NANOSILVER ECOTOXICITY: CHRONIC EFFECTS ON THE FRESHWATER GASTROPOD, *Physa acuta*, AND INFLUENCE OF ABIOTIC FACTORS

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF SCIENCE

BY

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MUNCIE, INDIANA

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# TABLE OF CONTENTS

TABLE OF CONTENTS .................................................................................. II  
ABSTRACT .................................................................................................................. 1
ACKNOWLEDGEMENTS ................................................................................................. 6

## CHAPTER 1

ABSTRACT .................................................................................................................. 7
INTRODUCTION ............................................................................................................. 8
METHODS ...................................................................................................................... 11
RESULTS ....................................................................................................................... 13
DISCUSSION .................................................................................................................. 15
REFERENCES ............................................................................................................... 18
FIGURE LEGENDS ........................................................................................................ 22
FIGURES ...................................................................................................................... 23

## CHAPTER 2

ABSTRACT .................................................................................................................. 27
INTRODUCTION ............................................................................................................. 28
METHODS ...................................................................................................................... 30
RESULTS ....................................................................................................................... 33
DISCUSSION .................................................................................................................. 35
REFERENCES ............................................................................................................... 39
FIGURE LEGENDS ........................................................................................................ 43
FIGURES ...................................................................................................................... 45

## CHAPTER 3

ABSTRACT .................................................................................................................. 54
INTRODUCTION ............................................................................................................. 55
METHODS ...................................................................................................................... 57
ABSTRACT

THESIS: Nanosilver Ecotoxicity: Chronic Effects on the Freshwater Snail, *Physa acuta*, and Influences of Abiotic Factors

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Freshwater ecosystems will likely become sinks for future silver loadings as a result of increased nanosilver (n-Ag) use in industrial and commercial applications. A series of bioassays was performed to assess how n-Ag toxicity may be influenced by abiotic factors associated with natural freshwater ecosystems. Additionally, these bioassays provide insight into how environmentally relevant concentrations of n-Ag may sublethally affect the freshwater benthic gastropod, *Physa acuta*, that plays pivotal roles in maintaining the structure and function of freshwater ecosystems. In sediment with no benthic organic carbon (BOC), gastropod vital rates decreased in treatments containing any n-Ag, gastropods in sediment with relatively low BOC appeared to trade off growth for reproduction at high n-Ag treatments, while gastropod vital rates in high BOC sediment remained unaffected at all nanosilver treatments. Sediment type may abate nanosilver toxicity as a result of organic carbon content. Effects of n-Ag on gastropod vital rates were not dependant on pH, suggesting aqueous pH does not directly influence n-Ag toxicity. Nanosilver (0.2 µg/L) stressed gastropods, altering their growth and reproduction tradeoff dynamics. Nanosilver concentrations modeled to exist in natural freshwaters, disrupted gastropod ability to detect and respond to a natural predator, while greater n-Ag concentrations stimulated gastropods to exhibit contaminant avoidance behavior and thereby attempted to flee their habitat. This study provides direction in understanding how adverse n-Ag effects may be influenced by abiotic parameters, while assessing sublethal effects of n-Ag on freshwater
gastropods that are likely to occur in natural freshwater ecosystems, given current estimates of environmental n-Ag concentrations.

INTRODUCTION

Nanotechnology is a rapidly expanding field that produces engineered nanomaterials (defined as having two or more dimensions between 1 and 100 nm) used in industrial and commercial applications including medical technologies, microelectronics, and antimicrobials (ATSM 2006). This increased nanomaterial production and use makes discharge of nanomaterials into aquatic ecosystems inevitable (Moore 2006, Farré et al. 2009). The high surface to volume ratio of nanomaterials results in high reactivity and a host of unique physiochemical properties, creating particles previously unseen to aquatic or terrestrial ecosystems (Nowack et al. 2011). Therefore, nanomaterial toxicity in freshwater ecosystems is unpredictable, even as nanomaterials present one the most critical challenges to freshwater ecosystems in future years (Auffan et al. 2009).

Nanosilver (n-Ag) is the fastest growing nanomaterial in consumer product applications today. The beneficial attributes of n-Ag to society are well understood, however n-Ag is likely to infiltrate freshwater ecosystems where its potential adverse effects, as well as how abiotic factors may influence nanosilver toxicity remain unknown. (Gao et al. 2002, Oberdörster et al. 2005, Moore 2006, Klaine et al. 2008). Acute n-Ag toxicity tests distinguish specific tolerance levels of freshwater organisms to n-Ag over short-term exposure periods (Griffitt et al. 2008, Bilberg et al. 2010, Scown et al. 2010). However, chronic toxicity tests designed to assess the potential long-term effects of environmentally relevant n-Ag concentrations on freshwater ecosystems remain limited. Furthermore, future chronic n-Ag toxicity tests should include abiotic factors of freshwater ecosystems to provide potential insights about n-Ag behavior and toxicity in real world freshwater ecosystems (Klaine et al. 2008).

The purpose of this study was twofold. First, we determined how chronic n-Ag toxicity on the freshwater gastropod, Physa acuta, was influenced by benthic organic carbon (BOC) and
pH using two 28 day bioassays. We hypothesized that increasing both BOC and pH will reduce chronic effects of n-Ag on gastropod growth and reproduction by providing organic or ionic ligands respectively, that will complex with and reduce bioavailable n-Ag. Second we determined how n-Ag may interfere with chemical ecology in aquatic systems by assessing the ability of freshwater gastropods to detect and respond to natural predator chemical cues generated from pumpkinseed sunfish (*Lepomis gibbosus*), in the presence of environmentally relevant n-Ag concentrations. We hypothesized that n-Ag would eliminate the ability of gastropods to sense predator presence by inhibiting gastropod chemoreception, similar to the effects of bulk heavy metals including lead, zinc, and copper on freshwater organisms (Lefcort et al. 1998, Beyers and Farmer 2001, McPherson et al. 2004).

This study documented how chronic effects of n-Ag on gastropod growth and reproduction may be influenced by environmental parameters and describes how gastropod chemoreception may be inhibited by concentrations of n-Ag modeled to exist in natural freshwaters. Because freshwater gastropods are ubiquitous, facilitate nutrient cycling, and serve as an important foodweb link between primary producers and higher tropic levels (Dillon 2000, Turner and Montgomery 2009), the potential chronic effects of n-Ag on gastropod fitness described in our study may alter the structure and function of real world freshwater ecosystems (Bernot and Turner 2001).

This thesis is a collection of manuscripts intended for publication. Each chapter is specifically formatted for publication in a peer reviewed scientific journal. Chapter three entitled, “Nanosilver: an emerging contaminant that inhibits freshwater gastropod (*Physa acuta*) ability to assess predation risk,” was submitted to *American Midland Naturalist* for publication on April 9, 2013.

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CHAPTER 1: INTERACTIVE EFFECTS OF NANOSILVER AND BENTHIC ORGANIC CARBON ON GASTROPOD GROWTH AND LIFE HISTORY

Abstract: As use of nanosilver (n-Ag) increases, the potentially adverse environmental effects of n-Ag remain largely unknown. Exposing aquatic organisms to a range of n-Ag concentrations across various environmental parameters, such as organic carbon presence, may yield results most applicable to real world situations. A 28 day exposure bioassay examined the interactive effects of five environmentally relevant n-Ag concentrations, cross-factored with three sediments consisting of different benthic organic carbon (BOC) contents, on the growth and reproduction of freshwater gastropods (*Physa acuta*). When BOC was low, gastropods appeared to trade off growth for reproduction at high nanosilver treatments. Gastropod vital rates when BOC was high remained unaffected at all nanosilver treatment levels, suggesting sediment type may abate nanosilver toxicity as a result of organic carbon content. Furthermore, environmentally relevant nanosilver concentrations reduced gastropod growth and reproduction, in the absence of BOC. This study also provided baseline information needed to assess the possible sublethal effects of n-Ag on benthic grazers in real world freshwater ecosystems.

*Keywords*: Nanosilver, *Physa acuta*, Organic Carbon, Long-term, Life History, Growth, Ecotoxicology
INTRODUCTION

Engineered nanomaterials display unique physiochemical properties which are used in commercial product applications that present technological, chemical, and medical benefits to society (Moore 2006, Fabrega et al. 2011). The nanomaterial market is progressing and expected to be valued at $3.1 trillion by 2015 (Lux 2008). Increased nanomaterial production and use will lead to the discharge of nanomaterials into biological systems, where the potential adverse effects of nanomaterials on freshwater ecosystems remain unknown (Moore 2006).

