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Analysis of Nanoparticle Exposure on Legionella Pneumophila Biofilm Morphology and Host-Bacteria Interactions

Tara Diane Raftery
Clemson University

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ANALYSIS OF NANOPARTICLE EXPOSURE ON LEGIONELLA PNEUMOPHILA BIOFILM MORPHOLOGY AND HOST-BACTERIA INTERACTIONS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

by
Tara Diane Raftery
August 2012

Accepted by:
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ABSTRACT

An increase in the amount of money spent on nanotechnology has led to an increase in funding to support nanoparticle (NP) research. Due to their stable, beneficial properties, NPs are found in daily consumer products such as bike frames, socks, cosmetics, and sunscreens. As a result of this, these NPs have the potential to enter the environment and interact with various organisms that play integral roles in the ecosystem as a whole. The effect that NPs have on aquatic systems is largely unknown and understudied. Biofilms are an important structural and functional part of aquatic ecosystems, and comprise various microorganisms from bacteria to protozoa. NP use will increase the concentration of NPs in the environment, making them more likely to interact with biofilms. NP contamination of aquatic environments may lead to adverse effects on environmental organisms. *Legionella pneumophila* is a biofilm forming bacterium that can survive in a wide range of temperatures and pH. First, NPs were characterized in their stock solutions and the exposure media, moderately hard water (MHW). Biofilms were exposed to NPs with various core compositions and surface chemistries, and at two different concentrations. Changes in biofilm morphology, bio-volume and roughness coefficient were observed after exposure to low concentrations of 4 and 18 nm AuNPs, 4 nm PtNPs, and low and high concentrations of 8 nm Fe$_3$O$_4$ NPs. Larger 50 nm AuNPs, 8 nm AgNPs, or PSBs did not result in changes to the biofilm morphology. In addition, the number of viable cells being released from the biofilm after NP exposure increased as NP size and concentration decreased, but treatments were not statistically different from controls. Biofilms were found to have similar numbers of viable cells after treatment with
PtNPs, suggesting that the NPs are not killing the cells. Furthermore, when planktonic \textit{L. pneumophila} were exposed to these NPs, no significant difference in cell biomass, pigment production, or cell viability was shown. Due to this decrease in biofilm bio-volume, we next examined how NP exposure affects the host-pathogen interaction between \textit{L. pneumophila} and amoebae. Biofilms exposed to 4 and 18 nm, citrate capped, spherical AuNPs significantly altered grazing ability of amoebae, which was not observed in biofilms exposed to 24 nm PSBs. Uptake and replication of NP exposed planktonic \textit{L. pneumophila} within amoebae were not altered regardless of NP size or core chemistry. Nanomaterial effects on the interaction of benthic organisms and bacteria may be direct or indirectly dependent on bacterial morphology. NP contamination therefore may alter interactions in normal ecosystem function.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Tamara McNealy, for giving me the opportunity to conduct research in her lab, and for her patience and guidance in mentoring me over the past 3 years. I would also like to thank my committee members, Dr. Steve Klaine, Dr. Chris Kitchens, and Dr. Mike Henson for their knowledge and willingness to help.

Thanks to Dr. Terri Bruce, the manager of the Jordan Hall Imaging Facility, where all of my fluorescent images were taken, for her encouragement and expertise in the field of microscopy. To my fellow graduate students, Uma Mahajan, Jordan Burbage, and Jonathan Gravgaard, thank you for assistance in daily lab tasks, and especially Uma and Jon, for their friendship and inspiration. Thank you to all of the undergraduates who helped with the daily maintenance of the lab, specifically Heidi Lindler who completed some of the initial amoebae experiments. Thanks to my fellow graduate student colleagues, Austin Wray, Brad Glenn, Aaron Edgington and Joe Bisesi, for their amity and guidance. I would like to acknowledge Clemson University, the Clemson University Research Fund, Creative Inquiry, and the Calhoun Honors College for funding and support. Finally, I am very grateful for my kind and loving family and boyfriend who have consistently supported me through their reassurance and motivation, helping me to succeed as a graduate student.
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INTRODUCTION

