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Silver Nanoparticles: Reducing Environmental Toxicity Through Shape Control

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ABSTRACT

The increasing usage of silver nanoparticles (AgNPs) in consumer and antibacterial products without regulation raises concerns about their environmental toxicity. Silver nanospheres have been shown to have toxic effects to a variety of organisms but the ecotoxicology of other AgNP shapes has not been established. However, different shapes that could help solve the problem of the environmental toxicity of AgNPs have not been sufficiently studied. This study found that silver nanocubes and nanoplates were significantly less toxic than silver nanospheres to the model plant species *Lemna minor* when exposed for 7 days. The shapes had no significant difference in growth inhibition to the model bacteria *Escherichia coli* using the agar disk diffusion method (modified CLSI procedures). More specifically, for fresh weight, silver nanospheres were up to 15.12% more toxic than silver nanoplates and nanocubes. These findings could help in creating new consumer products in the growing industry of nanotechnology that have less environmental toxicity after their intended use but similar antibacterial effects.

KEYWORDS

Silver nanoparticles | Ecotoxicology | E. coli, | L. minor

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Introduction

The global nanotechnology market reached $39.2 billion in 2016 and is expected to reach $90.5 billion by 2021 (McWilliams, 2016). Currently, there are approximately 1,814 consumer products from 622 companies in 32 countries that utilize nanoparticles. Silver is the most frequently used nanomaterial, with 435 products, or 24%, harnessing its capabilities (Vance *et al.,* 2015). Consumer products that contain silver have the potential to release silver ions into the environment, which could be not only devastating to the environment but also to humans with ingestion through water systems (Yu, Yin, and Liu, 2013).

When used and disposed of properly, the dangers of AgNP's to the environment are minimized. However, because nanotechnology is emerging, regulations and proper disposal techniques are not used. This results in AgNP's affecting ecosystem health through leaking into water systems. Several studies show AgNP's potentially being released into the environment through sewage treatment plants, where the resulting "sludge" is then spread onto soil as a fertilizer (Center for the Environmental Implications of Nanotechnology [CEINT], 2013). Additionally, the particles could be accidentally released through a factory's waste stream through synthesis into a nearby water stream (Lohse, 2015). Because silver nanoparticles range from 1-100 nanometers, they are often not filtered out in water treatment plants.

Recently, more research has been conducted concerning the effect of particle shape on toxicity and properties. In particular, new methods have been proposed to change the shape of AgNP's. Generally, AgNP's are spherical due to the increased stability over other shapes, as well as typical interaction with stabilizers during synthesis. With these new methods, it is believed that both planar (triangles, 5 or 6 diagonal, round surfaces, etc.) and three dimensional (cubic, pyramid, etc.) AgNP's could be produced (Khodashenasa & Ghorbanib, 2015).

The effect that the different AgNP's have on the environment has not been evaluated, with the exception of silver nanocubes and silver nanowires (Gorka *et al.,* 2015). The study found lower toxicity of silver nanocubes of approximately 34.3% with the model plant species *Lolium multiflorum*, while showing similar toxicity toward other environmentally relevant bacterial species. However, this study only conducted tests on three variants of AgNP's, nanocubes and nanowires compared to the traditional nanospheres. Further research is needed to determine the effects of nanorods, triangular AgNP's, nanoprisms, flower-shaped AgNP's, and nanobars in environmental conditions. If one of these variants is found to have lower toxicity to bacterial and plant species, it could assist in the development of new silver nanoparticle consumer products that cause less environmental toxicity after their intended use.

In order to further determine the environmental toxicity of different shapes of AgNP's, this study will evaluate two model species and three different shapes. The three shapes examined will be nanoplates (40-60 nm), nanocubes (75 nm), and nanospheres (50 nm) (Nanocomposix). To try and control all outside characteristics except for shape, AgNP's will be used that have the same coatings. To evaluate the toxicity of the shapes for antibacterial properties, *Escherichia coli* (*E. coli*) K-12 will be used. For ecosystem health and plant growth, *Lemna minor* will be examined.

Materials and Methods

Synthesis/Characterization of AgNPs. The silver nanoparticles were bought from Nanocomposix. The nanocubes were 75 nm, the nanospheres were 50 nm, and the nanoplates were 40-60 nm. All of the nanoparticles were coated in polyvinylpyrollidone (PVP). Additional characteristics are described in Table 1.

