Nanosilver Inhibits Freshwater Gastropod (Physa acuta) Ability to Assess Predation Risk

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ABSTRACT.—Freshwater gastropods represent common and important members of aquatic ecosystems, but emerging chemical contaminants may challenge gastropod fitness. As the use of nanosilver (n-Ag) increases, the potentially adverse effects of n-Ag on aquatic ecosystems remain largely unknown. In aquatic ecosystems, we hypothesized that n-Ag at trace concentrations may affect the ability of organisms to sense predation by interfering with chemoreception. We performed a microcosm experiment to assess the freshwater gastropod, Physa acuta, to detect and respond to natural predator cue derived from pumpkinseed sunfish (Lepomis gibbosus), at environmentally relevant nominal n-Ag concentrations over 24 h. For the first 6 h exposure, gastropod predator avoidance behavior measured 30–47% less in treatments with environmentally relevant n-Ag concentrations compared to treatments without n-Ag. Regardless of predator cue presence or absence, the proportion of gastropods occupying near surface habitat in 30 μg/liter n-Ag appeared 20–26% greater relative to no n-Ag treatments for the first six exposure hours, indicating contaminant avoidance behavior. These results suggest that nonlethal concentrations of emerging contaminants affect animal behaviors. These behaviors, in turn, may have consequences for species interactions and ecosystems. Thus, future research efforts need to address the ecology in ecotoxicology by understanding how environmentally relevant concentrations of emerging contaminants may adversely affect the chemoreception, growth, and fecundity of organisms essential to the structure and function of freshwater ecosystems.

INTRODUCTION
Freshwater gastropods serve as important food web links between sediment microbial communities and higher tropic levels, playing vital roles in nutrient cycling and energy flow (Bernot and Turner, 2001; Hall et al., 2003; Thorp and Covich, 2009; Turner and Montgomery, 2009). Among freshwater gastropods, pulmonate snails within the family Physidae commonly occur in both lentic and lotic ecosystems (Dillon, 2000). These gastropods graze on periphyton, which is composed of microbes, bacteria, algae, diatoms, and detritus, and serve as food resources for fish, crayfish, turtles, and many invertebrate predators (Kessler and Munns, 1989; Covich, 2010). Many pulmonate gastropods detect predation risk by sensing chemical cues associated with predators and respond by altering habitat use, feeding behavior, growth, and reproduction (Alexander and Covich, 1991; Turner, 1996; Bernot and Turner, 2001). For example, in the presence of molluscivorous fish, gastropods seek refuge under covered benthic habitat, while in the presence of a benthic predator, such as a crayfish, gastropods seek refuge at the surface of the water (Turner et al., 2000). Moreover, anthropogenic impacts, such as the discharge of heavy metals and emerging contaminants may lead to changes in gastropod traits, populations, and communities, leading to cascading indirect effects on the broader ecosystem (Bernot and Turner, 2001). Freshwater gastropods are therefore model ecotoxicology test organisms for studies aimed at understanding the effects of emerging contaminants, such as nanomaterials, on ecosystem level processes.

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The emerging field of nanotechnology presents technological, chemical, and medical benefits to society (Moore, 2006). Because manufactured nanoparticles exhibit physiochemical properties that differ from the bulk materials used to derive them, nanoparticles have versatile applications in many commercial products (Fabrega et al., 2011). The market for consumer products using the distinct properties of nanoparticles continues to grow, with a predicted value of $3.1 trillion by 2015 (Lux, 2008). Because nanomaterials are novel, the potential adverse environmental effects of large-scale nanoparticle production and use remain largely unknown. Furthermore, because of their small size, nanoparticles probably possess a greater likelihood than larger molecules of successfully infiltrating biological systems, resulting in possible effects on freshwater ecosystems (Moore, 2006).