Nanotechnology overview and importance

The application of nanotechnology in consumer products and biological applications has continually increased over the last decade. Global funding spent on nanotechnology research and development averaged $10 billion for 2011, and the amount of money spent is expected to increase by 20% by the year 2015 (Figure 1) (8). In the US alone, across 15 federal departments and agencies, funding requests for 2012 are $2.13 billion (8). Environmental, health and safety research and development funding requests in 2012 is $124 million, 38% higher than actual funding in 2010 (49). The number of products containing nanomaterial on the market is estimated to be near 1,800 by 2015 (59). This innovative material has opened new avenues for research, industry and
commercial applications. Nanoparticles (NPs) are currently used most often in health and fitness including sporting goods products such as tennis rackets and bike frames, cosmetics, personal care, clothing and sunscreen (59). NPs themselves are not new. Natural NPs are widely known to be found ubiquitously throughout the environment and are formed through processes such as nanobiomineralization and mineral weathering (66). NPs have been identified in natural waters, acid mine drainage sites and wastewater treatment plants (66). But, this rise in nanotechnology funding will lead to increased NP use, which will result in an increased concentration of NPs concentration in natural environments. This will change the balance of ecosystems and alter the ability of organisms to deal with NPs. Currently manufacturing and release of engineered NPs is only somewhat controlled. This means that NPs have the potential to be released into the environment, where they are especially reactive due to unique properties such as their small size and increased surface to volume ratio. Engineered NPs are expected to behave dynamically in the environment, where they are transformed from their original pristine starting condition through aggregation, reaction with organic material, and interaction with various chemicals. These transformations are one key component that must be assessed to determine potential environmental impacts. Nanomaterial is difficult to characterize and the characteristics of NPs can sometimes change drastically. Transformation of NPs when they enter aquatic systems will affect NP interactions and their toxicity in the environment. NPs have a high risk of entering aquatic systems directly through surface and ground water, rainwater runoff, spills associated with transportation from production facilities, and intentional release for environmental
Nanoparticle interactions with aquatic organisms

To date, there have been numerous nanomaterial toxicity studies done on aquatic organisms. Most of the work focuses on *Daphnia* and selected fish species. In a study by Roberts *et. al.* (48), single-walled carbon nanotubes were ingested through normal feeding behavior and acute mortality was only found at concentrations greater than 5 mg/L. The particles were coated with lysophosphatidylcholine, which *Daphnia magna* was able to use as a food source, ultimately biomodifying the carbon nanotube and altering its properties in freshwater. In another study, when *Daphnia* were exposed to titanium dioxide (TiO$_2$) NPs in a 96 hour acute toxicity test, a concentration dependent effect on mortality was observed with both anatase and rutile forms of TiO$_2$ (2). In addition, smaller anatase NPs (21 nm) were more toxic than larger 250 nm NPs. In toxicity tests on zebrafish, bovine serum albumin-coated multi-walled carbon nanotubes caused immune responses in zebrafish embryos, but could be eliminated after 96 hours. However, a reduced survival rate was noted in the second generation of zebrafish, suggesting that these particles might be negatively disrupting reproduction (6). In adult male zebrafish, a 24 hour LC50 of approximately 250 mg/L silver nanoparticles (AgNPs) was noted, and at half of this concentration, AgNPs were found as agglomerates in liver tissue and apoptosis was observed in liver cells (7).
Studies examining the interaction of NPs with protozoa, which constitute a large part of aquatic ecosystems, are limited and have mostly focused on the model organism *Tetrahymena*. Mortimer *et al.* (41) examined the effects of zinc oxide (ZnO) and copper oxide (CuO) NPs compared to the corresponding metal salts on *Tetrahymena thermophila* and found that CuO NPs were 10 times more toxic than bulk CuO, although toxicity in both cases was attributed to the solubilized fraction (41). CuO NPs were found within the food vacuoles of the protozoa and attached to cellular debris on the outside of the cell (Figure 2). In contrast, ZnO NPs had the same toxic effect as bulk ZnO on the protozoa. Exposure to CuO NPs also resulted in a change in proportion of fatty acids and increase in reactive oxygen species (ROS) generation compared to controls (42). Ghafari *et al.* demonstrated that single walled carbon nanotubes are internalized by *T. thermophila*, leading to decreased mobility and aggregation of the protozoa (18). Furthermore, TiO$_2$ NPs were observed to damage the cell membrane of *Tetrahymena*. Control cells were ellipsoid in shape, covered with cilia, and had regular lengthwise wrinkles on the surface, typical of this type of cell. However, after exposure to TiO$_2$ NPs, the cells became more pear-shaped, shrunken, and twisted. Regular wrinkles were
damaged and small, shallow holes were seen on the surface of the cell. A decrease in cell membrane permeability was also observed, leading to a reduced ability to provide a selective barrier against extracellular material (45).