L. minor and E. coli materials. Lemna minor (duckweed) was purchased commercially from Pond Plants Inc. Plant cultures were then kept in clear plastic containers under natural sunlight. The temperature was kept at 24 ± 2 °C. Approximately 7 days before testing, sufficient colonies were transferred aseptically into containers with Steinberg medium (Table 2) under test conditions (Organisation for Economic Cooperation and Development [OECD], 2006). The initial pH was ad-

justed to 5.5 ± 0.2. E. coli K-12 was purchased from Carolina Biological Supply Company. It was suspended in nutrient broth. The growth medium used was Mueller-Hinton agar from Thermo Fisher Scientific.

L. minor test design

A stock solution of 20 mg/L AgNPs was prepared for all three shapes. Five treatments were used for each shape, .01 mg/L, 0.1 mg/L,1.0 mg/L, and 5.0 mg/L in addition to a control (0 mg/L). There were three replicates of each treatment. The tests were performed in petri dishes containing 25 mL of media (Steinberg medium and AgNP). Steinberg medium was mixed according to OECD Guidelines (Table 2).

All of the petri dishes were kept in a Biotronette Mark III Environmental Chamber adjusted to a temperature of 24 ± 2 °C. All of the dishes were placed randomly around the incubator to reduce spatial differences in light intensity or temperature. The duration of the test was 7 days, with measurements at 0, 3, 5, and 7 days.

Nine samples (double or triple fronded) were chosen for each dish. The frond area and fresh weight were the data collected. Pictures were taken of all samples at 0, 3, 5, and 7 days with a meter stick visible. Computer software (ImageJ) was used to calibrate the images with a centimeter in the picture. From this, frond area was determined in cm^2. The fresh weight was determined by blotting the fronds dry and weighing with accuracy to .1 milligrams (measured at 0 and 7 days). Significant changes in visual appearance was also noted.

E. coli test design

The agar disk diffusion method was the method used here and has also been well established in past studies with AgNPs and in the CLSI guidelines (Shameli et al., 2012; Clinical Laboratory Standards Institute [CLSI], 2015). Four treatments were used for each shape, .5, 1.0, 5.0, and 10.0 mg/L in addition to a control (0 mg/L). All treatments were done with 5 replicates (5 disks).

The stock *E. coli* solution was adjusted to a .5 McFarland standard (1.5×10^8 colony–forming units (CFU)/mL). Filter paper disks were prepared (diameter .5 cm) and briefly submerged in .01 mL of AgNP treatments. Then, approximately .15 mL of the adjusted *E. coli* solution was evenly streaked across the surface of the agar with a sterilized glass rod. The dish was left to dry for 5 minutes with the lid on. Each disk was placed onto the surface of the agar and pressed gently. The duration of the test was 24 hours and the plates were held constant at 35℃ ± 2℃. All of the plates were incubated away from direct light. At the end of the 24 hours, the diameters of the zones of inhibition were measured in millimeters using image analysis.

Results and Discussion

Experiment 1

Lemna minor Shapes. In both cases of frond area and fresh weight, there was a significant difference in the shape of nanoparticle used after allowing for effects of concentration. For frond area, there was significance for both Spheres vs. Plates and Spheres vs. Cubes (Tukey test; P = .002, P = .006 respectively). This effect is modelled in Figure 1. This was also the result for fresh weight, with significance for both Spheres vs. Plates and Spheres vs. Cubes (Tukey test; $P < 0.001$, $P < 0.001$). This is indicated in Figure 2. However, there was no significant interaction between shape and concentration in either frond area or fresh weight (Tukey test; P = 0.278, P = 0.136 respectively), indicating the effects of the different shapes were not dependent on the concentrations tested.

The significant difference between Spheres vs. Cubes and Spheres vs. Plates but not Cubes vs. Plates (P = .006, P = .002, P = .635 respectively) indicates that both nanoplates and nanocubes are less phytotoxic than nanospheres. More specifically, the percent inhibition difference for frond area between spheres and plates was 6.573% and between spheres and cubes was 8.228%. This correlates with a previous study that found that silver nanocubes had 34.3% less root length reduction than silver nanospheres and overall was less phytotoxic (Gorka *et al*., 2015).

One reason why the shapes could have different effects lie within their characterization and fabric-

ation. It is generally agreed that the Ag+ ions are the cause of cell death. Because the different shapes have different rates of release of Ag+ ions, the toxicity could be different. Additionally, in aquatic environments over time, nanocubes are significantly more stable than nanospheres and aggregate. The increased stability could lead to less release of Ag+ ions.