Nanosilver (n-Ag) represents the most widely used nanoparticle globally and currently receives a great deal of scrutiny from academia and government regulators for its potential to affect ecosystems (Luoma, 2008; Nowack et al., 2011). Nanosilver acts as an antimicrobial agent which is applied to surfaces of consumer products where growth of microbes may occur, such as cosmetics, implants, plastics, soaps, T-shirts, textiles, and swimming pools (Klaine, 2008; Fabrega et al., 2011; Lee et al., 2011; Nowack et al., 2011). Aquatic ecosystems will likely become sinks for additional silver loadings as a result of widespread n-Ag use and discharge, primarily through wastewater effluent and rainwater runoff of solid waste (Oberdörster, 2005; Klaine et al., 2008). Expected nanosilver concentrations range from 0.02 μg/liter to 0.04 μg/liter in sewage effluent and natural freshwaters (Mueller and Nowack, 2008; Gottschalk et al., 2009).

Although n-Ag will unlikely exist at lethal concentrations (2.0 μg/liter; Bernot and Brandenburg, 2013) in freshwater ecosystems, current environmental n-Ag concentrations (0.03 μg/liter n-Ag; Mueller and Nowack, 2008) may chronically affect freshwater organisms. For instance, most aquatic organisms use chemoreception to sense their surroundings, locate food, search out mates, and detect predators (Dodson et al., 1994; Bernot and Turner, 2001; Derby and Sorensen, 2008). Changes in the chemical environment of aquatic ecosystems, such as elevated pesticide and heavy metals concentrations, may hinder chemoreception in many freshwater organisms including fish, gastropods, crustaceans, and amphibians (Blaxter and Hallers-Tjabbes, 1992; Lefcort et al., 1999; Lüring and Scheffer, 2007).

Chemoreception disruptions may result in profound effects on species interactions, leading to the potential reorganization of aquatic ecological communities (Weissburg et al., 2002). Constraints on chemoreception as a result of anthropogenic pollutants such as nanomaterials have only been minimally studied. The focus of our study included determining if environmentally relevant concentrations of n-Ag impaired the ability of the freshwater gastropod, Physa acuta (Draparnaud, 1805), to detect and respond to the presence of a natural predator cue. To address this, we quantified changes in gastropod behavior in the presence of a fish predator cue and n-Ag over 24 h in a laboratory behavioral assay.

**METHODS**

**Organisms.**—We obtained Physa acuta (hereafter Physa) from batch cultures (cultured for 8–11 mo, free of parasites) of offspring from parents originally collected from the White River (Muncie, IN, Longitude 40.1805°N, Latitude −85.432°E). Physa populations lived in synthetic spring water (US EPA, 2002) in aquaria (gastropod density <1.5 individuals/liter) at 22°C ±3°C with a 16:8 h light:dark photoperiod and received boiled spinach ad libitum, which decomposed and provided bacteria for gastropods to graze on, similar to periphyton (Warner, 1976). We changed the culture water twice weekly and provided continual aeration.
Pumpkinseed sunfish (*Lepomis gibbosus*; Linnaeus, 1758) serve as a natural predator to *Physa* (Mittlebach, 1984). We purchased pumpkinseed sunfish from Smith Creek Fish Farm (Bliss, NY, U.S.A.) and housed them in 37.85 liter synthetic spring water filled aquaria at 22 ± 2°C with a 16:8 h light:dark photoperiod. Sunfish received cultured *Daphnia magna* (Straus, 1820), pulmonate gastropods, and flake food (Hikari, Hayward, CA, U.S.A.) daily. We changed the sunfish culture water weekly and provided continual aeration.

**Nanosilver characterization.**—All experimental n-Ag treatment concentrations consisted of nominal n-Ag treatments derived from n-Ag stock solution purchased from Sciventions Inc. (Toronto, Canada; Product Number: 13021L). The manufacturer described nanosilver particles as carboxy-functionalized silver nanoparticles stabilized by sodium polyacrylate, between 1.0 and 10.0 nm in size, and of 97% purity. We prepared n-Ag stock solutions by sonicating n-Ag solution into synthetic spring water in an ice bath for 45 min. Before any experimental exposure, we confirmed silver nanoparticle size by subsampling a drop of n-Ag stock solution, placing it on a copper grid and viewing it using a Jeol JEM-1400 Transmission Electron Microscope (Joel Ltd., Tokyo, Japan). Sizes of all silver nanoparticles viewable on transmission electron microscopy (TEM) images were measured using Gaten Microscopy Suite DigitalMicrograph (Gatan Inc., Pleasanton, CA, U.S.A.) software. Over 40% of observable n-Ag particles within the purchased n-Ag stock solution measured between 5.0–10.0 nm in their longest dimension (mean n-Ag particle length = 10.2 nm, Range = 2.7–40.9 nm, n = 281; Fig. 1).