Lastly, bacterial biofilms in aquatic environments have an increased risk of being exposed to NPs, but very little research has been done examining the effects of NP exposure on biofilms. Most bacterial studies so far have focused on NP interactions with planktonic cells, and less emphasis has been placed on biofilm interaction with NPs. For instance, antimicrobial activity of Cu, Ag, and TiO$_2$ NPs on planktonic cells has been demonstrated, on *Escherichia coli*, *Bacillus* sp., and *Staphylococcus aureus* (43). Li et al. examined the effects of AgNPs on *E. coli* and *S. aureus*. For *E. coli*, concentrations of 1.25, 2.5, and 5 µg/ml caused a lag in the time to reach exponential phase by 12, 36, and 48 hours, respectively (32). *S. aureus* was more resistant to the AgNPs and showed lag times of 2, 12, 30 and 96 hours for concentrations of 1.25, 2.5, 5, and 10 µg/ml (31). Cerium oxide NPs have been found to have a size-dependent inhibition effect on the growth of planktonic *E. coli* and *B. subtilis* (44). Selenium NPs inhibited growth of *S. aureus* and decreased the percentage of live bacteria in culture compared to controls (60).

Of the few studies conducted that investigate the effects of NPs on biofilms, most are focused on the integration of NPs into surface material to prevent biofilm formation or contribute to general anti-microbial action. One study examined the interaction of AgNPs with *Pseudomonas putida* biofilms in the presence and absence of natural organic matter, and showed sloughing of biofilms in both instances (14). The AgNPs were found to be aggregated within the biofilm as seen in the TEM section in Figure 3. The same
group examined the interaction of AgNPs with marine biofilms as well (15). The biofilms were grown in situ for 3 days and then exposed to varying concentrations of AgNPs over 24 hours. A concentration-dependent reduction in volume to surface area of the biofilm was observed, as well as a concentration-dependent increase in AgNP uptake within the biofilm. Interestingly, the relative abundance of major taxonomic groups of bacteria remained the same, but the community structure was altered and normal biofilm development was reduced. Kalishwaralal analyzed formation of biofilms on Brain heart infusion agar, supplemented with Congo Red, with or without AgNPs (26). The production of exopolysaccharides (EPS), an indicator of biofilm formation, was inhibited at 50 nM AgNPs, demonstrating that the NPs impede biofilm formation. Complete inhibition of growth and EPS production was observed at 100 nM. The observed effects are likely due primarily to silver ions, which possess an antimicrobial effect. Dimkpa et al. studied the effects of AgNPs on the environmental bacterium, Pseudomonas chlororaphis O (11). These authors discovered that toxicity of AgNPs depended on the
amount of EPS present. Growth inhibition was observed when 1.5 mg/L AgNPs was added to EPS-normal and EPS-deficient cells. However, the addition of EPS during exposure to the same concentration of AgNPs reversed the growth inhibition that was observed. It should be noted that once AgNP concentrations reached 10 mg/L, the EPS did not continue to confer protection to the bacteria. But, the concentrations of AgNPs in the environment are never likely to reach 10 mg/L, so environmental biofilms producing a lot of EPS will likely be protected against these AgNPs. Lastly, Sheng and Liu demonstrated that wastewater biofilms containing cultivable heterotrophic bacteria are highly tolerant to AgNPs, as measured by cell viability per unit area (51). The authors speculate that the activity of these particles is likely due to silver ions rather than the AgNPs, but make no attempt to differentiate this in their system. Although the antimicrobial effects of AgNPs are likely due to silver ions, the effects of other more stable metallic NPs may be different. These studies provide some insight into the types of interactions that might be occurring when biofilms are exposed to various NPs.

Trophic transfer of nanoparticles in aquatic systems

Studies investigating the potential effect of NPs on trophic level interactions are of utmost importance in order to help us understand these interactions. A study by Lewinski et. al. showed that quantum dots (QDs) can be transferred from zooplankton to Danio rerio, zebrafish, through dietary exposure (30). The QDs did not bioaccumulate. Zhu et. al. determined that TiO₂ NPs were transferred to D. rerio through dietary exposure of Daphnia magna, but that bioaccumulation was not observed (73). In addition, no abnormality or mortality was observed in any of the experiments. Though
bioaccumulation was not observed in these studies, examination of trophic level interactions with multiple types of NPs is needed in order to predict what will happen if a large scale environmental NP contamination were to occur.