Nanosilver (n-Ag) is the most widely used and scrutinized nanomaterial and is the premier antimicrobial agent used in plastics, water purification systems, soaps, clothing and, cosmetics (Klaine 2008, Luoma 2008, Fabrega et al. 2011, Lee et al. 2012, Nowack et al. 2011). As a result of large scale n-Ag production and use, freshwater ecosystems are likely susceptible to future silver loadings (Oberdörster 2005), with n-Ag concentrations modeled to approach 0.03 µg/L (Mueller and Nowack 2008).

Aquatic organisms are several orders of magnitude more sensitive to n-Ag than mammals and humans (Nowack et al. 2011). Nanosilver at trace concentrations affects early stage life development of freshwater fishes, penetrating into the skin and circulatory system of zebrafish (Danio rerio) embryos (Yeo and Yoon 2009), while greater n-Ag concentrations alter gene pathways (Yeo and Pak 2008), and can be acutely toxic to zebrafish adults at 40 µg/L (Griffitt et al. 2008). Nanosilver is also acutely toxic to brown trout (Salmo trutta; Scown et al. 2010) and Eurasian perch (Perca fluviatilus; Bilberg et al. 2010) by inducing respiratory stress. Future studies examining acute and chronic impacts of n-Ag on aquatic organisms need to consider chemical characteristics of freshwater ecosystems to gain knowledge that is most applicable to real world situations.

Heavy metal toxicity to freshwater organisms is affected by abiotic parameters including pH, hardness, and dissolved organic carbon (DOC; Miller and Mackay 1980, Nelson et al. 1986, Sciera et al. 2004). Dissolved organic carbon can affect heavy metal speciation within the
environment, thereby determining bioavailability of heavy metals to freshwater organisms. Copper in freshwaters can bind with DOC to form complexes that are not available for biological uptake, resulting in reduced copper toxicity to fathead minnows (*Pimephales promelas*; Spry and Wiener 1991, Welsh et al. 1993). In freshwater systems, silver cations dissociated from bulk silver bind primarily to sulfide components of organic carbon based molecules. To a lesser extent in freshwater ecosystems, silver ions can also bind to additional chloride, bromide, and iodide ligands associated with organic carbon based molecules, reducing bioavailable silver in freshwater ecosystems (Lindsay and Sadiq 1978). Whether n-Ag behavior and fate is similar to the behavior and fate of bulk silver and other heavy metals in freshwater ecosystems is unknown (Luoma 2008).

Because of potential interactions between chemical compounds and n-Ag in the environment, actual bioavailable n-Ag and corresponding n-Ag toxicity may be different than concentrations that are theoretically modeled, although this relationship remains untested. Hypothetically, silver nanoparticles may shield themselves and prevent silver ions from binding to organic carbon-based molecules, allowing for direct delivery of ionic silver to the cytosol and/or membrane of cells (Louma 2008). If this n-Ag shielding from biogeochemical interactions occurs, then n-Ag bioavailability and toxicity in freshwater ecosystems would likely correspond to modeled aqueous n-Ag concentrations. Thus, n-Ag toxicity in freshwater ecosystems would be greater than comparable amounts of anthropogenic bulk silver. Unlike bulk silver, environmental interactions between DOC or benthic organic carbon (BOC) and n-Ag remain unknown. Future studies examining the effects of water and sediment chemistry on n-Ag toxicity are imperative in the development of water quality criteria and future n-Ag regulation policies.

Acute effects of n-Ag on individual organisms have been the focus of many studies (Zhu et al. 2006, Griffitt et al. 2008, Zhu et al. 2009); however, sublethal effects of n-Ag on broader ecological interactions remain unknown. Many contaminants may be acutely lethal and their effects noticeably apparent in a brief time period, while, others may slowly affect growth and
reproduction of freshwater organisms (Newman and Clements 2008). Chronic effects of contaminants during long-term toxicity tests reflect sensitive and ecologically important responses of organisms likely to occur in natural ecosystems. Because chronic toxicity studies are so rare, the United States Environmental Protection Agency (USEPA) has not developed criteria for protecting species from chronic silver exposure (Luoma 2008).

Benthic grazers, such as freshwater gastropods, are model organisms for n-Ag ecotoxicology tests because n-Ag accumulates in the upper-most sediment layer of aquatic ecosystems (Klaine et al. 2008, Bradford et al. 2009) where gastropod feeding occurs. Physids are the most common and abundant family of freshwater gastropods (Covich 2010), are ubiquitous (Dillon 2000), and link microbial communities with higher trophic levels (Thorpe and Covich 2009, Turner and Montgomery 2009). In the presence of natural stressors, including predation and overpopulation, freshwater gastropods are capable of trading off growth and reproduction (Crowl and Covich 1990). Anthropogenic stressors such as contaminants may alter gastropod growth and reproduction tradeoffs.

Understanding how trace n-Ag concentrations sublethally affect freshwater grazers in the presence of additional abiotic factors is more environmentally relevant than examining acute n-Ag toxicity alone. In a long-term microcosm experiment, we studied the effects of n-Ag on the growth and reproduction of *Physa acuta* in the presence of benthic sediment consisting of three BOC contents. The objective of this study was to examine how environmentally relevant n-Ag concentrations interacted with BOC to enhance or abate chronic n-Ag exposure to freshwater gastropods using growth rates, egg, and egg pouch production as response variables. We hypothesized that increasing n-Ag concentration will reduce gastropod response variables, and increasing BOC will reduce the negative effects of n-Ag on gastropod response variables.

**METHODS**

*Organisms*
Gastropods used to conduct this long-term bioassay were obtained from batch cultures of offspring collected from the White River (Muncie, IN). Gastropods were maintained in synthetic spring water (EPA 2002) filled aquaria at 22°C ± 3°C with a 16:8 hour light:dark photoperiod. Gastropods were fed boiled spinach *ad libitum*.

**n-Ag Characterization**

Nanosilver stock solution was purchased from Sciventions Inc. (Toronto, Canada). Nanosilver particles were described to be between 1.0 and 10.0 nm in size and of 97% purity. Silver nanoparticle size was confirmed by sub sampling 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, USA) software. Nominal n-Ag stock solutions were prepared by sonicating n-Ag solution into synthetic spring water in an ice bath for 45 minutes.

**Sediment Characterization**

Two sediments assumed to consist of various BOC percentages were collected from the White River (Muncie, IN). Commercial play sand was used as an assumed zero BOC treatment. Sediments were shoveled into respective buckets and homogenized and subsamples were then microwaved to sterilize. Inorganic and total carbon content of each sediment type was determined using the Solid Sample Module of a Shimadzu TOC-L Analyzer (Shimadzu Corporation, Kyoto, Japan). The difference between total and inorganic carbon yielded BOC of each experimental sediment. Shimadzu TOC-L analysis identified organic carbon content in each sediment type to be 0.23% (sand), 1.30% (low organic sediment), and 10.55% (high organic sediment).

**Bioassay**

A long-term bioassay was performed to quantify the interactive effects of BOC and n-Ag on the growth and fecundity of *Physa acuta*. Sand, low organic, and high organic sediment
types were cross-factored with five concentrations of n-Ag. Nanosilver concentrations (0.0, 0.02, 0.20, 20.0 µg/L) reflected a range of concentrations from those modeled to occur in natural freshwaters to concentrations associated with historic loadings of bulk silver (Luoma 2008, Mueller and Nowack 2008). This experimental design resulted in 15 treatment combinations, which were replicated four times, yielding 60 randomly assigned experimental microcosms. Each microcosm (400 mL glass jar) contained 5.0 g of sterilized experimental sediment, 125.0 mL of synthetic spring water consisting of a specific n-Ag concentration, and two gastropods. Gastropod shell length (Range: 4.1-9.2 mm, Mean: 5.8 mm) was measured using digital calipers prior to placing each gastropod into a microcosm. This bioassay was conducted at 22° C +/- 3° C with a 16:8 hour light:dark photoperiod.