**Behavioral assay.**—In a laboratory behavioral assay, we determined the effects of n-Ag on the the response of *Physa* to *L. gibbosus* chemical cues. We exposed *Physa* to one of three nominal n-Ag concentrations: a negative control (0 μg/liter), a concentration modeled to currently exist in natural freshwaters (0.03 μg/liter; Mueller and Nowack, 2008), and a concentration associated with historic silver loadings that would also not cause *Physa* lethality over 24 h based on our preliminary range finding studies (30 μg/liter; Luoma, 2008). We cross-factored these three n-Ag concentrations with predator cue presence (in the form of water from aquaria housing *L. gibbosus*) or absence, resulting in six treatment combinations, replicated eight times, yielding 48 randomly assigned experimental units. Experimental units consisted of a 1.9 liter plastic container (13.7 × 17.8 × 9.5 cm) containing 1.24 liters of synthetic spring water consisting of a specific n-Ag concentration, 10 *Physa* (average shell length of every third *Physa* = 6.8 mm, range = 3.5–10.5 mm, n = 48).

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**Fig. 1.**—Number of measured silver nanoparticles from a 1.0 μL sample of nanosilver stock solution (1.8 mg/ml), in a range of size classes (n = 281)
144), and an elevated unglazed ceramic tile (9.7 × 9.7 × 1.0 cm; see Turner, 1996), which served as refuge from predation.

Pulmonate gastropods serve as natural prey to *L. gibbosus* and *Physa* respond to sunfish chemical cue presence by moving to a predator safe microhabitat, typically seeking refuge under cover (Bernot and Turner, 2001). Following the addition of 10 *Physa* to each microcosm with a nominal n-Ag treatment concentration and a 1 h acclimation period, we added 10 ml of predator cue water or 10 ml of synthetic spring water as the negative predator cue control to each microcosm. Predator cue was obtained by feeding five crushed *Physa* to each of 24 *L. gibbosus* housed in individual aquaria (Turner, 1996; Turner and Montgomery, 2003). Each aquarium contained a single fish in 35.0 liters of synthetic spring water. Predator cue from each pumpkinseed sunfish was randomly paired with an experimental microcosm. We chose to derive predator cue from different fish and exclusively pair the predator cue with an individual microcosm to maintain statistical independence among replicates.

We recorded *Physa* habitat use 1, 3, 6, 18, and 24 h after the addition of predator cue. We defined *Physa* refuge use as the proportion of snails under each ceramic tile where it counted if at least 50% of its body appeared under the tile. We counted the proportion of *Physa* out of the water and near the surface of the water (≥50% of the snail body within 1.0 cm of the surface) to also quantify near surface habitat use, a behavioral response indicative of an attempt to escape benthic predators or contaminants (Bernot *et al.*, 2005). We also counted the proportion of *Physa* neither under the tile nor near the surface of the water as being in open habitat. This behavioral assay took place at 22 C ± 2 C over an 8:8:8 h light:dark:light photoperiod, with all habitat assessments performed during the lighted portions of the photoperiod.

**Statistical analysis.**—Proportional *Physa* habitat use data required an arcsine transformation to meet the assumptions of analysis of variance (ANOVA). We calculated a predator avoidance index as \( \frac{(P-NP)}{(1-NP)} \) where P = refuge use in predator cue microcosms and NP = mean refuge use in microcosms without predator cue, which resulted in index values between −1.0 and 1.0, with 0 matching habitat use in corresponding predator cue absence treatments (i.e., no antipredator behavior). We created two predator avoidance indices to analyze two aspects of predator avoidance separately, *Physa* seeking refuge under covered habitat and *Physa* seeking refuge in near surface habitat. *Physa* could occupy either covered habitat, near surface habitat, or open habitat.

The covered habitat predator avoidance index and the near surface habitat predator avoidance index, therefore, served as independent measures. We analyzed the effects of n-Ag on both predator avoidance indices, over time, using repeated measures analysis of variance \((\alpha = 0.05)\). We also analyzed the lone effects of n-Ag (considering all individual microcosms, regardless of predator cue presence or absence) on near surface habitat use by gastropods, over time, using repeated measures ANOVA \((\alpha = 0.05)\) to determine whether higher n-Ag concentrations induced a contaminant escape response. We used SYSTAT 11 software to perform all statistical analyses (Systat Software Inc., Chicago, IL, U.S.A.).