More specifically, interactions between bacteria and grazers are vital interactions to investigate, as bacteria play integral roles in aquatic ecosystems. There have been few, if any studies that have focused on the role of bacterial biofilms in this interaction, though some have studied planktonic bacteria and grazers. In a study examining the interaction of QDs with planktonic *E. coli* and *T. pyriformis*, it was found that the QDs associated with bacterial aggregates. Bacterial aggregates are not the desired prey of *T. pyriformis*, and they prefer to feed on individual *E. coli*. Therefore, the QDs were not transferred between the two species (24). However, Werlin *et al.* demonstrated bioaccumulation of QDs in *Pseudomonas aeruginosa* which were transferred to *T. thermophila* through protozoan feeding on the bacteria (65). This resulted in decreased protozoan motility, significantly slower growth of the protozoa, and inhibition of digestion of the bacteria, all of which could cause the QDs to remain bioavailable to the next higher trophic level.

Biofilms are found on many substrates in aquatic systems. They help sustain higher trophic level organisms (28), as they are common food source to numerous aquatic grazers such as protozoa and benthic invertebrates (4, 39). They are extremely stable, serve as the preferred niche for many bacteria in aquatic systems, and consist of multiple species. The importance of biofilms in ecosystems is widely known. Biofilms are rich in both prokaryotic and eukaryotic organisms that play important biogeochemical roles, including nutrient cycling in aquatic systems. More studies need to be focused on how
NPs might indirectly affect trophic level interactions through their interactions with biofilms. The fate and transfer of NPs in the environment is essential to characterize, as it will broaden our understanding and allow us to predict the toxic effects of these emerging contaminants in aquatic environments.

**Legionella in the environment**

*Legionella* is a bacterium found ubiquitously throughout freshwater systems and is endemic to lakes and rivers and other moist environments (58). It is also known for colonizing man-man environments such as cooling towers, whirlpools and plumbing systems, where it is a source of disease (50). Its preferred niche in both of these types of environments is the biofilm. In the environment, this bacterium commonly initiates attachment on top of already established biofilms. Biofilms are characterized by the production of EPS which provide a protective matrix for the bacteria. *Legionella* prefers warmer temperatures but can survive in the natural environment from temperatures ranging from 6°C-63°C and a pH of 5.5-9.2. However, it is only able to replicate between 25°C and 37°C and grows optimally between 32°C and 35°C (17). The ability of this organism to persist in many types of environments makes it a unique model organism to study. Additionally, the relationship between *Legionella* and amoebae plays a significant role in the persistence and dissemination of this pathogenic bacterium in the environment. *Legionella* uses amoebae as a host cell to replicate in the environment, and the interaction is well characterized (10). We utilized this model system to investigate relationships within microbial communities in the environment. The ability of *Legionella* to replicate within amoebae hosts increases virulence and resistance to antimicrobial agents, while
also concentrating, thereby increasing bacterial numbers (57). \textit{Legionella} use two systems in their relationship with amoebae. The dot/icm Type IV secretion system contains proteins that activate the secretion system in the \textit{Legionella} membrane for exportation of virulence factors. The other, a two-component sensing system known as Let A/S, controls a stringent response system that also regulates virulence factors. The bacteria have two distinct phases in the host-pathogen interaction, which can also be simulated in broth culture – the replicative phase (avirulent) and the transmissive phase (virulent). The transmissive phase is characterized by sodium-sensitivity, motility, cytotoxicity, osmotic resistance, and the ability to evade phagosome-lysosome fusion (57). When the bacteria express transmissive traits it enables them to infect amoebae and evade phagolysosome fusion and degradation by the host cell. They subsequently switch to the replicative phase to promote replication inside of amoebae, and then express transmissive traits to promote lysis of the host cell. Uptake of \textit{Legionella} by amoebae is thought to be a receptor-mediated event (62). This interaction is governed by the control of expression or access to ligands required for bacterial recognition by amoebae.

Previous work in our lab qualitatively characterized the interaction of gold nanoparticles with \textit{L. pneumophila} biofilms (54). A reduction in biofilm coverage was observed through fluorescence microscopy images after exposure to 0.7 µg/L 4 and 18 nm AuNPs, but not 50 nm AuNPs. In addition, exposure to 20 nm polystyrene beads did not cause a reduction in biofilm coverage (54). Therefore, we aimed to quantitatively characterize these interactions. Additionally, our goal was to quantitatively characterize the interaction of NPs with various core compositions, surface chemistries, and
concentrations with *L. pneumophila* biofilms and planktonic cells. The hypothesis is that NPs released into the environment will interact with organisms in biofilm communities, altering stability within the biofilm. Size, composition, and concentration of NPs are predicted to determine their interaction with microbial biofilms. Additionally, NP interaction with biofilm communities will alter interactions between *L. pneumophila* and amoebae. Therefore the effect of NPs on biofilms and the effect of NPs on the interaction of planktonic and biofilm phase *L. pneumophila* with amoebae is examined here.
MATERIALS AND METHODS