Concentrations

There was a significant difference between the different concentrations of the shapes in both frond area and fresh weight. For frond area, there was a significant difference with the values: 5.000 vs. 0.000, 5.000 vs. 0.010, 1.000 vs. 0.000, and 1.000 vs. 0.010 (Tukey test; $P < 0.001$, $P = .003$, $P = .004$, $P = .019$ respectively). This suggests the most notable difference in toxicity occurred with 0, .01 mg/L, and 1.000 mg/L to 5.000 mg/L, while the other dilution jumps did not have an effect on toxicity. For fresh weight, there was a significant different with the values: 5.000 vs. 0.000, 0.100 vs. 0.000, 1.000 vs. 0.000, 0.010 vs. 0.000, and 5.000 vs. 1.000 (Tukey test; all $P < .001$).

The significance of the concentrations with 5.000 vs. 0.000, 0.100 vs. 0.000, 1.000 vs. 0.000, 0.010 vs. 0.000, and 5.000 vs. 1.000 mg/L as before (Tukey test; all $P < .001$) all indicate that the lowest concentration tested had a significant effect on toxicity. This suggests the largest toxicity jumps occurred from 0.000 mg/L to .0100 mg/L, .10 mg/L, and 1.000 mg/L, which could mean the lowest toxicities tested could be one of the critical points where an amount above would not have a significantly different impact. Additionally, the lack of significance from 1.000 to 5.000 mg/L suggests that 1.0 mg/L could have been a concentration where close to maximum toxicity could occur. This supports other studies that found that concentrations as small as .05 mg/L were toxic (Pereira et al., 2017).

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$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_{i)})}{t}$$

where:

- μ_{i-j} : average specific growth rate from time i to j
- N_i : measurement variable in the test or control vessel at time i
- N_j : measurement variable in the test or control vessel at time j
- t : time period from i to j

$$\%\, I_r = \frac{(\mu c - \mu r)}{\mu c} \times 100$$

where:

- $\%\, I_r$: percent inhibition in average specific growth rate
- μ_C : mean value for μ in the control
- μ_T : mean value for μ in the treatment group

(Pereira et al., 2017).

Lemna minor exposed to AgNPs over a 7 day period experienced significant loss in both frond area and fresh weight in a dose dependent manner. This corresponds with previous studies that found significant inhibition in root growth and frond area for L. gibba with concentrations as small as .025 mg/L (Farrag, 2015).

Variable correlation. After looking separately at frond area and fresh weight, a Pearson correlation test indicated there was a significant relationship between the two variables. The coefficient of correlation as 0.523 indicates a mildly strong positive correlation between frond area and fresh weight. This is also expected, as when the frond area increases or decreases, the mass should as well.

Percent Inhibition. Percent inhibition was calculated using the following two equations.

For the first significant difference in shape (Spheres vs. Plates; $P = .002$), the largest difference in percent inhibition of frond area was in 5.0 mg/L. The Plates were 6.573% less inhibiting of frond area than the spheres. The other significant result for shape was Spheres vs. Cubes ($P = .006$), and the largest difference in inhibition for frond area was also in 5.0 mg/L. The Cubes were 8.228% less inhibiting of frond area than the Spheres.

The other variable percent inhibition was evaluated for was fresh weight. The first shape difference (Spheres vs. Plates; $P = .002$) yielded the largest percent inhibition difference in 5.0 mg/L,

The other shape difference (Spheres vs. Cubes; P = .006) had the largest percent inhibition difference in fresh weight in 1.0 mg/L, 15.307%. These values indicate that both Plates and Cubes were less inhibitory to the growth of Lemna minor.

Another notable observation was the chlorosis of the L. minor after 3 days in all shapes and concentrations except for the control. This could mean that the AgNPs induced an oxidative stress status in cells, thus ensuing upregulated enzymatic activity as a self-defense mechanism. Observed chlorosis further inhibiting the growth was comparable to another study that observed chlorosis at 7 days (Pereira *et al*., 2017).

Overall, frond area and fresh weight supported the fact that silver nanocubes and nanoplates are less phytotoxic than nanospheres. Although all of the dishes were kept in a consistent environment, it might not have been completely reflective of environmental conditions. This study design did not completely account for environmental transformations, as AgNPs could react with surrounding inorganic ligands or sulfur in wastewater treatments and reduce their toxicity (Levard, Hotze, Lowry, and Brown, 2012). It is important to note that shape is only one of the many factors that affect the final overall phytotoxicity of silver nanoparticles in the environment. Therefore, this study can only potentially conclude that silver nanocubes and nanoplates will be less toxic in the environment than nanospheres. However, understanding the shape dependent properties of AgNPs could contribute to eventually reducing overall phytotoxicity while maintaining their intended antibacterial properties.

