in ecotoxicology tests, *Hyallela azteca* and *Lymnea stagnalis.* The first objective of this study was to determine if AuNM bioaccumulate in a periphyton and macroinvertebrate food chain in controlled laboratory flume experiments. In turn, periphyton community make-up was investigated to determine if it affects AuNM behavior and uptake. It was hypothesized that macroinvertebrates would accumulate AuNM by feeding on periphyton exposed to AuNM in artificial streams. In addition, no significant difference would be seen in uptake or behavior of AuNM in mixed periphyton community versus single species *Oscillatoria prolifera.* Another objective was to determine fate of AuNM in laboratory flumes after five days of exposure. It was hypothesized periphyton would

uptake AuNM within hours of initial spiking, leaving little to no AuNM present in the water column.

CHAPTER 2: Manuscript

INTRODUCTION

Nanotechnology use is rapidly proliferating throughout numerous industries, creating concern for its potential environmental impacts. Natural environments may be exposed to nanomaterials through a variety of pathways, such as intentional (i.e., for remediation) or accidental exposure (Handy et. al. 2008; Klaine et. al. 2008; National Research Council 2012). Specifically, aquatic environments are at risk to NM exposure from atmospheric emissions, waste from production facilities, or runoff (Glenn et. al. 2012; Klaine et. al. 2008). Determining nanomaterial fate and behavior within the scope of natural aquatic ecosystems is a complex task yet crucial to estimating the potential risk of these novel pollutants.

Environmental conditions greatly influence nanomaterial fate and behavior in an aquatic setting. The presence and concentration of dissolved organic carbon (DOC) can affect NM agglomeration, aggregation, and settling, which can affect whether NM remain suspended in the water column or precipitate to the benthos (Klaine et. al. 2008; Keller et. al. 2010; Kennedy et. al. 2008; Nason et. al. 2012; Tourinho et. al. 2012). Periphyton communities (i.e., the matrix of algae, bacteria, and microorganisms attached to benthic surfaces) have a strong potential to be exposed to suspended nanomaterials because of their high capacity for solute uptake (Sabater et. al. 2007). Even without direct cellular uptake, agglomerated NM may settle into the periphyton matrix. Countless aquatic organisms rely on periphyton as a food source, and nanomaterial-contaminated periphyton may pose a risk of dietary exposure and trophic transfer of these contaminants.

This study focused on the movement of AuNM through an aquatic food chain in a recirculating flume system. Periphyton (both multi- and single-species), *Lymnaea stagnalis*, and *Hyalella azteca* were chosen to simulate a simple lotic food chain. It was hypothesized that periphyton would accumulate AuNM rapidly and little AuNM would be detected in the water column after just a few hours. In addition, we hypothesized that, after feeding on the periphyton, we would detect Au in the tissues of macroinvertebrates. However, we expected tissue concentrations to be lower than in periphyton suggesting no bioaccumulation of AuNM.

METHODS AND MATERIALS

Experimental Design

Experiments were conducted in which AuNM were added to recirculating flumes containing tiles seeded with periphyton and the tiles were then moved to a beaker to investigate trophic transfer to macroinvertebrates. Two separate experiments were performed using different periphyton communities: a single species monoculture and a field-collected mixed community. Once exposure to AuNM was complete, two aquatic macroinvertebrate species, *Lymnaea stagnalis* and *Hyalella azteca*, were allowed to feed on the tiles and then depurated before processing for total gold analysis.

Nanomaterial Characterization

AuNM were synthesized using the Turkevich method (Kimling et al. 2006) at Clemson University. Briefly, 0.01 M chloroauric acid was boiled in ultra pure water and then 1% sodium citrate was added. Sodium citrate caps assist in aqueous suspension. Zeta-sizer results indicated that the majority of the particles had a size distribution by intensity of 28 nanometers (Figure 1A) while majority of size distribution by number around 15 nanometers (Figure 1B). Discrepancies for this may be attributed to the fact that a few large particles can obstruct Zeta-sizer detection of numerous small nanomaterials.

Flume Design

Flumes were constructed using PVC downspout extensions (24 cm long), plastic sheets, flexible PVC tubing (3/16 inch inner diameter), 2000 mL plastic bottles, and submersible pumps. Plastic bottles serve as a “reservoir” at the end of each flume and a submersible pump returned water to the head of the flume (flow rate = 200 L/h). A small

piece of notched plastic was glued to the end of each channel to retain about 1500 mL of water within each flume. Flumes were randomly arranged on tables (Figure 2).

Periphyton Colonization

Separate recirculating streams were set up for algal colonization of unglazed clay tiles. In each colonization stream, forty unglazed tiles (26 cm^2) were placed in ion-enriched water (IEW) prior to addition of periphyton for colonization (Figure 3). IEW is Ann Arbor city water that has been amended with NaCl and CaCl (40 mg/L), and NaBr and KCl. Fluorescent grow lights (~2000 lux, Hydrofarm) on a 16-8h light/dark cycle provided light for maximum periphyton growth. Periphyton from the Huron River (Nichols Drive, Ann Arbor, MI) was collected in mid-September 2011 by scraping rocks with a stiff-bristled brush into containers, which were then transported back to the University of Michigan Aquatics Laboratory. The collected periphyton was then homogenized in a blender and poured into two colonization streams (80 total tiles). A single species of algae, *Oscillatoria prolifera*, was purchased from The University of Texas (UTEX: The Culture Collection of Algae, Strain B 1270) and cultured in IEW in incubators (Thermo Scientific Illuminated Incubator 818) on a 16-8 hour light/dark cycle at 25°C. After 4 weeks in the incubator, *Oscillatoria* cultures were transferred to two colonization streams. While in the incubator, beakers were kept separate, carefully handled, and covered with parafilm to discourage cross contamination of species (verified with microscopy). Both algae types were allowed to colonize the tiles for 6 weeks prior to initiation of the experiments.