Organisms and media. *Legionella pneumophila* Philadelphia 1 (ATCC 33152) and *Legionella pneumophila* JR32 were cultured on buffered charcoal yeast extract agar (BCYE) at 37°C and sampled from the plate after 3 days incubation. ACES ((N-(2-Acetamido)-2-aminoethanesulfonic acid)-buffered yeast extract broth (AYE) was used to make bacterial suspensions (pH=6.9). *Acanthamoeba polyphaga* was grown independently in tissue culture flasks at 25°C in tryptic soy broth. Moderately hard water (MHW, hardness = 80mg/L CaCO$_3$, alkalinity = 60 mg/L CaCO$_3$, pH = 7.8-8.) (13) was used as the exposure medium for all biofilm exposures.

Nanoparticles. Citrate capped, gold NPs (AuNPs), sizes 4, 18, and 50 nm were synthesized with modifications using protocols adopted from Gole and Murphy (19). Citrate capped platinum NPs (PtNPs), sized at 4 nm, and citrate capped 8 nm silver NPs (AgNPs) were synthesized by Dr. Christopher L. Kitchen’s lab (Clemson University). Iron oxide NPs (Fe$_3$O$_4$ NPs) synthesized by Dr. Thompson Mefford’s lab (Clemson University) were 8 nm in size and coated with nitroDOPA-PEG2000 (polyethylene glycol, 2000 kDa). All NPs were synthesized in deionized (DI) water. Carboxylate-modified 24 nm polystyrene beads (PSB) were purchased from Bangs Laboratories, Inc. (Fishers, Indiana). The carboxylate modification provides a negative surface charge, which allows comparison to the negative surface charge of the citrate coated AuNPs and helps prevent aggregation of the PSBs. Table 1 provides concise information on NP composition, surface chemistry, size, and shape.
Nanoparticle characterization. NPs were initially characterized in the stock solutions using zeta potential, dynamic light scattering (DLS), and transmission electron microscopy (TEM). They were also characterized in MHW and AYE that were used as exposure media. For TEM characterization, particles were added in a 1:1 ratio to either MHW or AYE and 50 µL was immediately pipetted onto a Formvar/carbon coated copper grid (Electron Microscopy Sciences). For characterization of solutions with low stock concentrations, the 1:1 solution was centrifuged at 8,000 rpm for 20 minutes at room temperature (RT) to concentrate NPs, then immediately re-suspended in 50 µL of the respective media and pipetted onto the copper grid. For zeta potential and DLS characterization, particles were added to MHW at concentrations up to 6500 µg/L. All samples were immediately read in duplicate. DLS measures the hydrodynamic diameter of the particles in solution by analyzing the fluctuations in the intensity of scattered light from particles as they undergo Brownian motion. These intensity fluctuations are analyzed and the Stokes-Einstein relationship is used to determine the size of the NPs (34). Zeta potential is determined by using the electric double layer formed outside of a particle in a liquid suspension; it is calculated by measuring the velocity of the particles

Table 1. Nanoparticle core composition, surface chemistry, size and shape used in biofilm assays.

<table>
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<th>Core Composition</th>
<th>Surface Chemistry</th>
<th>Core Size (nm)</th>
<th>Shape</th>
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<tbody>
<tr>
<td>Gold (Au)</td>
<td>Citrate</td>
<td>4, 18, 50</td>
<td>Sphere</td>
</tr>
<tr>
<td>Platinum (Pt)</td>
<td>Citrate</td>
<td>4</td>
<td>Sphere</td>
</tr>
<tr>
<td>Silver (Ag)</td>
<td>Citrate</td>
<td>8</td>
<td>Sphere</td>
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<tr>
<td>Magnetite (Fe₂O₄)</td>
<td>Polyethylene glycol (PEG)</td>
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<td>Sphere</td>
</tr>
<tr>
<td>Polystyrene Beads (PSB)</td>
<td>Carboxylate</td>
<td>24</td>
<td>Sphere</td>
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</table>


as they move across a laser beam. Zeta potential and DLS could not be used to characterize particles in AYE due to the complexity of the bacterial media.