Throughout 28 days, egg pouches were collected every three days and the number of eggs in each pouch was then counted. Additionally, on every third day, each microcosm received fresh treatment water and 6.0 mg of commercial algae wafers (Hikarit, Hayward, CA, USA). During water changes, experimental sediments were slightly swirled but were not removed from the microcosms. Total eggs and egg pouches from each microcosm were divided by the summed number of days both gastropods in each microcosm survived to yield both eggs and egg pouches per gastropod per day. Eggs per pouch for each microcosm was calculated by dividing total eggs produced by total egg pouches produced throughout the experiment. Gastropod growth rates were determined by subtracting the initial from the final gastropod shell length and dividing by the number of days the corresponding gastropod survived, resulting in growth per gastropod per day.

**Statistical Analysis**

Individual and interactive effects of n-Ag and sediment type (predictor variables) on gastropod eggs per pouch production, daily egg production, and growth rate (response variables) were analyzed with factorial analysis of covariance (ANCOVA) with average initial shell length of gastropods in each microcosm as the covariate. Statistical analyses were
performed using IBM SPSS 17.0 statistical software (IBM Corporation, Armonk, NY, USA). Alpha was set to 0.05 for all tests.

RESULTS

**n-Ag Characterization**

Nanosilver particles within the purchased n-Ag stock solution ranged between 2.7-40.9 nm in their longest dimension, with a mean length of 10.2 nm. Over 40% of observable n-Ag particles within the purchased n-Ag stock solution ranged between 5.0-10.0 nm in their longest dimension (Figure 1).

**Effects of Sediment Type and n-Ag on Gastropod Fecundity**

Nanosilver effects on the number of eggs per pouch produced by gastropods depended on sediment type (n-Ag x sediment effect: $F_{8,44} = 2.40, P = 0.03$, Figure 2). In sand, at all n-Ag treatments, mean gastropod eggs per pouch production was less than eggs per pouch production by gastropod in sand treatments without n-Ag, decreasing as much as 60% in the presence 0.02 µg/L n-Ag. In contrast to n-Ag effects on sand, mean gastropod eggs per pouch in low and high organic sediments at 0.2, 2.0, and 20.0 µg/L n-Ag were all greater than corresponding sediment treatments lacking n-Ag. Mean eggs per pouch production by gastropod in low organic sediment at 20.0 µg/L n-Ag was 42% greater than mean eggs per pouch production by gastropod in low organic sediment treatments without n-Ag.

Neither n-Ag concentration nor sediment type affected gastropod daily egg production ($F < 1.53, P > 0.23$, Figure 3). Although not statistically different, daily egg production by gastropods in sand without n-Ag produced 6.2 eggs per day, while gastropod daily egg production in sand treatments with n-Ag, only ranged from 3.1 to 5.7 eggs per day. In low organic sediment, gastropods appeared to increase daily egg production with increasing n-Ag concentration. Gastropods in low organic sediment, exposed to 2.0 and 20.0 µg/L n-Ag, produced 5.2 and 9.2 eggs per day respectively, while gastropods in low organic sediment without n-Ag produced only 4.3 eggs per day. In high organic sediment treatments, daily egg
production by gastropods followed no clear trend with respect to increasing n-Ag concentration and never significantly differed from high organic sediment treatments that lacked n-Ag.

**Effects of Sediment Type and n-Ag on Gastropod Growth**

Neither n-Ag concentration nor sediment type affected gastropod growth ($F < 1.57$, $P > 0.22$, Figure 4). Although not statistically different, trends show gastropod growth rate in sand treatments showed a mean decrease with increasing n-Ag concentration between 0.02 and 2.0 µg/L n-Ag, while at 20.0 µg/L n-Ag, mean growth rate did not differ from sand with 0.0 µg/L n-Ag treatments. In low organic sediment, growth rate appeared to decrease with increasing n-Ag concentration. Gastropods in low organic sediment, exposed to 2.0 and 20.0 µg/L n-Ag, grew 0.03 and 0.02 mm/d per day respectively, while gastropods in low organic sediment without n-Ag grew 0.06 mm/d. In high organic sediment treatments, gastropod growth rate followed no clear trend with respect to increasing n-Ag concentration and never significantly differed from gastropod growth in high organic sediment treatments without n-Ag.

**DISCUSSION**

Consistent with our hypothesis, in the absence of BOC, *Physa* reproduction and growth both were repressed by environmentally relevant concentrations of n-Ag, while increasing BOC may diminish the sublethal effects of n-Ag on *Physa* growth and life history. Mean gastropod reproduction and growth rates all decreased in the absence of BOC (0.26% BOC content) at relatively low n-Ag concentrations. At n-Ag concentrations reminiscent of historic silver loadings (2.0 and 20.0 µg/L) in the presence of relatively low BOC (1.25% BOC content), n-Ag increased eggs per pouch and daily egg production while decreasing growth. No chronic effects of n-Ag were displayed by gastropods in the presence of relatively high BOC (10.55% BOC content).

In sand sediment treatment, a decrease in mean gastropod vital rates in all n-Ag treatments (excluding gastropod growth in 20.0 µg/L n-Ag), relative to mean vital rates in no n-Ag treatments, suggests n-Ag was not retained by sand sediments and remained available to manifest chronic toxicity towards gastropods. In sand, mean gastropod growth at 20.0 µg/L n-Ag
was not less than mean gastropod growth in sand without n-Ag treatments, however this may be attributed to gastropods in this treatment displaying the smallest average initial shell size of any n-Ag treatment, resulting in increased allometric growth rates by these smaller individuals. In low organic sediments at higher n-Ag concentrations (2.0, 20.0 µg/L), sediment likely became saturated with n-Ag, resulting in bioavailable n-Ag that stressed gastropods, inducing a growth for reproduction tradeoff. The relatively high BOC content of high organic sediment treatments may have bound to n-Ag particles and/or dissociated silver ions, limiting bioavailable n-Ag and eliminating all n-Ag induced stresses on gastropod vital rates.

Our results suggest BOC in freshwater ecosystems may abate toxicity of silver when in the nanoparticle form. Benthic organic carbon within our treatments was not characterized, however most likely was in the form of humic acids (Haworth 1971, Senesi et al. 1991). Because bulk silver has a high binding affinity for reduced sulfur groups (thiol groups, thioethers, and disulfides) of humic acids, we suspect n-Ag in this long-term experiment bound to reduced sulfur components of organic carbon based molecules in experimental sediments (Maurer et al. 2012). However, this theory remains untested. In sand and low organic sediments, n-Ag may have only become bioavailable after all reduced sulfur components of humic acids became saturated with n-Ag and/or silver ions, allowing for the observed chronic n-Ag effects on gastropods in these treatments. No n-Ag effects were apparent in high organic sediment at all n-Ag treatments, suggesting a surplus of reduced sulfur components of humic acids continually bound to n-Ag and/or silver ions eliminating chronic n-Ag toxicity throughout all 28 days. Similar to our results, Sciera et al. (2004) and Bury et al. (2009) reported acute toxicity (LC50) of bulk heavy metals including silver and copper to freshwater fishes significantly decreases with increasing DOC. However, these studies did not examine copper or silver in the nanoparticle form, which may exhibit increased toxicity as well as differences in environmental behavior and speciation. Additionally, Sciera et al. (2004) nor Bury et al. (2009) examined how
environmentally relevant metal concentrations interact with DOC to result in chronic effects that are likely to occur in freshwater ecosystems.

Our results suggest that concentrations of n-Ag far below those examined in acute toxicity tests with bulk heavy metals (Sciera et al. 2004, Bury et al. 2009), may result in chronic effects on freshwater organisms. Freshwater gastropods serve as an important food web link between sediment microbial communities and higher tropic levels, and play vital roles in nutrient cycling (Hall et al. 2003). Therefore, effects of n-Ag on gastropod growth and life history in freshwater ecosystems with negligible BOC may be profound, altering trophic interactions that may lead to adverse effects at the ecosystem level (Bernt and Turner 2001).

Chronic effects of n-Ag on gastropod communities with limited BOC are likely to occur based on our results and current estimates of environmental n-Ag concentrations (Mueller and Nowack 2008, Gottschalk et al. 2009). Because analytical methods for measuring n-Ag in situ remain unavailable, in vitro studies coupling environmentally relevant n-Ag concentrations with abiotic factors of freshwater ecosystems provide our best understanding of potential n-Ag effects in natural ecosystems. Additionally, our data provide baseline information about how n-Ag effects may vary between ecosystems, aiding in the recognition of freshwater ecosystems most susceptible to the effects of future n-Ag contamination (Luoma 2008). Future studies should examine how pH or type and source of organic carbon based molecules enhance or abate n-Ag toxicity, and examine how n-Ag may potentially affect gastropod ability to detect and respond to chemical cues associated with natural predators.