Flume Experiments

Prior to each experiment, every flume (n = 30) had new tubing installed and was flushed with tap water for 24 hours. Fluorescent grow lights (=~1200 lux) on a 16-8h light/dark cycle were used to provide light during the five day exposure period. Expectations were AuNM would quickly fall out of solution (~less than 96 h) so this five day exposure period was chosen. Each reservoir was filled with 1500 mL of IEW and the recirculating pumps were turned on. Two periphyton tiles were haphazardly selected from the colonization streams and added to each flume (Figure 4). Background water samples were taken once prior to spiking, acidified with aqua regia (5%), and stored in acid-cleaned centrifuge tubes at room temperature for later analysis of total gold. One of three treatment groups (reference, low, or high) was assigned to each flume and low and high treatment flumes (n = 10 each) were spiked with AuNM at the top of each flume to a final nominal concentration of 100 µg/L and 500 µg/L, respectively. Nominal concentrations used in this study for low treatment (100µg/L) and high treatment (500µg/L) are reasonable natural concentrations and similar to those used in other studies (Glenn et. al. 2012; Nason et. al. 2012; Pan et. al. 2012). Time 0 water samples were taken immediately after spiking of all flumes was complete. Unfiltered water samples were taken at 1, 4, 12, 24, 48, 72, 96, and 120 h after the initial Au addition. After each water sampling period, samples were acidified with 5% aqua regia (Anderson et al. 2005) and stored at room temperature until analysis. Daily maintenance of flumes included randomized pH and temperature checks of 1 flume per treatment per sampling period. Some water loss was experienced due to high evaporation rates and reservoirs were refreshed with clean IEW as needed to maintain 1500 mL in each flume. All water

samples were analyzed for total Au content by inductively-coupled plasma mass spectrometry (ICP-MS) (detection limit of 0.5 µg/L).

Periphyton Analysis

After five days, the bottom tile from each flume was removed and analyzed for chlorophyll *a* and total Au content. Periphyton was collected by gently scraping tiles and homogenizing the resulting mixture. Subsamples of the homogenized mixture were collected on preweighed glass microfiber filters (Whatman GF/F, 25 mm diameter) for two analytical replicates each of chlorophyll *a* and total Au analysis. Prior to total Au analysis, filters were dried overnight at 70°C and acid digested in a Microwaved Accelerated Reaction System 5 (CEM Corporation) with 9 mL of nitric acid and 3 mL of hydrochloric acid (trace metal grade) (U.S. EPA 1996). Samples were quantitatively diluted and analyzed for total Au by ICP-MS. Periphyton chlorophyll *a* content was measured by fluorescence readings following ethanol extraction (Biggs & Kilroy 2000) on a fluorometer (Turner Designs TD-700).

Feeding Assays

Hyalella azteca and *Lymnaea stagnalis* were obtained from existing laboratory cultures based on EPA methods (EPA 2000). For the feeding assays, we used 7-14 day old *H. azteca* and 14-21 day old *L. stagnalis* (U.S. EPA 2010). At the end of the periphyton flume exposures, the top tile from each flume was removed and added to a 600 mL beaker filled with clean IEW. From each treatment (reference, low, and high), five beakers containing tiles had 10 *H. azteca* added to them and the other five received 10 *L. stagnalis.* Organisms were allowed to feed on the tile for 24 h before being transferred to clean IEW with no food for a 24h depuration period (Neumann et. al.

1999). After depuration, *H. azteca* were acid digested at ambient air temperature with a 5:4 combination of nitric acid and hydrogen peroxide while *L. stagnalis* were digested with a 5:2 combination of nitric acid and hydrogen peroxide (Norwood et al., 2006; Croteau and Luoma 2007). *L. stagnalis* soft tissue plus shells were processed together due to their small size and frail shells at this life stage. All samples were analyzed for total Au content by ICP-MS (Perkin Elmer Elan 6000). Replicates were taken at least one per every ten samples with standard reference calibration at 0 µg/L, 50 µg/L, 100 µg/L, and 500 µg/L (TraceCERT gold standard). The 0µg/L reference served as a continuing calibration blank throughout sample analysis.

Statistical Analysis

Results from chlorophyll *a* analysis, periphyton digestions, and invertebrate digestions, were analyzed using separate two-way ANOVAs ($\alpha = 0.05$) with algae type and nominal Au concentration as factors. Because all Au levels in control treatments were reported as non-detectable, statistical analysis of Au concentration data included just the low and high treatments. Dissolved Au in water samples (low and high treatments only) were analyzed using ANCOVA ($\alpha = 0.05$) with algae type, nominal Au concentration, and sample time as factors. In order to accommodate data normality in periphyton data, all control values (returned as 0µg/L) were removed from data as well as two outliers. For the Huron experiment, water column Au concentrations from early sampling times were highly variable with many non-detectible concentrations. These non-detects were possibly due to incomplete mixing or poor sampling and we have removed those data from our statistical analysis. Tukey post hoc tests were used to determine significant differences between treatments within significant factors. Shapiro-

Wilk tests (α=0.05) and residual plots were utilized to test assumptions of linear models. Natural log transformations were implemented when assumptions of linear models were not met. All statistical analyses were performed with R 2.15.0 (R Core Team 2012).