Further research is needed on other plant species to determine overall environmental toxicity. L. minor only reflects one of the areas of eukaryotic cells that are in nature. Additionally, environmental conditions should be recreated and taken into account. Because AgNPs will sulfidize in wastewater treatment plants, reducing toxicity because of silver sulfide insolubility, realistic concentrations of AgNPs should be tested. Wastewater "sludge" that is used as fertilizer conditions should also be recreated in order to look at more facets of how AgNPs can be released into the environment.

Experiment 2: E. coli Shapes. There was no significance between the different shapes of AgNPs tested. The Tukey test was P = 0.695. This is shown in Figure 3. This indicates that shape dependent toxicity may only be important in plants. Consequently, this could be useful in developing a new line of antibacterial consumer products.

If nanocubes and nanoplates are less toxic to plants but equally as effective in antibacterial properties as nanospheres, then replacing the widely used nanospheres with nanoplates and nanocubes could help reduce environmental toxicity while maintaining the same antibacterial effectiveness. This also supports a study that found no significant difference of nanocubes and nanospheres in bacteria growth but significant difference in root length (Gorka *et al*., 2015). In contrast, a study found that silver nanocubes, nanospheres, nanorods, and nanoplates did not have different cytotoxicity but different antibacterial effects, which is directly opposite to the findings presented here (Helmlinger *et al*., 2016). This could be due to the researchers looking only at human stem cells and not aquatic organisms or plants.

Concentrations There was significance between the concentrations tested. The overall Tukey test value was P < .001. Looking more specifically at the origin of this significance, the multiple comparison procedure showed significance between 10.000 vs. 1.000, 10.000 vs. 0.500, 10.000 vs. 5.000, 10.000 vs. 0.000, 5.000 vs. 1.000, 5.000 vs. 0.500, and 5.000 vs. 0.000. This indicates that concentrations between 0.000 and 1.000 would not have a difference in growth inhibition. No significance between the lower values indicates that concentrations between 0.000 and 1.000 would not have a significant difference in growth inhibition. It also indicates that concentrations 5.0 -10.0 mg/L would have the maximum toxicity. This contrasts a study that found silver nanosphe-

es to be toxic to *E. coli* at 1.0 mg/L (Paredes *et al*., 2014). However, this could be attributed to the different strain of *E. coli* in that study (O157:H7 instead of K-12).

Further research is needed to determine more realistic effects of the antibacterial shape dependent properties of AgNPs. One area is to test more types of bacteria, possibly algae and other environmentally beneficial bacteria. This would assess the impacts of different shapes not only on negative bacteria in the environment but also beneficial bacteria. Another way this experiment could be improved is through assessing the impacts of concentration more accurately. This could be done by directly mixing AgNP's with the bacterial suspension properties as nanospheres, then replacing the widely used nanospheres with nanoplates and nanocubes could help reduce environmental toxicity while maintaining the same antibacterial effectiveness. This also supports a study that found no significant difference of nanocubes and nanospheres in bacteria growth but significant difference in root length (Gorka *et al*., 2015). In contrast, a study found that silver nanocubes, nanospheres, nanorods, and nanoplates did not have different cytotoxicity but different antibacterial effects, which is directly opposite to the findings presented here (Helmlinger *et al*., 2016). This could be due to the researchers looking only at human stem cells and not aquatic organisms or plants.nanospheres to be toxic to E. coli at 1.0 mg/L (Paredes et al., 2014). However, this could be attributed to the different strain of E. coli in that study (O157:H7 instead of K-12).

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could be done by directly mixing AgNP's with the bacterial suspension.

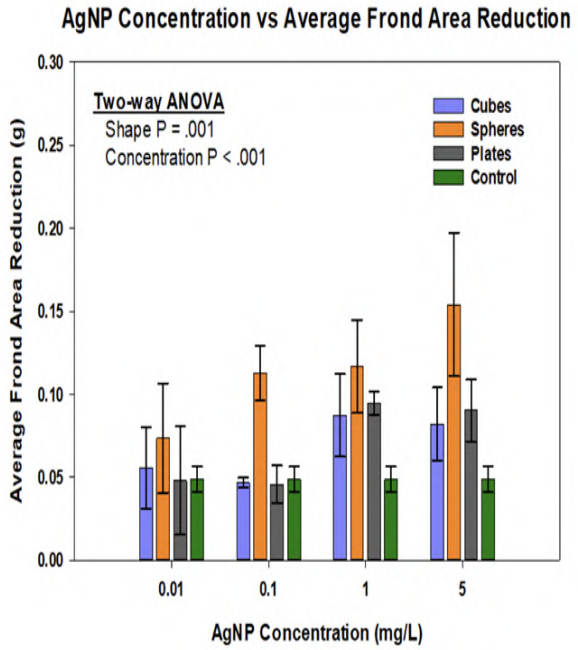

Fig. 1: Silver nanoparticle (silver nanocubes, nanospheres, and nanoplates) concentration compared to the average frond area reduction for *L. minor* over 7 days. Error bars represent ±1 SD