Overall, our results showed that concentrations of n-Ag modeled to occur in natural freshwaters may reduce gastropod fitness. However, these chronic effects may be reduced by the presence of BOC which may bind to and eliminate bioavailable n-Ag. Gastropods and other benthic invertebrates inhabiting freshwater ecosystems with limited BOC may be most susceptible to chronic n-Ag toxicity, in turn adversely affecting broad ecological interactions that shape and define ecosystem structure and function (Bernt and Turner 2001).
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FIGURE LEGENDS

Figure 1. Number of measured silver particles from n-Ag stock solution, in a range of size classes.

Figure 2. Eggs per pouch production by gastropods exposed to five n-Ag concentrations in the presence of three sediment types consisting of different BOC contents. Bars represent means + SE.

Figure 3. Daily egg production by gastropods exposed to five n-Ag concentrations in the presence of three sediment types consisting of different BOC contents. Bars represent mean effect size differences (calculated as Log(n-Ag Observation/No n-Ag Observation), yielding a scale in which x=0 matches observations in corresponding sediment treatments without n-Ag) between n-Ag treatments relative to corresponding sediment no n-Ag treatments + SE.

Figure 4: Growth rates of gastropods exposed to five n-Ag concentrations in the presence of three sediment types consisting of different BOC contents. Bars represent means + SE.
Figure 1
Figure 2

- Sand
- Low Organic
- High Organic

Nanosilver concentration (µg/L) vs. Physa eggs per pouch for different levels of nanosilver and organic content.
Effects on Physa eggs per day

Nanosilver concentration (µg/L)

-0.6
-0.4
-0.2
0.0
0.2
0.4

Sand
Low Organic
High Organic

Figure 3
Figure 4
CHAPTER 2: INTERACTIVE EFFECTS OF NANOSILVER AND pH ON GASTROPOD GROWTH AND LIFE HISTORY

Abstract: As use of nanosilver (n-Ag) increases, the potentially adverse environmental effects of nanosilver remain largely unknown. Exposing aquatic organisms to a range of nanosilver concentrations across various environmental parameters, such as pH, yields results most applicable to real world ecosystems. This 28 day exposure bioassay examined the effects of five environmentally relevant nanosilver concentrations, at two different H⁺ concentrations represented as pH 6.0 and 8.3, on the growth and reproduction of freshwater gastropods (Physa acuta). Effects of n-Ag on gastropod vital rates were not dependant on pH treatment, suggesting aqueous pH does not directly influence chronic n-Ag toxicity. Nanosilver at concentrations (0.2 µg/L) that may occur in localized areas of natural waters experiencing flushes of n-Ag, altered gastropod life history by inducing a growth for reproduction tradeoff. This study provides baseline information needed to assess the possible sublethal effects of n-Ag on benthic grazers in real world freshwater ecosystems, while displaying that n-Ag toxicity, at environmentally relevant concentrations, in freshwater ecosystems is not directly dependant on environmental pH.

Keywords: Nanosilver, Physa acuta, pH, Long-term, Life History, Growth, Ecotoxicology
INTRODUCTION

The technology of novel nanomaterials is resulting in an array of commercial products that present chemical, medical, and technological benefits to society on a large scale (Lux 2008). Nanomaterials are engineered to exhibit unique properties that differ from the larger particles used to derive them, and as a result of these unique properties, potential adverse environmental effects of nanomaterials remain unknown (Moore 2006, Fabrega et al. 2011). Additionally, nanomaterials are expected to enter biological systems (Moore 2006) and penetrate through cell membranes because of their small size (Lin 2007).

Nanosilver (n-Ag) is the most widely applied nanomaterial (Luoma 2008, Nowack et al. 2011), replacing traditional organic chemical agents as a premier antimicrobial in a host of products such as tires, cosmetics, water purification systems, clothing, and textiles (Klaine 2008, Fabrega et al. 2011, Lee et al. 2012). Freshwater ecosystems will likely experience increased silver loadings as a result increased n-Ag synthesis, use, and disposal (Oberdörster 2005). Nanosilver concentration in sewage treatment effluent is estimated to range from 0.02 and 0.04 µg/L (Mueller and Nowack 2008, Gottschalk et al. 2009). Future studies aimed at understanding the potential ecological impacts of n-Ag need to examine chronic organismal effects of n-Ag at environmentally relevant concentrations, while considering the potential synergistic and antagonistic effects of other chemical characteristics in freshwater.

Similar to heavy metal toxicity, n-Ag toxicity may be affected by abiotic parameters such as hardness, dissolved organic carbon, and pH (Miller and Mackay 1980, Nelson et al. 1986, Sciera et al. 2004). For example, increasing aqueous pH reduced ionic copper toxicity towards fathead minnows (*Pimephales promelas*; Erickson et al. 1996). Although, the effects of aqueous pH on n-Ag toxicity remain unknown, we can postulate possible mechanisms to describe how pH may influence n-Ag toxicity. Alkaline conditions, relative to acidic conditions, contain more hydroxide anions which may ionicly bind to n-Ag particles and/or dissociated silver cations, limiting bioavailability of n-Ag and, hence, n-Ag toxicity to freshwater organisms. In acidic
conditions, n-Ag will not complex with hydrogen cations, perhaps acting synergistically with n-Ag particles and/or dissociated silver cations to increase toxicity. Conversely, hydrogen cations in acidic conditions may compete with dissociated silver cations for binding sites on freshwater organisms, such as gills, thereby reducing n-Ag toxicity as a result of decreased aqueous pH (Meador 1991), however these potential mechanisms remain unstudied.

The chemical behavior of n-Ag in freshwater ecosystems remains largely unknown and, therefore, estimating the potential influence of aqueous pH on n-Ag toxicity proves difficult. In freshwater ecosystems, n-Ag may dissociate into silver cations, where effects of pH on n-Ag toxicity would likely be comparable to the effects of pH on ionic silver. Contrarily, silver in the nanoparticle form, may shield itself from biogeochemical interactions, preventing silver cations from complexing with anions, allowing for direct delivering of ionic silver to the cytosol and/or membrane of cells (Louma 2008). Future understanding of the effects of water chemistry on n-Ag toxicity will be crucial in the development of water quality criteria and future n-Ag regulation policies.

The use of gastropods in ecotoxicology tests is increasing (Bernot et al. 2005, Lefcort et al. 2013, Bernot and Brandenburg 2013) because freshwater gastropods can be easily collected from natural ecosystems, cultured in a laboratory setting (Dillon 2000), and are ecologically crucial for maintaining ecosystem structure in function by serving as an important link between microbial food webs and higher trophic levels (Hall et al 2003). Because freshwater gastropods inhabit the benthos, which likely acts as a large compartment for n-Ag, gastropods are more likely to encounter and be adversely affected by n-Ag than freshwater organisms within the water column of aquatic ecosystems (Klaine et al. 2008, Bradford et al. 2009). Furthermore, gastropods can display effects of stressors by trading off growth and reproduction, a chronic effect of contaminants that can be easily quantified in a laboratory setting and is also likely to occur in natural freshwater ecosystems (Crowl and Covich, 1990). Therefore, freshwater gastropods serve as model organisms for chronic aquatic ecotoxicology studies with broad
Short-term, acute effects of n-Ag on individual organisms have been the focus of many studies (Zhu et al. 2006, Griffitt et al. 2008, Zhu et al. 2009), while sublethal effects of n-Ag on broader ecological interactions remain unstudied (Luoma, 2008). Nanosilver is acutely toxic to freshwater organisms, including crustaceans macroinvertebrates, and algae (Griffitt et al. 2008), resulting in death over a brief time period. Nanosilver inhibits gill function of freshwater fishes, causing acute toxicity to zebrafish (Danio rerio) at n-Ag concentrations as low as 40 µg/L (Griffitt et al. 2008), with similar effects on Eurasian perch (Perca fluviatilis; Bilberg et al. 2010) and brown trout (Salmo trutta; Scown et al. 2010) at equal concentrations. Future studies should include a variety of water quality parameters to best mimic freshwater ecosystems in order to understand real world n-Ag behavior, fate, and toxicity. To the best of our knowledge, how chemical characteristics of freshwater ecosystems interact with n-Ag, at environmentally relevant concentrations, to sublethally affect aquatic organismal fitness remains unstudied.