Figures

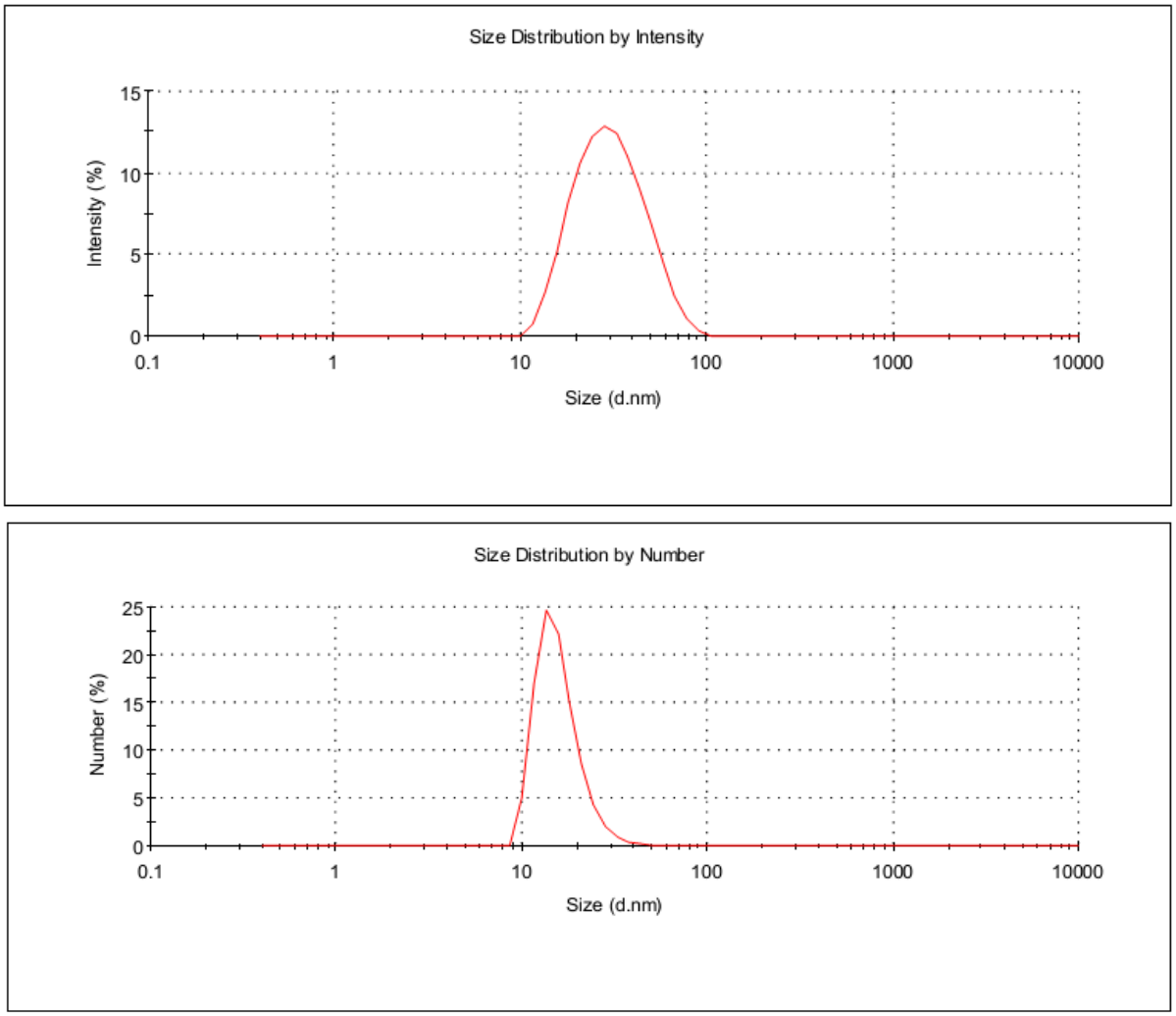

Figure 1 A and B: Size Distribution of gold nanomaterials by intensity (A) and number (B) using a Zeta sizer.

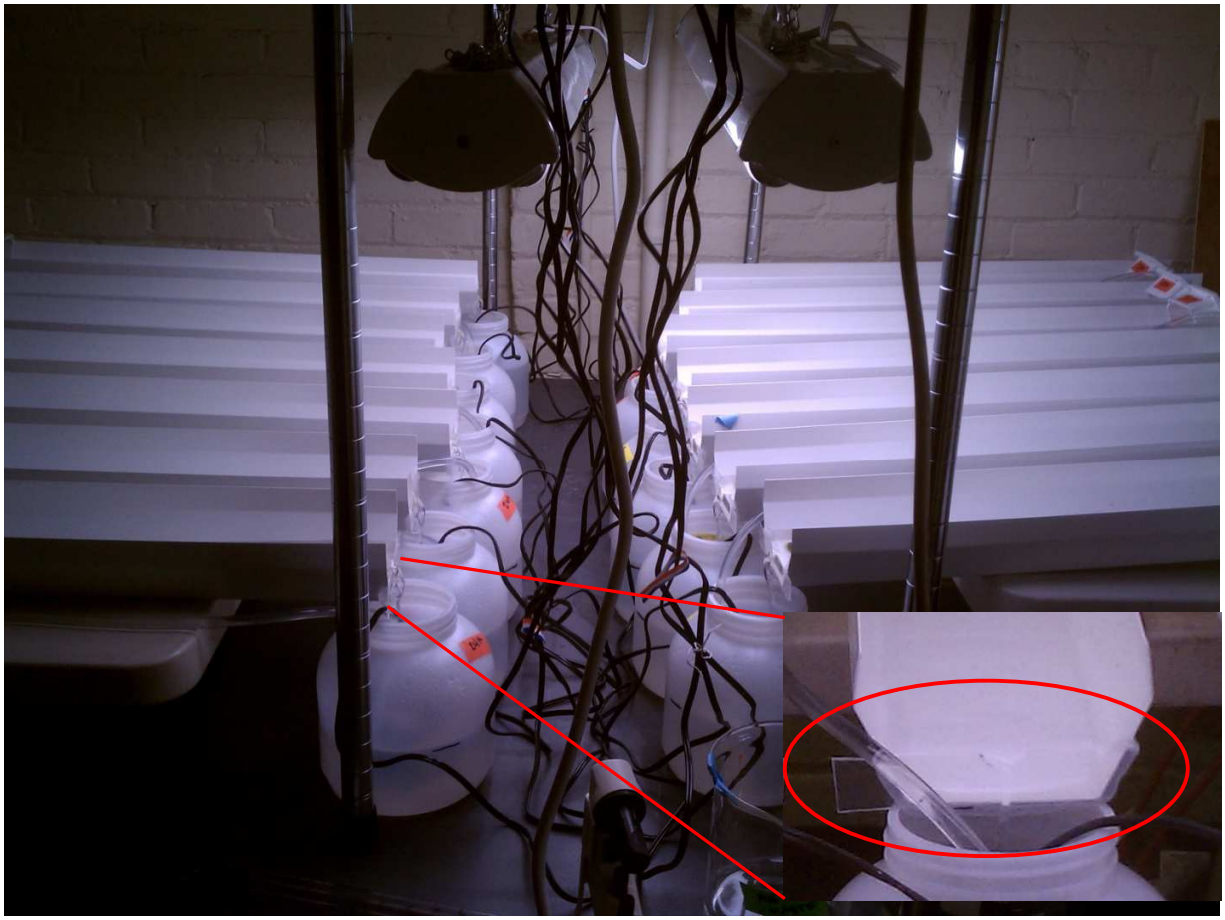

Figure 2: Photograph of the flume setup used to expose periphyton to gold nanomaterials. Inset shows a zoom of the notched dam retaining water in the channel.

Figure 3: Image of the unglazed tiles in colonization streams during the 4-week colonization period

Figure 4: Image of periphyton tiles placed at the base of the artificial flumes prior to addition of the gold nanomaterials.

RESULTS

Water Samples

Total Au concentrations in water were similar to nominal concentrations at time zero and then declined exponentially (Fig. 5). There was no significant difference between the mixed periphyton (Huron River) community compared to single strain (*Oscillatoria)* species treatments ($p = 0.14$; Table A1). However, Au concentrations declined significantly through time ($p < 0.001$) and the rate of decline differed between

high and low Au treatments ($p < 0.05$). Around 48 h into the experiment in both high and low treatments almost no Au was detectible in the water column (Fig. 5).