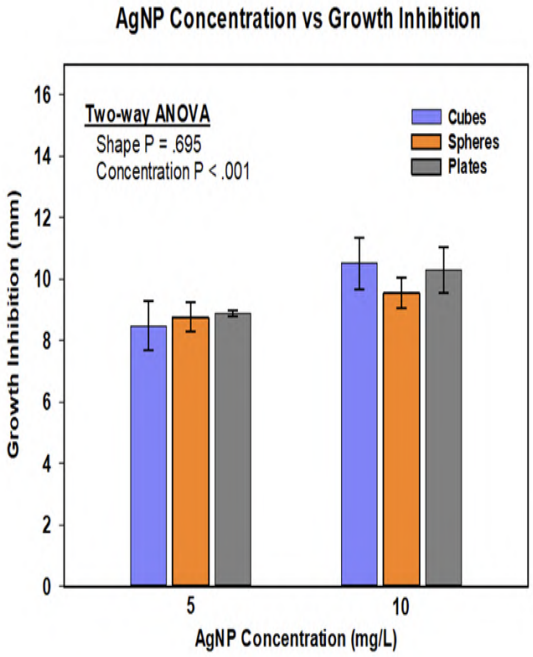

Fig. 2. Silver nanoparticle (silver nanocubes, nanospheres, and nanoplates) concentration compared to the average fresh weight reduction for L. minor over 7 days. Error bars represent ±1 SD

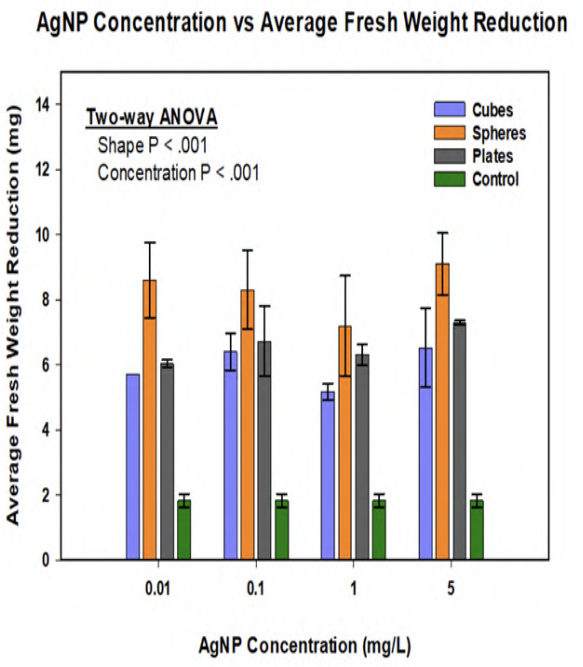

Fig. 3. Silver nanoparticle (silver nanocubes, nanospheres, and nanoplates) concentration compared to the zones of inhibition of *E. coli* over 24 hours. Error bars represent ±1 SD.

Table 1: AgNP Characteristics

Size (nm)	Mass Concentration (mg/mL)	Atomic (Ag) Molarity (mmol/L)	Particle Concentration (particles/mL)	Ag Mass Percent (%)	Max Optical Density (cm^{-1})	Peak Wavelength (nm)
50	0.02	0.185	2.9×10^{10}	0.002	2.3	420
60	0.02	0.185	1.7×10^{10}	0.002	1.9	430
70	0.02	0.185	1.1×10^{10}	0.002	1.6	445
80	0.02	0.185	7.1×10^{9}	0.002	1.2	460

Substance	Concentration (g/L)
KNO_3	17.50
KH_2PO_4	4.50
K_2HPO_4	0.63
$MgSO_4 \cdot 7H_2O$	5.00
$Ca(NO_3)_2 \cdot 4H_2O$	14.75
H_3BO_3	120.0
$ZnSO_4 \cdot 7H_2O$	180.0
$Na_2MoO_4 \cdot 2H_2O$	44.0
$MnCl_2 \cdot 4H_2O$	180.0
$FeCl_3 \cdot 6H_2O$	760.00
EDTA Disodium-dihydrate	1500.0

Table 2: Composition of the modified Steinberg me-

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