**RESULTS**

In the presence of predator cue, covered habitat use by *Physa* occurred 30–47% less in treatments containing 0.03 μg/liter n-Ag relative to treatments without n-Ag (n-Ag effect: \( F_{2,21} = 17.12, P < 0.01, \) Fig. 2). *Physa* covered habitat use in treatments without n-Ag peaked 1 to 6 h after predator cue addition. However, differences diminished by 18 h and 24 h after predator cue addition (n-Ag × Time effect: \( F_{4,8} = 2.29, P = 0.03, \) Fig. 2). Predator
avoidance by *Physa* occupying covered habitat did not differ between microcosms with 0.03 µg/liter or 30 µg/liter n-Ag over 24 h.

Effects of n-Ag on *Physa* near surface habitat use depended on the amount of time after exposure to predator cue (n-Ag × Time effect: F_{4,8} = 2.49, P = 0.02, Fig. 3). The greatest near surface habitat use due to predator cue occurred 6 h after predator cue additions in treatments with 30 µg/liter n-Ag, but did not differ from other treatments at any other time period. In the presence of fish predator cue, n-Ag did not directly affect the proportion of gastropods occupying near surface habitat (n-Ag effect: F_{2,21} = 1.19, P = 0.32, Fig. 3).

More *Physa* used near surface habitat immediately after and up to 6 h after the exposure to 30 µg/liter of n-Ag (n-Ag × Time effect: F_{4,8} = 4.28, P < 0.01, Fig. 4). The proportion of gastropods occupying near surface habitat measured 20%–26% greater during the first 6 exposure hours in 30 µg/liter n-Ag compared to 0 µg/liter n-Ag treatments, while the proportion of *Physa* occupying near surface habitat in 0.03 µg/liter n-Ag did not differ from 0 µg/liter n-Ag treatments at any time.

**DISCUSSION**

The ability of *Physa* to sense and respond to predator cue appeared compromised by both an environmentally relevant concentration of n-Ag and a concentration associated with historic silver loadings. *Physa* in treatments with a low n-Ag concentration did not alter habitat use in the presence of a predator cue. Higher concentrations of n-Ag caused *Physa* to occupy near surface habitat. Greater near surface habitat use by *Physa*, in a high n-Ag concentration, may indicate their attempts to crawl out of the water. Because we observed this behavior for the first six exposure hours in treatments with high n-Ag, and not solely from induction by the presence of a predator cue, gastropods may have sensed the presence of the contaminant and actively attempted to evade the high n-Ag concentration by fleeing their habitat, thus exhibiting contaminant avoidance behavior.

Nanosilver is toxic to a number of freshwater organisms, including macroinvertebrates, algae, crustaceans, and fish (Griffitt *et al*., 2008), with apparent adverse effects shortly after exposure. Nanosilver concentrations between 0.01–0.02 µg/liter (Yeo and Yoon, 2009) affect early stage life development of zebrafish embryos (*Danio rerio*, Hamilton, 1822) while
n-Ag concentrations between 40–4,000 mg/liter alter zebrafish gene pathways (Yeo and Pak, 2008). Such concentrations also impede gill function of freshwater fishes, including zebrafish (Griffitt et al., 2008), eurasian perch (*Perca fluviatilis*; Linnaeus, 1758; Bilberg et al., 2010) and brown trout (*Salmo trutta*; Linnaeus, 1758; Scown et al., 2010), causing acute toxicity to zebrafish at n-Ag concentrations as low as 40 µg/liter (Griffitt et al., 2008). However, the potential long-term and sublethal effects of environmentally relevant n-Ag concentrations on organismal growth, reproduction, and predator risk assessment remain understudied. Possible unknown n-Ag effects on freshwater organisms warrant study because they may subsequently lead to adverse effects on broader ecological interactions.