Periphyton

The Huron River periphyton community make-up was dominated by the cyanobacteria *Limnothrix* and diatoms. No significant differences were seen between periphyton biomass (as chlorophyll a) for different periphyton communities ($p = 0.28$) (Fig. 8), nominal Au treatment type ($p = 0.89$), or the interaction between periphyton community and treatment type ($p = 0.84$). (Figure 6 and Table A2). Gold was found in periphyton in both algae types and in both high and low treatments (Fig. 6). Results of the 2-way ANOVA indicated a significant difference between treatment type ($p = 0.02$). Nominal Au treatment had strong significant affect on periphyton Au content ($p < 0.001$) with low and high treatments differing from one another for both Huron and *Oscillatoria* treatments. In addition, no significant differences ($p = 0.29$) were reported in AuNM concentration between mixed-culture and single species periphyton.

Feeding Assays

We measured striking differences in Au concentrations between our two grazers as we measured Au in *L. stagnalis* but no Au was detected *H. azteca.* No significant mortality was reported in either species during 24h exposure period or 24h depuration period (Table A4). Au was found in the tissues of *L. stagnalis* in both the Huron high and low Au treatments (2.4 and 2.1µg/g dry weight, respectively) as well as in the *Oscillatoria* high and low treatments (2.2 and 1.45 µg/g, respectively) (Figure 7). Between different nominal Au treatments, *L. stagnalis* tissues differed in Au concentration ($p < 0.001$) with control treatments having significantly lower Au body

burden than low and high treatments. However, we found no significant difference between low and high treatments, no interaction between nominal Au concentration and periphyton community ($p = 0.8551$), and no significant difference was seen between periphyton type ($p = 0.25$) (Table A3). No statistical analysis was done for *H. azteca* as all results were returned as non-detectable.

Nanomaterial Fate

A mass balance was used to estimate the fate of AuNM for three compartments: water, periphyton, and attached to the flume. We estimated that the majority of AuNM spiked during experiments was not measured in the water and periphyton, and likely ended up in flume tubing and reservoir (Table A6). For the low and high treatments, on average 79.7% and 80.3%, respectively, of the Au was unaccounted for and likely aggregrated to physical structures in the mesocosm. Assuming even settling of AuNM, every square centimeter of the flume surfaces should have 0.66 µg Au/cm^2 for high treatments and 0.13µg Au/cm^2 for low treatments. Results from *Oscillatoria* and Huron River periphyton high treatments were 0.17 µg Au/cm^2 and 0.38 µg Au/cm^2, respectively, while low treatment results were 0.04 µg Au/cm^2 and 0.07 µg Au/cm^2, respectively.

Figures

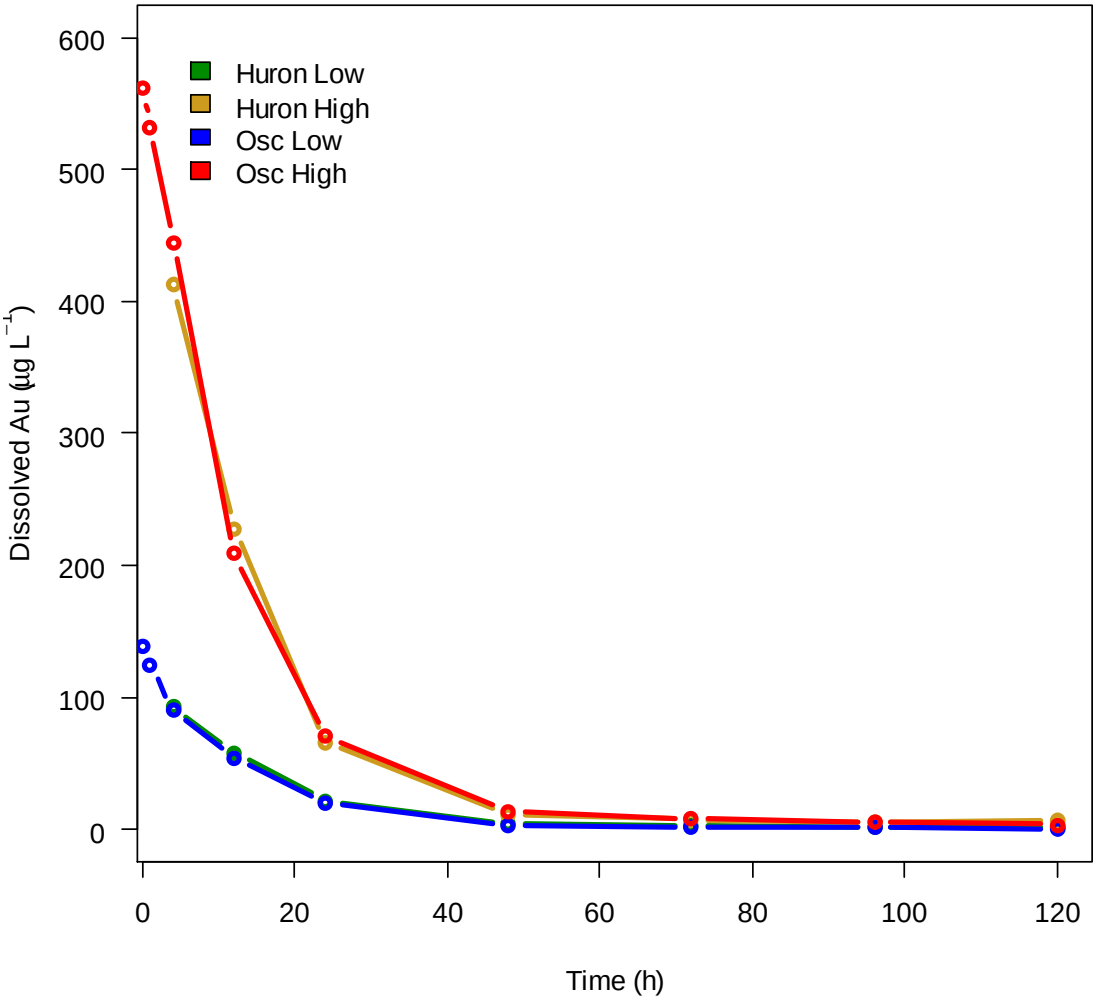

Figure 5: Dissolved gold concentrations in water samples over time. High and low treatments received 500 and 100 µg/L Au nanomaterials at time zero.