**Biofilm establishment.** *L. pneumophila* from a 3 day old BCYE plate was used to make a *L. pneumophila* suspension in AYE at an OD$_{600}$=0.6. Biofilms were established on glass slides in petri dishes or plastic chambers. Bacteria were added to dishes/chambers in a 10% AYE solutions for 24 hours to allow for initial establishment and attachment of the biofilm to the substrate at 26°C. After 24 hours the supernatant was removed, replaced with 100% AYE, and incubated for 4 additional days at 26°C to ensure a mature biofilm was formed. On day 5 AYE was removed and biofilms were washed twice with sterile ultrapure water (UPW) and the media replaced with MHW with or without NPs (control). Biofilms were exposed to all NPs listed in Table 1. NPs were added at concentrations of 0.7 (Au only), 1, or 100 µg/L (equivalent to 1.2x10$^7$ or 1.7x10$^9$ PSBs/ml). After 48 hours the supernatant was removed and biofilms were washed with UPW. Slides were removed, air dried, fixed in methanol for 10 minutes, then stained with 3 µM Syto 11 nucleic acid stain (Invitrogen) for 30 minutes. Slides were rinsed and coverslips were mounted using Vectashield or a 50/50 v/v solution of glycerol:phosphate buffered saline (PBS). Images of biofilm morphology were taken on a Nikon TiE Eclipse laser scanning confocal microscope (Jordan Hall Imaging Facility, Clemson University). At least 3 independent replicates were completed for each NP.

**Image Analysis.** COMSTAT (22), a biofilm analysis program that quantifies bio-volume, surface area, average and maximum thickness and roughness, was used to analyze 3-dimensional biofilm images. It uses the basic concept of setting thresholds to
determine pixels containing bacteria and background pixels. Quantitative analysis via COMSTAT estimates the biomass of the biofilm through bio-volume, which is determined by the number of biomass pixels in all images of a stack multiplied by the voxel size, divided by substratum area of the stack. The surface area is determined by analyzing any biomass pixels that are exposed to the background, which is all of the bacteria directly exposed to nutrients on the top of the biofilm. The average thickness provides a measure of the spatial thickness of the biofilm, taking into account any pores or voids in the biofilm. The maximum thickness of the biofilm is the thickness of the stack from bottom to top, ignoring any pores and voids that might be present inside the biofilm. The roughness coefficient of a biofilm can be calculated using the thickness distribution. This calculation provides a measure of how much the thickness of the biofilm varies and is an indicator of biofilm heterogeneity. For all 3D confocal images, bio-volume and roughness are presented.

For amoebae experiments where single plane images were used, images were taken in a two-dimensional plane of view at the surface of the biofilm and analyzed using the surface area analysis in COMSTAT (22). The data presented is a quantification of the surface area coverage of the biofilm.

**Colony forming units (CFUs) in biofilm supernatant.** Biofilms were grown as stated above with a slight variation. Biofilms were established in 4 well chambers (Lab-Tek) that required an initial attachment phase of 2 days rather than 1 day due to the difference in surface area. Medium was switched to 100% AYE on day 2 and allowed to incubate for 3 additional days. AuNPs or PtNPs were added on day 5 at 0.7 (Au), 1 (Pt), or 100
(Au and Pt) µg/L. On day 7, the supernatant plus two washes was collected and centrifuged at 4,000 rpm for 20 minutes at 23°C. The pellet was re-suspended in 500 µL of UPW and serial dilutions were plated on BCYE to determine the number of viable cells (CFUs/ml) being released from the biofilm after NP exposure. At least four independent replicates were completed for each NP.

**Biofilm viability assay.** Biofilms were grown as described above and exposed to PtNPs at concentrations of 1 and 100 µg/L. On day 7 after biofilms were washed, a cell scraper was used to scrape the biofilms into UPW, and slides were rinsed vigorously to remove all bacteria. All washes for each treatment were added to the same tube. Each tube was centrifuged at 4,000 rpm for 20 minutes at 23°C and the pellet re-suspended in 250 µL of UPW. Serial dilutions were plated on BCYE to determine the number of CFUs/ml in control vs. exposed biofilms. It is important to note that CFUs only determine the number of viable cells in the biofilm. Three independent replicates were completed.

**Planktonic assay.** Previous observations that demonstrate that AuNPs cause a reduction in biofilm bio-volume led us to examine the effects of NPs on planktonic *L. pneumophila* (55). Since similar bio-volume results were obtained with other NPs, planktonic assays were completed for each NP used in biofilm assays. *L. pneumophila* from a 3 day old BCYE plate was used to make a suspension in AYE at OD$_{600}$=0.05. NPs were added to each flask at concentrations of 1, 100, 1000 or 10,000 µg/L, which varied depending on NP stock concentration. Cultures were grown for 78 hours at 37°C, 150 rpm, and samples were taken at 0, 18, 30, 48, 66, and 78 hours. At each time point 1 ml was removed from each sample, centrifuged at 8,000 rpm for 5 minutes at 23°C, and the supernatant was
removed and read at an OD of 400 nm to determine pigment production. Pigment production is related to the virulence of the bacteria, as the ability of *Legionella* to reduce iron comes from homogentisic acid and is needed to successfully infect a host cell (5, 53). The pellet was then resuspended in PBS and biomass was determined by measuring the OD at 600 nm. Cell viability (CFUs/ml) was also determined by serial dilution plating. At least two independent replicates were completed for each NP.