The objective of this study was to examine how environmentally relevant n-Ag concentrations interacted with aqueous pH to enhance or abate chronic n-Ag exposure to freshwater gastropods using growth rates, egg, and egg pouch production as response variables. We hypothesized that increasing n-Ag concentration will reduce gastropod growth rates and fecundity, and increasing pH will reduce the negative effects of n-Ag on these gastropod vital rates.

METHODS

Organisms

Gastropods used to conduct this long-term bioassay were obtained from batch cultures of offspring originally collected from the White River (Muncie, IN). Gastropods were maintained in aquaria filled with synthetic spring water (EPA 2002) at 22°C +/- 3°C with a 16:8 hour light:dark photoperiod. Gastropods were fed boiled spinach ad libitum.
**n-Ag Characterization**

Nanosilver stock solution was purchased from Sciventions Inc. (Toronto, Canada). Nanosilver particles were described to be between 1.0 and 10.0 nm in size and of 97% purity. Silver nanoparticle size was confirmed by sub sampling 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, USA) software. Nanosilver stock solutions were prepared by sonicating n-Ag solution into synthetic spring water in an ice bath for 45 minutes.

**Bioassay**

A 28 day bioassay was performed to quantify the effects of pH and n-Ag on the growth and fecundity of *Physa acuta*. Two pH treatments (6.0, 8.3) spanning the range of pH commonly occurring in natural freshwater ecosystems were cross-factored with five concentrations of n-Ag. Nanosilver also reflected a range of concentrations (0.0, 0.02, 0.20, 2.0, 20.0 µg/L), from those likely to occur in natural freshwaters to concentrations associated with historic loadings of bulk silver (Luoma 2008, Mueller and Nowack 2008). Synthetic spring water without treatment served as the pH 8.3 treatment water. By adding H$_2$SO$_4$, the pH of synthetic spring water was reduced to create test water of pH 6.0. This experimental design resulted in 10 treatment combinations, replicated four times, yielding 40 randomly assigned experimental microcosms. Each microcosm (400 mL glass jar) contained two gastropods and 125.0 mL of synthetic spring water consisting of a specific n-Ag concentration and pH. Gastropod shell length (Range: 4.1-5.9 mm, Mean: 5.0 mm) was measured using digital calipers prior to placing each gastropod into a microcosm. This behavioral assay was conducted at 22° C +/- 3° C with a 16:8 hour light:dark photoperiod.

Egg pouches were collected and the number of eggs in each pouch were counted every three days for 28 days. Additionally, on every third day, each microcosm received fresh
treatment water and 6.0 mg of commercial algae wafers (Hikarit, Hayward, CA, USA). Prior to the first two water changes, the pH of treatment water in each microcosm was measured to assess changes in water pH between treatments over 3 day water change intervals. Gastropod growth rates were determined by subtracting the initial from the final gastropod shell length and dividing by the number of days the corresponding gastropod survived, resulting in growth per gastropod per day. Total eggs and egg pouches from each microcosm were divided by the summed number of days both gastropods in each microcosm survived to yield both eggs and egg pouches per gastropod per day. Eggs per pouch for each microcosm was calculated by dividing total eggs produced by total egg pouches produced throughout the experiment.

**Statistical Analysis**

Individual and interactive effects of n-Ag and pH (predictor variables), over time, on gastropod daily egg, daily egg pouch, eggs per pouch production, and growth (response variables) were analyzed using repeated measures factorial analysis of covariance (RM-F-ANCOVA).

Effects of n-Ag, regardless of pH treatment, and effects of pH, regardless of n-Ag treatment, on gastropod daily egg, daily egg pouch, and eggs per pouch production, as well as growth were both analyzed using analysis of covariance (ANCOVA). For all RM-F-ANCOVA and ANCOVA analyses, the covariate was average initial shell length of gastropods in each microcosm. Furthermore, relationships between all measured aspects of gastropod reproduction and growth were determined using linear and non-linear regression. Alpha was set at 0.05 for all tests.

Linear and non-linear regressions were performed using IBM SPSS 17.0 statistical software (IBM Corporation, Armonk, NY, USA) and all other statistical analyses were performed using SYSTAT 11 software (Systat Software Inc., Chicago, IL, USA).

**RESULTS**

*n-Ag Characterization*
Length of n-Ag particles contained within the purchased n-Ag solution ranged between 2.7-40.9 nm, with a mean length of 10.2 nm (SE=0.42nm). More than 40% of observable n-Ag particles within the purchased n-Ag stock solution were between 5.0-10.0 nm in their longest dimension (Figure 1).

**pH Treatment Characterization**

Mean water pH in microcosms containing pH 6.0 treatment water increased from 6.0 to 6.55 over the first two 3 day water change intervals. Mean pH in microcosms containing pH 8.3 treatment water decreased from 8.3 to 7.66 over the first two 3 day water change intervals. Changes in pH between pH treatments were not dependant on n-Ag concentration and actual pH differed between pH 6.0 and 8.3 treatments after 3 day water change intervals ($F_{1,30} = 1,222.9$, $P < 0.01$, Figure 2).

**Effects of n-Ag and pH on Gastropod Vital Rates**

Nanosilver effects on gastropod daily egg production were not influenced by pH (n-Ag x pH effect: $F_{4,23} = 0.78$, $P = 0.55$) nor were they influenced by pH over time (n-Ag x pH x time effect: $F_{32,184} = 1.0$, $P = 0.50$). Nanosilver effects on gastropod daily egg pouch production were not affected by pH (n-Ag x pH effect: $F_{4,23} = 0.75$, $P = 0.57$) nor were they influenced by pH over time (n-Ag x pH x time effect: $F_{32,184} = 1.28$, $P = 0.16$). Nanosilver effects on gastropod eggs per pouch production were not influenced by pH (n-Ag x pH effect: $F_{4,23} = 1.48$, $P = 0.24$) nor were they affected by pH over time (n-Ag x pH x time effect: $F_{32,184} = 1.10$, $P = 0.33$). Nanosilver effects on gastropod growth also remained unaffected by pH (n-Ag x pH effect: $F_{4,29} = 1.42$, $P = 0.25$).

Although not statistically significant, regardless of pH treatment, n-Ag at 0.2 µg/L influenced gastropod reproduction and growth. Gastropods exposed to 0.2 µg/L n-Ag produced 6.5 eggs per day, while gastropod daily egg production in all other n-Ag treatments ranged from 4.1 to 4.8 eggs per day (Figure 3). Additionally, gastropods exposed to 0.2 µg/L n-Ag produced 0.60 egg pouches per day, while daily egg pouch production by gastropods in all other n-Ag
treatments only ranged from 0.36 to 0.45 egg pouches per day (Figure 4). Contrary to increased gastropod reproduction trends in 0.2 µg/L n-Ag relative to all other n-Ag treatments, gastropod growth rate in 0.2 µg/L n-Ag was only 0.39 mm/d, while gastropod growth rate in all other n-Ag treatments ranged from 0.42 to 0.60 mm/d (Figure 5). A decreasing linear relationship between gastropod daily egg production and growth existed in treatments with 0.2 µg/L n-Ag ($F_{1,6} = 8.1$, $P = 0.03$, $R^2 = 0.57$, Figure 6), regardless of pH treatment, with no additional gastropod reproduction and growth relationships existing at any other n-Ag treatment.

pH treatment influenced all measured aspects of gastropod reproduction, regardless of n-Ag treatment. Daily egg production by gastropods was affected by pH treatment (pH effect $F_{1,31} = 5.20$, $P = 0.03$, Figure 7), resulting in a 33% increase in daily egg production by gastropods in pH 6.0 treatment relative to gastropods in pH 8.3 treatment. pH effects on gastropod daily egg production, were influenced by time (pH x time effect: $F_{8,248} = 8.6$, $P < 0.01$), resulting in decreased daily egg production by gastropods in pH 8.3 treatment relative to 6.0 pH treatment from days 19 through 28. Daily egg pouch production by gastropods was affected by pH treatment over time, decreasing mean gastropod daily egg pouch production by 7% to 22% between days 22 and 28 respectively in pH 8.3 treatment relative to pH 6.0 treatment (pH x time effect: $F_{8,248} = 5.32$, $P < 0.01$, Figure 8). Overall, mean eggs per pouch production was affected by pH treatment ($F_{1,37} = 6.94$, $P = 0.01$), causing gastropods in pH 8.3 treatment to produce 18% less eggs per pouch, on average, than gastropods in pH 6.0 treatment, with this effect not dependent on exposure time.