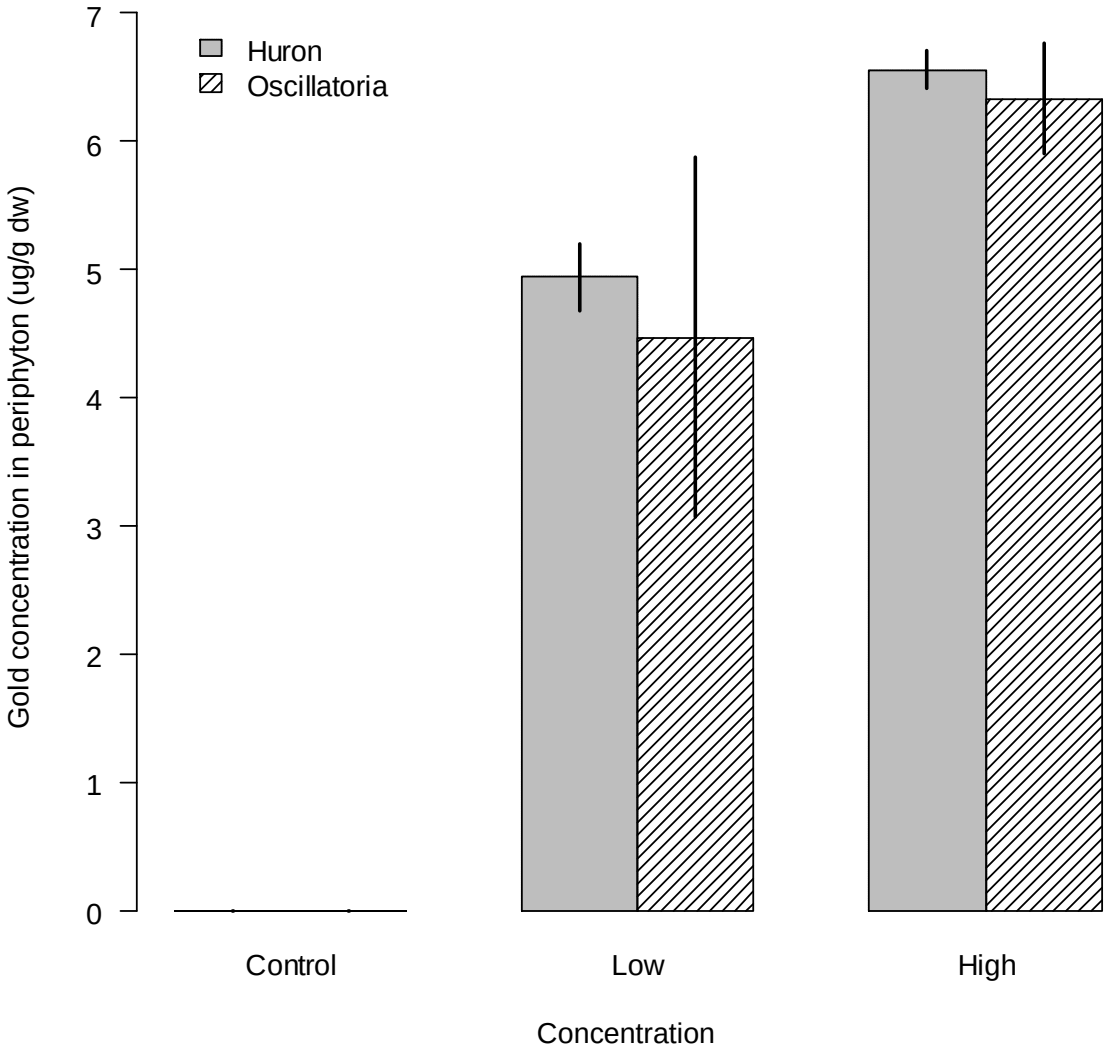

Figure 6: Average total gold concentration in periphyton from two experiments using different periphyton communities (Huron R. (mixed) and Oscillatoria (single-species) and three nominal Au nanomaterial concentrations (0, 100, and 500 µg/L, respectively). Data were log+1 transformed for statistical analysis. Errors are present as standard deviations.