**Amoebae-Legionella interaction.**

**Planktonic interaction assay.** Infection assays using planktonic *Legionella* were conducted as previously described (38). 4 and 18 nm AuNPs were chosen for this assay, as these AuNPs had an effect on *L. pneumophila* biofilm morphology. 24 nm PSBs were used as a control as these had no effect on biofilm morphology in previous experiments. *A. polyphaga* were seeded into 6 well plates at 10⁶/well and *Legionella* added at a multiplicity of infection (MOI) = 100 either alone, simultaneously with NPs or after incubating bacteria overnight with NPs. Overnight incubation results in adsorption and uptake of AuNPs by the bacteria (55). The 6 well plates were centrifuged at 500 rpm for 10 minutes at 23°C to force the bacteria to settle to the bottom of the plate. After 2 hours, amoebae were washed four times with PBS to remove remaining extracellular bacteria. For the 2 hour time point, amoebae were immediately collected, lysed, and the lysate was plated for CFU determination. A second set of plates was incubated for 48 hours to determine bacterial replication. Treatments included: 1) amoebae only; 2) NPs only; 3) *L. pneumophila* only; 4) *L. pneumophila* + simultaneous addition of NPs; and 5) *L.
_pneumophila_ + NPs after an overnight incubation. Four independent replicates were completed for both uptake and replication for each NP.

**Biofilm interaction assay.** For biofilm assays, biofilms were established as described above. On day 5, NPs (4 and 18 nm Au) were added in MHW to biofilms at a concentration of 0.7 µg/L (or the equivalent number of PSBs/ml (1.2 x 10^7 PSB/mL). On day 7, biofilms were washed twice with sterile UPW to remove non-adherent bacteria and any NPs not integrated within the biofilm. _A. polyphaga_ were added to the biofilm at a concentration of 10^6/dish in MHW. Biofilm samples included: 1) _L. pneumophila_ only, 2) _L. pneumophila_ + _A. polyphaga_, 3) _L. pneumophila_ / NP exposed, and 4) _L. pneumophila_ / NP exposed + _A. polyphaga_. Biofilms were incubated for an additional 48 hours at 26°C. On day 9, amoebae were collected from each biofilm by first collecting biofilm supernatant. Biofilm were then washed twice with sterile UPW to remove loosely attached amoebae and wash solutions were added to the original supernatant. Finally, to release any remaining amoebae from the biofilm, fresh MHW was added to the biofilms which were then placed on ice for 20 minutes. The supernatant was removed and added to the original supernatant. The supernatant containing all the amoebae was centrifuged at 4,000 rpm for 12 minutes at 23°C and the pellet resuspended in 1 ml MHW. Amoebae survival was analyzed using a trypan blue assay. This assay uses the trypan blue dye to determine whether amoebae are alive or dead. Live, metabolically active amoebae are able to expel the dye and appear transparent. Dead amoebae are metabolically inactive and unable to remove the dye so they remain blue, making it easy to differentiate between live and dead organisms. Surface area was determined by removing the glass slides with
the remaining biofilms and fixing them in methanol for 10 minutes. The slides were then stained with 0.1% crystal violet, rinsed and imaged at 60x magnification using a Nikon TE2000 microscope and analyzed via COMSTAT to estimate the amount of surface area covered by the biofilm. Six independent replicates were completed for each NP.

**Statistical Analysis.** For all biofilm assays (*Legionella* only and amoebae-*Legionella* interactions), a one-way analysis of variance (ANOVA) was used to compare cell bio-volume surface area, roughness, or number (log) of amoebae. A one-way ANOVA was also used to compare supernatant CFU’s, biofilm viability, and all NP treatments for each time point in *Legionella* planktonic assays. When significant differences were found among treatments, Fisher’s Least Significant Difference (LSD) was used to determine which treatments significantly differed from the others. A significance level of $p<0.05$ was used for all tests, and differences were marked on graphs with an asterisk. For planktonic phase amoebae-*Legionella* interaction assays, a one-way ANOVA was used to compare the three *Legionella* treatments.
RESULTS

NP characterization by transmission electron microscopy, zeta potential, and dynamic light scattering shows size and stability of NPs in stock solutions and exposure media