**DISCUSSION**

Contrary to our hypothesis, pH did not affect n-Ag toxicity to *Physa acuta* throughout the 28 day bioassay. Consistent with our hypothesis, nanosilver did affect aspects of *Physa acuta* reproduction and growth. Gastropods can display effects of stressors, such as over population, predation risk, and contaminants by trading off growth and reproduction (Crowl and Covich 1990), a chronic effect of nanosilver that occurred in 0.2 µg/L. Our results suggest n-Ag effects
on gastropod growth and reproduction tradeoff dynamics may occur in localized areas of freshwater ecosystems that may experience n-Ag flushes associated with sewage effluent.

pH may not have affected n-Ag toxicity because relative hydrogen ion concentration differences between pH treatments was not great enough, only varying between 2.3 to 1.1, or because microcosms in our study, having contained no sediment, were limited in organic carbon based molecules. pH may indirectly affect n-Ag toxicity in natural ecosystems by altering functional groups on organic carbon based molecules (Goyer 1996). Increased pH in freshwater ecosystems deprotonates organic carbon based molecules, thereby increasing the degree to which cations dissociated from heavy metals will bind to organic carbon based molecules, thus reducing bioavailable heavy metals and perhaps silver cations dissociated from n-Ag (Di Toro et al. 2000). Future studies should examine possible interactive effects on pH and organic carbon on chronic n-Ag toxicity.

Nanosilver at 0.2 µg/L sublethally stressed gastropods, inducing a growth for reproduction tradeoff, with no effects on gastropod reproduction and growth at higher n-Ag concentrations (2.0 and 20.0 µg/L). In real world ecosystems, gastropods increase biological fitness by sensing stressors such as predator presence and delay reproduction to increase growth in an attempt to reduce predation risk (Covich 2010). Such changes in gastropod life history events can alter gastropod populations which would likely affect species interactions and ecosystems processes that may drastically influence plant and animal populations within freshwater ecosystems (Bernot and Turner 2001). Higher n-Ag concentrations (2.0 and 20.0 µg/L) may have affected other aspects of gastropod fitness other than growth and reproduction. Gastropods may have sensed higher n-Ag concentrations and increased near surface habitat use in an attempt to avoid n-Ag, limiting the time gastropods were exposed to n-Ag. Such contaminant avoidance behavior by gastropods has been observed as increased gastropod
movement rates when exposed to contaminants such as ionic liquids (Bernot et al. 2005) or nanosilver (Bernot and Brandenburg 2013), however this theory remains untested.

Although pH in the pH treatments did change over three day water change intervals, pH, regardless of n-Ag treatment, influenced gastropod reproduction. Changes in pH over three day water change intervals may be attributed to differences in redox potential between pH treatments, leading to an oxidizing environment in pH 6.0 treatments and a reducing environment in pH 8.3 treatments, ultimately driving in an overall push towards neutral pH in both treatments. Because pH in freshwater ecosystems is largely determined by bicarbonate buffering, calcium carbonate, an essential compound required for gastropod shell growth (Hasse et al. 2000), is limited in acidic conditions. Therefore, pH 8.3 treatment likely had more available calcium carbonate to be allocated towards gastropod shell growth than pH 6.0 treatment. However, overall gastropod growth between pH treatments did not differ, even though gastropods in pH 6.0 treatment displayed higher rates of reproduction than gastropods in pH 8.3 water during the final six exposure days. pH difference between treatments was relatively low, with a minimal apparent effect on gastropod fitness during the first 22 days of exposure. However, increased gastropod reproduction in pH 6.0 treatment during the last six exposure days could be a subtle effect of acidified conditions, with decreased calcium carbonate relative to pH 8.3 treatment, indicating a possible growth for reproduction tradeoff by gastropods in pH 6.0 treatment after 22 exposure days. Concurrently, decreased gastropod reproduction in pH 8.3 treatment during the last six exposure days, could be a subtle effect of alkaline conditions, with increased calcium carbonate, relative to pH 6.0 treatment, indicating a possible reproduction for growth tradeoff by gastropods in pH 8.3 treatment after 22 exposure days. However, unlike gastropod reproduction, we only measured gastropod shell length at the start and completion of this 28 day bioassay, thus alterations to gastropod growth and reproduction dynamics that may explain temporal differences in gastropod reproduction between pH treatments over time remain untested.
Overall, our results show n-Ag toxicity is not influenced by pH when pH differed between a range of 6.0 to 8.3 and 6.5 to 7.6. Because our study did not include additional environmental characteristics such as benthic sediment, effects of pH on n-Ag toxicity in may differ in real world ecosystems. Future studies need to incorporate various environmental parameters when examining nanomaterial toxicity to best understand behavior, fate, and toxicity of novel nanomaterials in real world freshwater ecosystems. However, such data remain rare (Klaine et al. 2008) even though nanomaterials are likely to present one of the most critical challenges for freshwater ecosystems in future years (Auffen et al. 2009). Our study provides valuable framework in understanding how n-Ag toxicity may vary between freshwater ecosystems, but may not be directly dependant on pH. Such data prove imperative in the development of future n-Ag regulation policies and remediation efforts. Moreover, our data provide a novel understanding of how n-Ag, at environmentally relevant concentrations, may sublethaly affect aquatic organisms over long-term exposure periods. Effects of n-Ag at relatively low concentrations over long-term periods are likely to influence broader ecological interactions, adversely affecting ecosystem structure and function (Bernot and Turner 2001).

REFERENCES CITED


FIGURE LEGENDS

Figure 1: Number of measured silver particles from nanosilver stock solution used in laboratory bioassays, in a range of size classes.

Figure 2: Observed pH in each pH treatment at five n-Ag concentrations following three day water change intervals. Bars represent mean pH + SE.

Figure 3: Daily egg production by gastropods exposed to five different n-Ag concentrations, regardless of pH treatment. Bars represent mean daily egg production + SE.

Figure 4: Daily egg pouch production by gastropods exposed to five different n-Ag concentrations, regardless of pH treatment. Bars represent mean daily egg pouch production + SE.

Figure 5: Growth rate by gastropods exposed to five different n-Ag concentrations, regardless of pH treatment. Bars represent mean growth rate + SE.

Figure 6: Gastropod growth rate relative to gastropod daily egg production in five n-Ag treatments, regardless of pH treatments. Linear regression line represents the relationship between gastropod growth and reproduction, displaying a decreasing trend in gastropod growth relative to increasing gastropod reproduction in 0.2 µg/L n-Ag. No trends at any other n-Ag treatment exist. Symbols represent individual microcosm observations of gastropod growth and daily egg production.

Figure 7: Cumulative eggs per gastropod in pH 6.0 and 8.3 treatments, regardless of n-Ag treatment, over 28 days. Symbols represent mean cumulative eggs per gastropod ± SE.

Figure 8: Cumulative egg pouches per gastropod in pH 6.0 and 8.3 treatments, regardless of n-Ag treatment, over 28 days. Symbols represent mean cumulative egg pouches per gastropod ± SE.

FIGURES
Figure 1
Figure 2

Nanosilver concentration (µg/L) vs pH after three days

- pH 6.0
- pH 8.3

Nanosilver concentration (µg/L)

0 0.02 0.2 2 20

pH 6.0
pH 8.3

Figure 2
Figure 3
Figure 4

Nanosilver concentration (µg/L)

Physa egg pouches per day
Figure 5
Figure 6
Figure 7
Figure 8
CHAPTER 3: Nanosilver: An Emerging Contaminant that Inhibits Freshwater Gastropod (Physa Acuta) Ability to Assess Predation Risk

Abstract.- As the use of nanosilver (n-Ag) increases, the potentially adverse effects of n-Ag on aquatic ecosystems remain largely unknown. In aquatic ecosystems, n-Ag at trace concentrations may affect the ability of organisms to sense predation by interfering with chemoreception. We performed a mesocosm experiment to assess the ability of freshwater gastropods (Physa acuta) to detect and respond to natural predator cue derived from pumpkinseed sunfish (Lepomis gibbosus), at environmentally relevant n-Ag concentrations over 24 h. Gastropods avoiding predation by occupying covered habitat was 30-47% less in treatments with environmentally relevant n-Ag compared to treatments without nanosilver, during the first 6 exposure hours. Regardless of predator cue presence, the proportion of gastropods occupying near surface habitat in 30.0 µg/L n-Ag was 20-26% greater relative to no n-Ag treatments for the first 6 exposure hours. Environmentally relevant n-Ag concentrations disrupted gastropod ability to detect and respond to a natural predator while greater n-Ag concentrations stimulated gastropods to exhibit contaminant avoidance behavior and thereby attempted to flee their habitat.