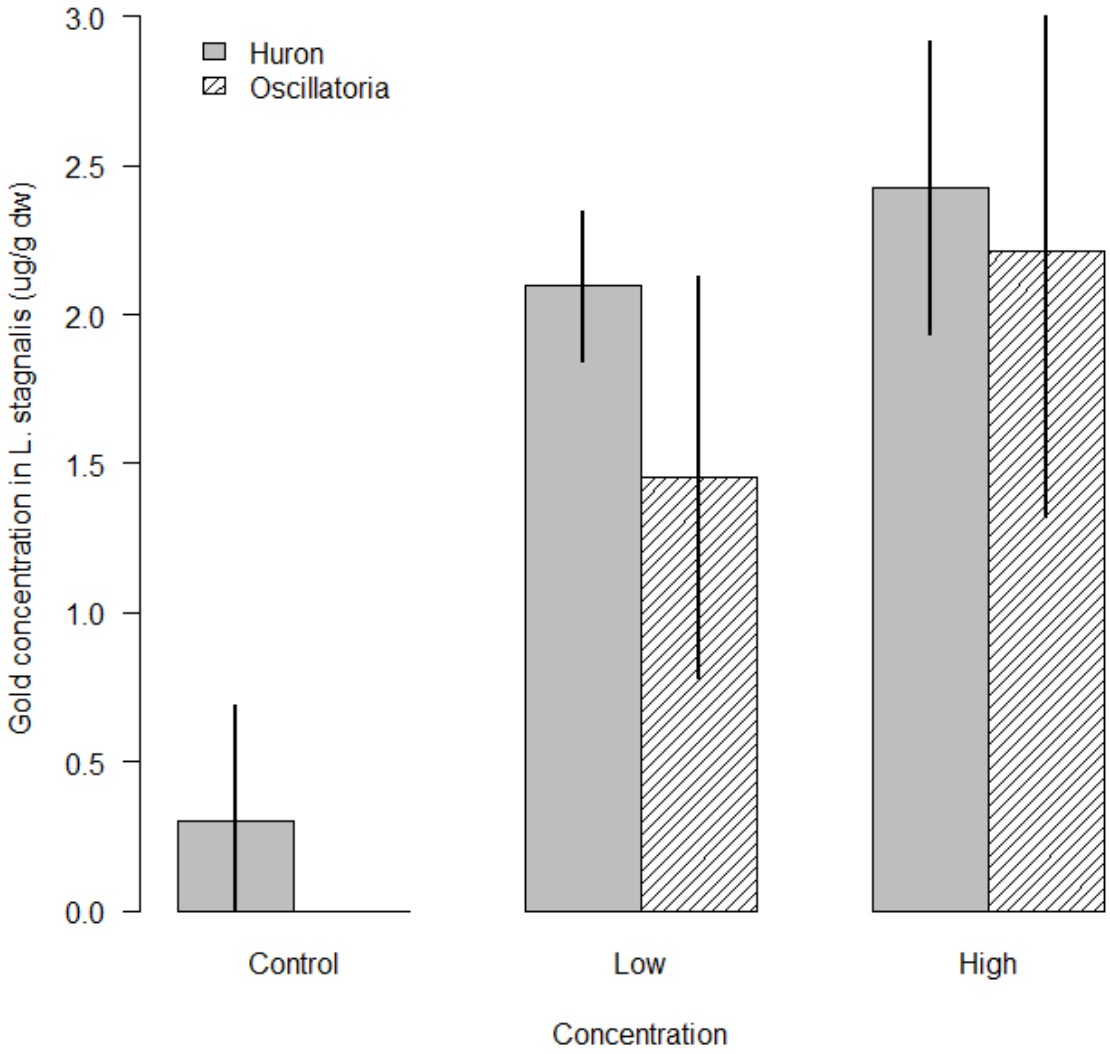

Figure 7: Average total gold concentration in *L. stagnalis* from two experiments using different periphyton communities (Huron R. (mixed) and Oscillatoria (single-species) and three nominal AuNMconcentrations (0, 100, and 500 µg/L, respectively). Data were log+1 transformed for statistical analysis. Errors are present as standard deviations.

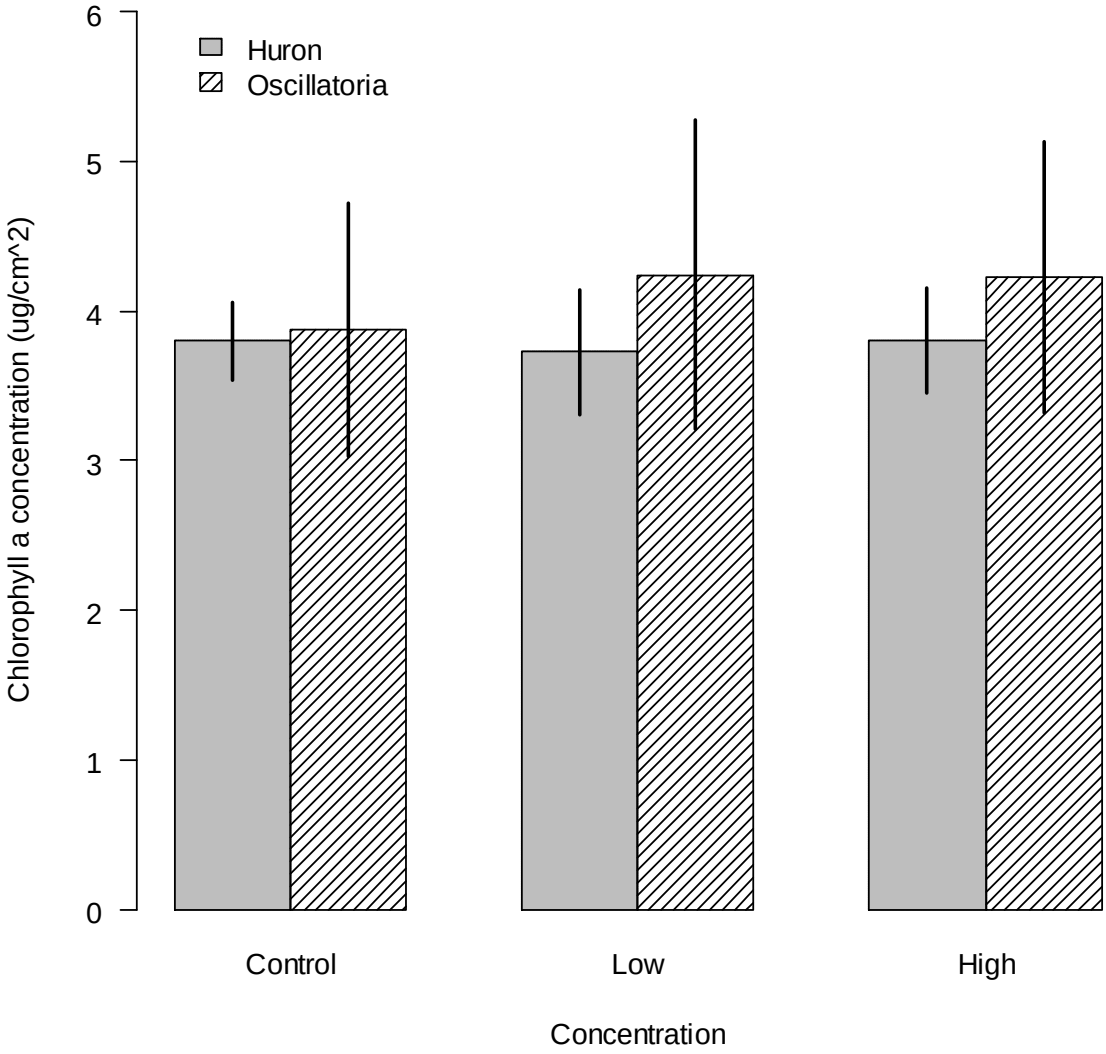

Figure 8: Biomass results (µg/cm^2 chlorophyll *a*) from two experiments using different periphyton communities (Huron R. (mixed) and Oscillatoria (single-species) and three nominal AuNMconcentrations (0, 100, and 500 µg/L, respectively). Data were log transformed for statistical analysis. Error bars represent standard deviations.

DISCUSSION

Nanomaterials are known to have unique properties that affect their behavior, partitioning, and exposure in aquatic systems (Handy et. al. 2012; Holbrook et. al. 2010; Keller et. al. 2010; Kennedy et. al 2008; Klaine et. al. 2012). Based on results of the current study, we suggest that suspended AuNM quickly aggregated and precipitated out of the water column. The highest Au concentrations occurred at 1 hour after spiking and then declined exponentially until no Au was measured in any treatment after 48 hours. This may be attributed to nanomaterial agglomeration, aggregation, or precipitation (Tourinho et. al. 2012). Rapid aggregation and precipitation of AuNM was noted in this study, similar to other NM studies (Glenn et. al. 2012; Manusadzianas et. al. 2012; Nason et. al. 2012). Upon flume examination during the experiments, it was evident AuNM had attached to the inside of plastic tubing, reservoirs, and possibly flume pumps. However, AuNM aggregated throughout each mesocosm including on periphyton tiles, which allowed for examination of dietary exposure. Surface area calculations indicate uneven settling of AuNM within each system, specifically proportionally less settling on periphyton tiles. This may be explained by tile location in fast moving water of flumes, in addition to AuNM affinity for plastic surfaces. Due to rapid AuNM aggregation, organism exposure to dissolved Au is unlikely. In the environment, even without thick biofilm communities, AuNM may settle out just as quickly as in the current study as it is dominated by physical aggregation.