Studies aimed at understanding the effects of contaminants, including nutrient loading (Turner and Chislock, 2010), pesticides (Moore and Waring, 1998), and hydrocarbons (Pearson et al., 1981) on chemical ecology in aquatic ecosystems continue to grow. Similar,
studies on novel nanomaterials remain limited, even though nanomaterials may present a crucial threat to freshwater ecosystems in future years (Auffan et al., 2009). Silver nanomaterials may reduce the ability of gastropods to assess predation risk by disrupting or altering receptor sites of ion channels located on neuronal membranes, similar to the effects of heavy metals such as lead, zinc, and copper (Rozsa and Salonki, 1990; Pyle and Mirza, 2007). Alternatively, n-Ag may bind to and alter kairomones released by sunfish predators, thereby blocking predator cue effectiveness, or n-Ag may modify the conformation of gastropod olfactory receptors (Wojtasek and Leal, 1999). However, these mechanisms remain unstudied.

Higher concentrations of n-Ag induced Physa to exit the water, while predator cue presence did not induce gastropods to use near surface habitat. The greater proportion of gastropods occupying near surface habitat during the first 6 h exposure in 30 µg/liter n-Ag can be directly attributed to n-Ag. Therefore, gastropods may have occupied near surface habitat to evade n-Ag, demonstrating contaminant avoidance behavior. Previous studies have documented this behavior by Physa. For example, Physa crawled faster in the presence of high concentrations of ionic liquids in an effort to escape the contaminant (Bernot et al., 2005).

Contaminant avoidance behavior induced by n-Ag, in turn, increased the amount of time gastropods remained exposed to predation risk. Additionally, movement induced by flushes of contaminants, such as n-Ag, in freshwater ecosystems may reduce gastropod fitness by using energy at the expense of growth or reproduction throughout their life history. These results showed that regardless of predator cue presence or absence, gastropod near surface habitat use did not differ between n-Ag treatments after 18 h. Diminishing contaminant avoidance behavior after 18 h suggests that n-Ag may have bound to ligands associated with the ceramic tile, abating n-Ag effects, or suggests that gastropods became habituated to the n-Ag treatments. Because treatment conditions included basic solutions (pH range at 6 h: 8.4–9.1, n = 12) at room temperature and lacked in dissolved organic ligands, n-Ag used in this study likely remained in the nanoparticle form, as opposed to dissociating into silver cations (Luoma, 2008; Navarro et al., 2008). Future studies investigating how abiotic characteristics of freshwater ecosystems, such as pH or organic matter variation, may enhance or abate n-Ag toxicity would be most relevant in determining n-Ag fate in freshwater systems. However, analytical methods for measuring n-Ag in situ remain difficult or unavailable.

Anthropogenic activities have resulted in discharges of manmade contaminants and subsequent alterations to the chemical characteristics of ecosystems that freshwater organisms were adapted to previously. Beyond sensing predation risk, aquatic organisms use chemoreception to detect and respond to a host of environmental characteristics that enhance their fitness (Dodson et al., 1994; Derby and Sorensen, 2008). Additionally, chemoreception aids in maintaining ecologically important indirect species interactions across multiple trophic levels. For example, freshwater organisms, such as benthic gastropods, upon detecting the presence of predation through chemical cues will commonly deploy antipredator behavior at the expense of foraging (Lima, 1998), reducing gastropod growth and reproduction (Turner, 1996), while indirectly increasing periphyton biomass. Chemosensory perception drives such higher order interactions that are pivotal in maintaining freshwater ecosystems. However, n-Ag induced alterations to species interactions may drastically alter ecosystem structure and function (Bernot and Turner, 2001).

Both the distribution and detection of many emerging contaminants in lentic and lotic freshwater ecosystems are increasing (Veatch and Bernot, 2011; Bernot et al., 2013; Ferguson et al., 2013). Future studies aimed at understanding how trace concentrations of emerging
contaminants influence organismal and ecosystem health are vital. Our results provide potential insight into how emerging nanomaterials may affect organismal fitness in a sublethal manner, in turn influencing species interactions and ecosystem structure and function. The effects of both chemoreception inhibition and induction of contaminant avoidance behavior could potentially be ecologically extensive, leading to disruptions in predation and consumption that may cascade throughout aquatic communities resulting in adverse effects at the ecosystem level (Brönmark and Hansson, 2012).

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LITERATURE CITED

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