Keywords.- Nanosilver, Physa acuta, Sublethal, Chemosensory Impairment, Ecotoxicology, Gastropod, Freshwater Snail
INTRODUCTION

The emerging field of nanotechnology presents technological, chemical, and medical benefits to society. Because manufactured nanoparticles exhibit physiochemical properties that are different from the bulk materials used to derive them, nanoparticles have versatile applications in many commercial products (Moore, 2006; Fabrega et al., 2011). The market for consumer products using the distinct properties of nanoparticles is progressing and expected to be valued at $3.1 trillion by 2015 (Lux, 2008). Because nanomaterials are so novel, the potential adverse environmental effects of large-scale nanoparticle production and use remain largely unknown. Moreover, because of their smaller size, nanoparticles are more likely to infiltrate biological systems than larger molecules, resulting in possible effects on freshwater ecosystems (Moore, 2006).

Nanosilver (n-Ag) is the most widely used nanoparticle and because of possible environmental risks associated with n-Ag, it may currently be receiving the most scrutiny of any engineered nanoparticle (Luoma, 2008; Nowack et al., 2011). Nanosilver is an antimicrobial agent which is applied to surfaces of consumer products where growth of biological microbes may occur. Products that contain n-Ag include 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 (Oberdörster, 2005). Nanosilver concentrations are expected to range from 0.02 µg/L to 0.04 µg/L in sewage effluent and natural freshwaters (Mueller and Nowack, 2008; Gottschalk et al., 2009).

Nanosilver is acutely toxic to freshwater organisms, including macroinvertebrates, algae, and crustaceans (Griffitt et al., 2008), with apparent adverse effects in a brief time period. Nanosilver concentrations between 0.01-0.02 µg/L affect early stage life development of zebrafish (Danio rerio; Hamilton, 1822) embryos (Yeo and Yoon, 2009) while higher concentrations alter zebrafish gene pathways (Yeo and Pak, 2008) and 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/L (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, which may subsequently lead to adverse effects on broader ecological interactions, remain largely unstudied.

Although n-Ag is not modeled to exist at lethal concentrations in freshwater ecosystems, current environmental n-Ag concentrations may chronically affect freshwater organisms. Sublethal effects of n-Ag could include chemosensory impairment of freshwater organisms. Changes in the chemical environment of aquatic ecosystems, such as elevated pesticide concentrations and heavy metals, can hinder chemoreception of freshwater organisms including fish, gastropods, crustaceans, and amphibians (Blaxter and Hallers-Tjabbes, 1992; Lefcort et al., 1999; Luring and Scheffer, 2007). Effects of n-Ag on the chemoreception of aquatic organisms may be a common chronic occurrence in polluted freshwater ecosystems because most aquatic organisms use chemoreception to sense their surroundings, locate food, search out mates, and detect predators (Dobson et al., 1994; Bernot and Turner, 2001; Derby and Sorensen, 2008). Disruptions in the transfer of chemical cues may result in profound effects at population levels leading to the potential reorganization of aquatic ecological communities (Weissburg et al., 2002). However, constraints on chemoreception as a result of anthropogenic pollutants such as nanomaterials have only been minimally studied.

Nanisolwer accumulates in the upper-most sediment layers of aquatic ecosystems (Klaine et al., 2008; Bradford et al., 2009). As a result, benthic grazers, particularly freshwater gastropods, are ideal organisms to study when examining the sublethal effects of n-Ag on freshwater organisms. Among freshwater gastropods Physids are the most common and abundant family, found in both lentic and lotic ecosystems (Dillon, 2000), serving as an
important link between primary producers and higher trophic levels within freshwater food webs (Bernot and Turner, 2001; Thorp and Covich, 2009; Turner and Montgomery, 2009). Pulmonate gastropods detect predation risk by sensing chemical cues associated with natural predators and respond by altering habitat use, feeding behavior, growth, and reproduction (Alaxander and Covich, 1991; Turner, 1996; Bernot and Turner, 2001). For example, in the presence of molluscivorous fish, gastropods will seek refuge under covered benthic habitat, while in the presence of a benthic predator, such as a crayfish, gastropods will seek refuge at the surface of the water (Bernot and Turner, 2001).

The objective of our study was to determine 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. Changes in gastropod behavior in the presence of a natural predator cue and n-Ag over 24 h were quantified in a laboratory behavioral assay. We hypothesized that nanosilver will inhibit the ability of *Physa acuta* to detect and respond to a natural predator cue.

**METHODS**

*Organisms.* - *Physa acuta* used to conduct this behavioral assay were obtained from batch cultures of offspring originally collected from the White River (Muncie, IN, Longitude 40.1805°N, Latitude -85.432°E). *Physa acuta* were maintained in synthetic spring water (EPA, 2002) filled aquaria at 22°C +/- 3°C with a 16:8 h light:dark photoperiod and fed boiled spinach ad libitum.

Pumpkinseed sunfish (*Lepomis gibbosus*; Linnaeus, 1758) are a natural predator to *P. acuta* (Mittlebach, 1984). Pumpkinseed sunfish were purchased from Smith Creek Fish Farm (Bliss, NY, USA) and housed in 37.85 L, synthetic spring water filled aquaria at 22°C +/- 3°C with a 16:8 h light:dark photoperiod. Sunfish were fed cultured *Daphnia magna* (Straus, 1820), pulmonate gastropods, and flake food (Hikari, Hayward, CA, USA) daily.
Nanosilver Characterization. - Nanosilver stock solution was purchased from Sciventions Inc. (Toronto, Canada). Nanosilver particles were described to be between 1.0 and 10.0 nm in size and of 97% purity. We prepared nanosilver stock solutions by sonicating n-Ag solution into synthetic spring water in an ice bath for 45 minutes. Before any experimental exposure, we confirmed silver nanoparticle size by sub-sampling 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, USA) software.

Behavioral Assay. - In a laboratory behavioral assay, we directly tested the effects of n-Ag on the ability of freshwater gastropods to detect and respond to a natural predator, L. gibbosus. We exposed gastropods to three n-Ag concentrations: a negative control (0.0 \(\mu\)g/L), a concentration modeled to currently exist in natural freshwaters (0.03 \(\mu\)g/L; Mueller and Nowack, 2008), and a concentration associated with historic silver loadings (30.0 \(\mu\)g/L; Luoma 2008). We cross-factored predator cue presence/absence with three n-Ag concentrations resulting in six treatment combinations, replicated eight times, yielding 48 randomly assigned experimental units. Experimental units consisted of a 1.9 L plastic container (13.7x17.8x9.5 cm) containing 1.24 L of synthetic spring water consisting of a specific n-Ag concentration, 10 gastropods (average shell length of every third gastropod=6.8 mm; Range=3.5-10.5 mm), and an elevated, unglazed ceramic tile (9.7x9.7x1.0 cm; see Turner, 1996) to serve as gastropod refuge from predation.

Pulmonate gastropods serve as natural prey to L. gibbosus and therefore respond to sunfish presence by moving to a predator safe microhabitat, typically seeking refuge under covered habitat (Bernot and Turner, 2001). Following the addition of 10 gastropods to each mesocosm 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 mesocosm. Predator cue was created by feeding five crushed *P. acuta* to each of 24 pumpkinseed sunfish housed in individual aquaria. Each aquarium contained a single feeding fish in 35.0 L of synthetic spring water. Predator cue from each pumpkinseed sunfish was randomly paired with an experimental mesocosm.

We assessed gastropod habitat use 1, 3, 6, 18, and 24 h after the addition of predator cue. Gastropod refuge use was determined to be the proportion of gastropods under each ceramic tile. To be considered seeking refuge under the ceramic tile, a gastropod had at least 50% of its body under the ceramic tile. Additionally, we measured the proportion of gastropods out of the water and near the surface of the water (≥ 50% of the gastropod body within 1.0 cm of the surface) to quantify near surface habitat use, a behavioral response indicative of benthic predator presence. This behavioral assay was conducted at 22°C ± 3°C with a 16:8 h light:dark photoperiod.