Water chemistry, including the amount of DOC and ionic strength, in natural waters may play an important role in AuNM fate. Aquatic environments with high levels of DOC may be less at risk to AuNM aggregation and settling due to DOC's stabilizing ability once bonded to AuNM (Nason et. al. 2012 and Hyung et. al. 2006). Importantly,

our experimental IEW waters were very low in DOC (<1 mg/L; DM Costello pers. obs.), perhaps contributing to the rapid rate of nanomaterial settling. Low DOC waters are known to have higher aggregates and less stable suspensions. Similarly, increasing ionic strength encourages NM aggregation (McLaughlin and Bonzongo 2012; Nason et. al. 2012).

Since periphyton actively take up solutes for growth, their chances for dissolved contaminant uptake are great (Sabater et. al. 2007). Previous research has illustrated uptake of nanomaterials by both aquatic and terrestrial plants (Glenn et. al. 2012; Hull et. al. 2011; Renault et. al. 2008). Either cellular uptake or agglomeration is responsible for the measureable Au in periphyton tissues; however, since microscopy was not performed, it is not possible to determine whether or not AuNM actually crossed cell membranes. Although we could not determine if cellular uptake occurred in our periphyton, the difference between uptake and aggregation is not important for many grazers as they consume the entire periphyton matrix while feeding. Despite difference in community composition, both periphyton communities experienced accumulation of AuNM at similar loading rates. This suggests there was no difference in efficiency between single species community, *Oscillatoria,* and the mixed Huron River community. This was unexpected as research on phytoplankton has shown greater uptake of titanium NM in a mixed community (Kulacki et. al. 2012). For other dissolved solutes (e.g., nitrogen) there is a positive relationship between increased uptake rates and species richness (Cardinale 2011, Baker et. al. 2009). Likely, physical aggregation and settling of AuNM is the dominant force responsible for periphyton AuNM accumulation, not biology or ecology.

When attempting to assess potential toxic effects of nanomaterials in the environment, most studies highlight uncertain exposure scenarios, uncertain effects thresholds, and difficult risk characterization. These issues stem mostly from NM transport (both particle and molecular) and NM behavior in environmental matrices (Klaine et. al. 2012). Many studies reporting negative effects on organisms used concentrations orders of magnitude higher than those in the present study (Judy et. al. 2011; McLaughlin and Bonzongo 2012; Manusadzianas et. al. 2012; Unrine et. al. 2012). However, numerous studies have illustrated the potential for trophic transfer of nanomaterials *in vivo* and *in vitro* with measured adverse effects on aquatic and terrestrial organisms from dietary exposure (Gaiser et. al. 2011; Griffit et. al. 2011; Werlin et. al. 2011). Adverse effects include biomagnification (Werlin et. al. 2011), thickening of gill tissue and altered gene expression in sheepshead minnows (Griffit et. al. 2011), and significant mortality in *D. magna* and trout hepatocytes (Gaiser et. al. 2011). In contrast, many studies have also shown that organisms with nanomaterial exposure do not experience any negative toxicological effects (Petersen et. al. 2008; Galloway et. al. 2010; Parks et. al. 2013).

Based on current information, environmentally relevant concentrations of NM are not known. Although given what is known about contaminant concentration in general our chosen concentrations are environmentally and ecologically relevant. Many studies reporting negative effects on test subjects used concentrations orders of magnitude higher than those in the present study (Judy et. al. 2011; McLaughlin and Bonzongo 2012; Manusadzianas et. al. 2012; Unrine et. al. 2012). If NM exposures are more similar to pharmaceuticals we might expect environmentally relevant concentrations in the

nanogram/L range. As NM research evolves, we may discover concentrations used in this study were not environmentally relevant.

Although existing studies offer equivocal results about the importance of dietary exposure and trophic transfer of NM, this potential exposure route has implications for nanomaterial use, environmental exposure and possible human exposure. The current study shows trophic transfer of AuNM from periphyton to *L. stagnalis* but no elevated gold levels were seen in *H. azteca.* Although we have demonstrated the potential for trophic transfer of AuNM, it is important to explore why our results differed for our two grazers. First, *L. stagnalis* and *H. azteca* have slightly different feeding mechanisms; Although both are grazers, *L. stagnalis* is a more general feeder as it removes the entire periphyton matrix with its radula as it feeds rather than selectively grazing like *H. azteca* (Balog et. al. 2012). *L. stagnalis* spent most of the 24 hour exposure time on top of and moving around the periphyton tile (where all the periphyton was located) whereas many *H. azteca* were recovered from the underside of the tiles where no periphyton had grown. Being located under the tiles can also be attributed to behavioral differences between our grazers as *H. azteca* are smaller, more mobile, and often hide under cover. Further, differences between our grazers could have been a result of differential sensitivies; the dose of Au in periphyton may have triggered reduced feeding in *H. azteca* but may not have been substantial enough to alter *L. stagnalis* feeding rates. In studies with other metals, the feeding rates of both grazers have been shown to be sensitive to chemical contaminants (Croteau and Luoma 2009, Nguyen et. al. 2012). Future studies should explore how different dietary doses of NM alter feeding rates of grazers.

Although the current study has demonstrated trophic transfer no biomagnification occurred as Au concentrations in snail tissues were lower than those in periphyton. Detection of AuNM fate within the snails was not carried out due to their small size and frail shells. The LC_{50} of gold in freshwater for *H. azteca* is 3,150 ug/L while the LC_{50} of gold for *L. stagnalis* is unknown (U.S. EPA 2013). If the LC_{50} of gold in *L. stagnalis* is similar to that of *H. azteca*, we can surmise levels reported in this study will not have significant lethality. The levels of gold observed in *L. stagnalis* were not high enough to cause any noticeable damage to the organism. However, higher concentrations or a longer exposure period to AuNM may have adverse effects on *H. azteca* and *L. stagnalis*.

In theory, artificial flumes are an exemplary tool as they provide a small scale realistic approach to complex real world scenarios. However, based on results of this study, we often find laboratory artifacts in simplistic mesocosm experiments. This stresses the need for learning how to measure NM in the environment to perform *in situ* experiments. Advancing environmental chemistry in regards to NM will help make this a reality.

CONCLUSION

This research investigated the movement of AuNM through an aquatic food chain. Regardless of community composition, periphyton communities exposed to AuNM in closed flume systems displayed elevated Au concentrations, most likely due to physical aggregation and settling of AuNM. Dietary exposure to contaminated periphyton led to elevated Au tissue concentrations in *L. stagnalis* while Au was not detected in *H. azteca* tissues. Differences between *L. stagnalis* and *H. azteca* body burden may be attributable to feeding mechanisms. These results suggest selective feeding by macroinvertebrates,

NM settling, and NM aggregation are important when considering NM fate in the environment and their movement throughout the food chain.

APPENDIX A

Table A1:

Water ANCOVA Results			
Factor	**df**	**F-value**	**p-value**
Algae	1	3.251	0.0821
Treatment	1	8.4	0.0072*
Time (h)	1	20.262	0.0001*
Algae:Treatment	1	2.337	0.1375
Algae:Time (h)	1	3.195	0.0847
Treament:Time (h)	1	5.802	0.0228*
Algae:Treatment:Time (h)	1	2.22	0.1474
Shapiro-Wilk (p-value)			
0.523			

*Statistically Significant

Table A2:

Periphyton Digestion Results [a]			
Factor	**df**	**F-value**	**p-value**
Algae	1	0.7123	0.4026
Treatment	2	172.5154	<2.0e-16
Algae:Treatment	2	0.2244	0.7998
Shapiro-Wilk (p-value)			
0.2543			

[a] Log+1 Transformation

Table A3:

***L. Stagnalis* Exposure Results** [ab]			
Factor	**df**	**F-value**	**p-value**
Algae	1	1.4072	0.2476
Treatment	2	15.5493	5.348e-05*
Algae:Treatment	2	0.1576	0.8551
Shapiro-Wilk (p-value)			
0.1194			

Table A4: *H.azteca* and *L. Stagnalis* mortality counts from 24 h exposure period.

Flume	Algae	Species	Alive	Dead
1	Huron	H.A.	9	1
2	Huron	H.A.	9	1
3	Huron	H.A.	10	0
4	Huron	H.A.	10	0
5	Huron	H.A.	9	1
6	Huron	L.S.	9	1
7	Huron	L.S.	10	0
8	Huron	L.S.	10	0
9	Huron	L.S.	9	1
10	Huron	L.S.	8	2
11	Huron	H.A.	10	0
12	Huron	H.A.	7	3
13	Huron	H.A.	10	0
14	Huron	H.A.	10	0
15	Huron	H.A.	10	0
16	Huron	L.S.	10	0
17	Huron	L.S.	10	0
18	Huron	L.S.	10	0
19	Huron	L.S.	10	0
20	Huron	L.S.	10	0
21	Huron	H.A.	10	0
22	Huron	H.A.	10	0
23	Huron	H.A.	10	0
24	Huron	H.A.	10	0
25	Huron	H.A.	9	1

26	Huron	L.S.	10	0
27	Huron	L.S.	10	0
28	Huron	L.S.	10	0
29	Huron	L.S.	10	0
30	Huron	L.S.	10	0
1	Osc	H.A.	10	0
2	Osc	H.A.	10	0
3	Osc	H.A.	10	0
4	Osc	H.A.	10	0
5	Osc	H.A.	10	0
6	Osc	L.S.	8	2
7	Osc	L.S.	10	0
8	Osc	L.S.	8	2
9	Osc	L.S.	10	0
10	Osc	L.S.	10	0
11	Osc	H.A.	10	0
12	Osc	H.A.	10	0
13	Osc	H.A.	10	0
14	Osc	H.A.	8	2
15	Osc	H.A.	9	2
16	Osc	L.S.	10	0
17	Osc	L.S.	10	0
18	Osc	L.S.	9	1
19	Osc	L.S.	10	0
20	Osc	L.S.	10	0
21	Osc	H.A.	10	0
22	Osc	H.A.	10	0
23	Osc	H.A.	10	0
24	Osc	H.A.	8	2
25	Osc	H.A.	10	0
26	Osc	L.S.	10	0
27	Osc	L.S.	9	1
28	Osc	L.S.	9	1
29	Osc	L.S.	8	2

Table A5:

Biomass Results [a]			
Interaction	**df**	**F-value**	**p-value**
Algae	1	1.211	0.2761
Treatment	2	0.1143	0.8923
Algae:Treatment	2	0.1726	0.842
Shapiro-Wilk (p-value)			
0.3543			

[a] Log transformation

Table A6: Nanomaterial Budget Breakdown by Flume

Flume	Treatment	Algae	% in Water	% in Periphyton	% in Tubing
11	Low	Huron	2.60	8.52	88.88
11	Low	Osc	1.72	0.00	98.28
12	Low	Huron	2.77	18.66	78.57
12	Low	Osc	0.00	0.00	100.00
13	Low	Huron	2.11	16.74	81.15
13	Low	Osc	1.36	7.41	91.23
14	Low	Huron	3.92	17.44	78.64
14	Low	Osc	1.28	16.48	82.24
15	Low	Huron	2.41	43.81	53.78
15	Low	Osc	0.00	25.29	74.71
16	Low	Huron	0.00	26.65	73.35
16	Low	Osc	0.00	18.55	81.45
17	Low	Huron	1.89	12.42	85.69
17	Low	Osc	0.00	26.76	73.24
18	Low	Huron	0.00	18.10	81.90
18	Low	Osc	0.00	5.06	94.94
19	Low	Huron	0.00	31.37	68.63
19	Low	Osc	0.00	12.46	87.54
20	Low	Huron	1.16	35.00	63.84
20	Low	Osc	0.00	31.75	68.25
21	High	Huron	0.52	24.93	74.55
21	High	Osc	0.73	0.67	98.61
22	High	Huron	1.46	22.21	76.33
22	High	Osc	0.00	8.04	91.96
23	High	Huron	0.47	15.55	83.97

23	High	Osc	0.58	10.23	89.20
24	High	Huron	1.69	28.30	70.01
24	High	Osc	0.56	2.09	97.36
25	High	Huron	0.83	21.94	77.23
25	High	Osc	0.73	9.29	89.98
26	High	Huron	0.78	43.72	55.50
26	High	Osc	0.44	36.61	62.95
27	High	Huron	1.30	31.99	66.71
27	High	Osc	1.39	8.46	90.14
28	High	Huron	1.46	30.04	68.50
28	High	Osc	1.00	12.67	86.33
29	High	Huron	0.77	24.93	74.30
29	High	Osc	0.80	24.97	74.24
30	High	Huron	3.46	17.39	79.15

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