*Statistical Analysis.* Proportional gastropod habitat use data were arcsine transformed to meet the assumptions of analysis of variance (ANOVA). We then calculated a predator avoidance index as \((P-NP)/(1-NP)\) where \(P=\) refuge use in predator cue mesocosms and \(NP=\) mean refuge use in mesocosms without predator cue, which results in index values between -1.0 and 1.0, with 0 matching habitat use in corresponding predator cue absence treatments. We created two predator avoidance indices to analyze two aspects of predator avoidance separately, gastropods seeking refuge under covered habitat and gastropods seeking refuge in near surface habitat. We analyzed the effects of n-Ag (predictor variables) on both predator avoidance indices (response variables), over time, using repeated measures analysis of variance. We further analyzed the effects of n-Ag, regardless of predator cue presence, on near surface habitat use by gastropods (response variable), over time, using repeated measures ANOVA (\(\alpha=0.05\)). All analyses were performed using SYSTAT 11 software (Systat Software Inc., Chicago, IL, USA). Alpha was set to 0.05 for all tests.
RESULTS

Over 40% of observable n-Ag particles within the purchased n-Ag stock solution were between 5.0-10.0 nm in their longest dimension (mean n-Ag particle length=10.2 nm, Range=2.7-40.9 nm; Fig. 1).

In the presence of predator cue, covered habitat use by gastropods was 30-47% less in treatments containing 0.03 µg/L n-Ag, and 66% to 74% less in treatments containing 30.0 µg/L n-Ag, relative to treatments without nanosilver (n-Ag effect: $F_{2,21} = 17.12, P < 0.01$, Fig. 2). The increase in covered habitat use by gastropods in treatments without n-Ag was greatest 1 to 6 h after predator cue addition, however, differences diminished by 18 h and 24 h (n-Ag x Time effect: $F_{4,8} = 2.29, P = 0.03$, Fig. 2). Predator avoidance by gastropods occupying covered habitat did not differ between mesocosms with 0.03 µg/L or 30.0 µg/L n-Ag over 24 h.

Effects of n-Ag on gastropods avoiding predation by occupying near surface habitat depended on the amount of time after exposure to predator cue (n-Ag x Time effect: $F_{4,8} = 2.49, P = 0.02$, Fig. 3). Predator avoidance by gastropods occupying near surface habitat was greatest 6 h after predator cue additions in treatments with 30.0 µg/L n-Ag but did not differ from other treatments at any other time period. In the presence of predator cue, n-Ag did not directly affect the proportion of gastropods occupying near surface habitat in an attempt to avoid predation (n-Ag effect: $F_{2,21} = 1.19, P = 0.32$, Fig. 3).

Nanosilver affected the proportion of gastropods occupying near surface habitat regardless of predator cue presence/absence, with this effect diminishing over time (n-Ag x Time effect: $F_{4,8} = 4.28, P < 0.01$, Fig. 4). The mean proportion of gastropods occupying near surface habitat was 20%-26% greater during the first six exposure hours in 30.0 µg/L n-Ag compared to no n-Ag treatments, while the proportion of gastropods occupying near surface habitat in 0.03 µg/L n-Ag did not differ from no n-Ag treatments at any hour observed.

DISCUSSION
Consistent with our hypothesis, the ability of gastropods to sense and respond to predator cue was eliminated by both an environmentally relevant concentration of n-Ag and a concentration associated with historic silver loadings. Predator cue presence without n-Ag caused gastropods to avoid near surface habitat and occupy covered habitat, while gastropods in treatments with a n-Ag concentration modeled to occur in natural freshwaters did not alter habitat use in the presence of a predator cue. Higher concentrations of n-Ag caused gastropods to occupy near surface habitat. Increased near surface habitat use by gastropods, in a high n-Ag concentration, regardless of predator cue presence, may be the result of gastropods attempting to crawl out of the water. Because this behavior was observed for the first 3 exposure hours in treatments with high n-Ag, and not induced 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.

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 are growing, while similar studies on novel nanomaterials remain limited, even though nanomaterials may present one of the most crucial threats to freshwater ecosystems in future years (Auffan et al., 2009). Silver nanomaterials most likely reduce gastropod ability to assess predation risk by disrupting or altering receptor sites of ion channels located on neuronal membranes, similar to the effects of heavy metals including lead, zinc, and copper (Rozsa and Salonki, 1990; Pyle et al., 2007). However, this hypothesis remains untested and additional mechanisms of n-Ag induced chemosensory impairment exist. Nanosilver may alter kairomones released by sunfish predators, eliminating the function of predator cue, or n-Ag may modify the conformation of gastropod olfactory glands used to sense chemical surroundings, effects which are both induced by acidified water (Wojtasek and Leal, 1999; Brown et al., 2002).
Higher concentrations of n-Ag induced gastropods to exit their habitat, with this effect dependant on predator cue presence only at hour 6. Thus, the higher proportion of gastropods occupying near surface habitat during the first 3 exposure hours in 30.0 µg/L n-Ag at can be directly attributed to n-Ag. Therefore, gastropods were occupying near surface habitat in an effort to evade n-Ag, demonstrating contaminant avoidance behavior. Previous studies have documented this behavior by *P. acuta*. For example, gastropods were shown to increase movement rates 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 were exposed to predation risk. Additionally, movement induced by flushes of contaminants, perhaps n-Ag, in freshwater ecosystems may reduce gastropod fitness by using energy at the expense of growth and/or reproduction throughout gastropod life history. This experiment showed that regardless of predator cue treatment, 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 and/or dissociated silver ions may have bound to the ceramic tile and/or organic ligands associated with gastropod tissue or excrements. Further understanding of n-Ag fate in freshwater systems will be valuable in assessing n-Ag toxicity in real world ecosystems. 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 unavailable.

Beyond sensing predation risk, chemosensory perception is used by aquatic organisms to detect a host of environmental characteristics aimed at increasing biological fitness (Dodson *et al.*, 1994; Derby and Sorensen, 2008). Freshwater organisms, such as benthic gastropods, commonly deploy antipredator behavior at the expense of foraging (Lima, 1998), indirectly increasing periphyton biomass (Bernot and Turner, 2001), while reducing gastropod growth and
reproduction (Turner, 1996). Such interactions are pivotal in maintaining freshwater ecosystems, however contaminant induced alterations to species interactions may drastically alter ecosystem structure and function (Bernot and Turner, 2001).

Overall, our results showed that concentrations of n-Ag modeled to occur in natural freshwaters eliminated *P. acuta* ability to sense predation risk while higher concentrations induced contaminant avoidance behavior by *P. acuta in vitro*. The effects of both chemoreception inhibition and induction of contaminant avoidance behavior could potentially be ecologically profound, leading to disruptions in predation and consumption that may cascade throughout aquatic communities resulting in possible adverse effects at the ecosystem level (Brönmark and Hansson, 2012).

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FIGURE LEGENDS

Figure 1: Number of measured silver nanoparticles from nanosilver stock solution, in a range of size classes.

Figure 2: *Physa acuta* predator avoidance by occupying covered habitat in experimental mesocosms in the presence of 3 n-Ag concentrations following exposure to predator chemical cues from pumpkinseed sunfish, *L. gibbosus*, symbols represent mean predator avoidance ± SE.

Figure 3: *Physa acuta* predator avoidance by occupying near surface habitat in experimental mesocosms in the presence of 3 n-Ag concentrations following exposure to predator chemical cues from pumpkinseed sunfish, *L. gibbosus*, symbols represent mean predator avoidance ± SE.

Figure 4: Proportion of *Physa acuta* occupying near surface habitat for each hour observed in the presence of 3 n-Ag concentrations regardless of predator cue presence, symbols represent mean proportion of gastropods occupying near surface habitat ± SE.
Figure 1: Distribution of silver particle sizes (nm).

- 0-5 nm: 0 particles
- 5-10 nm: 140 particles
- 10-15 nm: 80 particles
- 15-20 nm: 40 particles
- 20-25 nm: 20 particles
- 25-30 nm: 10 particles
- 30-35 nm: 5 particles
- 35+ nm: 1 particle
Figure 2

Covered habitat use as predator avoidance index

- n-Ag 0.0 µg/L
- n-Ag 0.03 µg/L
- n-Ag 30.0 µg/L
Near surface habitat use as predator avoidance index

Figure 3
Figure 4