TEM analysis demonstrated that all AuNP, PtNP, AgNP, Fe$_3$O$_4$ NP, and PSB stock solutions (DI water) contained NPs that were uniform in size and well-dispersed (Figure 1). When the particles were placed in MHW or AYE exposure media and imaged on the TEM, single NPs and clusters of NPs were seen for all NP types, however the clusters could be due to the drying process required before imaging on the TEM. DLS was therefore used to further confirm the size determined by TEM. It is expected that the size of the particles using DLS will be slightly larger than the size determined by TEM, since the total particle size will include any surface coating as well as hydration layer, whereas TEM only measures the core size. DLS of 4, 18 and 50 nm AuNPs confirmed their respective sizes (Table 2), with average hydrodynamic diameters of 10.69 nm, 20.09 nm, and 26.13 nm, respectively.
nm, and 59.89 nm respectively. Single volume peaks were at 7.05 nm, 17.84 nm, and 49.24 nm. DLS of the 4 nm PtNPs reported an average size of 11.68 nm with a single volume peak at 8.98 nm (Table 2). AgNPs demonstrated an average hydrodynamic diameter of 17.93 nm, with a single volume peak at 5 nm. The average size takes into account any NP clusters/aggregates or dust particles that may be present in the solution. Larger dust particles reaching sizes over 1000 nm can increase the true size of the particles; therefore sometimes the volume peaks are a more reliable measurement than the average diameter. Fe₃O₄ NPs, planned to have an 8 nm core, demonstrated an average size of 73.11 nm with a single peak on DLS. This increase in size was expected due to the large PEG polymer used to coat the surface of the NP. Lastly, 24 nm PSBs had an average hydrodynamic diameter of 26.62 nm which is expected due to the carboxylate coating on the surface. These particles showed a single volume peak at 22.36 nm.

Table 2. Dynamic light scattering results of nanoparticle stock solutions.

<table>
<thead>
<tr>
<th>Nanoparticle Core Size (nm)</th>
<th>Approximate size of surface coating (nm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>% intensity peak</th>
<th>% volume peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 nm AuNP</td>
<td>2 nm</td>
<td>10.69</td>
<td>11.68</td>
<td>7.05</td>
</tr>
<tr>
<td>18 nm AuNP</td>
<td>2 nm</td>
<td>20.09</td>
<td>21.65</td>
<td>17.84</td>
</tr>
<tr>
<td>50 nm AuNP</td>
<td>2 nm</td>
<td>59.89</td>
<td>67.67</td>
<td>49.24</td>
</tr>
<tr>
<td>4 nm PtNP</td>
<td>2 nm</td>
<td>11.68</td>
<td>12.77</td>
<td>8.98</td>
</tr>
<tr>
<td>8 nm AgNP</td>
<td>2 nm</td>
<td>17.13</td>
<td>51.26</td>
<td>5.0</td>
</tr>
<tr>
<td>8 nm Fe₃O₄ NP</td>
<td>40 nm</td>
<td>73.11</td>
<td>86.89</td>
<td>56.91</td>
</tr>
<tr>
<td>24 nm PSB</td>
<td>2 nm</td>
<td>26.62</td>
<td>29.67</td>
<td>22.36</td>
</tr>
</tbody>
</table>

Zeta potential (ZetaSizer Nano ZS, Malvern Instruments Ltd) was measured to determine the stability of the particles and to characterize the surface charge on the particle. A suspension of NPs is generally considered stable if the value is below -30 mV.
or above $+30 \text{ mV}$ (35). However, factors such as pH and conductivity can affect the zeta potential (35). The zeta potential was measured in the stock solution and in MHW for all nanoparticles. All of the AuNP and PtNP stock solutions were stable as demonstrated by their zeta potential (Table 3). When AuNPs were added to MHW their zeta potential shifted closer to zero (-17 mV to -20 mV), indicating that they are only fairly stable in MHW. This change is mostly likely due to the various ions in MHW, which causes electrostatic screening and changes the interactions between charges. However, the 4 nm PtNP zeta potential remained at -29.8 mV, suggesting that these NPs are still moderately stable when initially added to MHW. AgNPs had an initial zeta potential of -64.5 mV, indicating stability. When added into MHW the zeta potential increased to -9.7 mV, suggesting aggregation in the ionic MHW exposure medium. The Fe$_3$O$_4$ NPs are stabilized by a neutral PEG coating which provides steric stabilization in contrast to ionic stabilization. Therefore the zeta potential is expected to be close to 0 because there is no charge on the particle. The Fe$_3$O$_4$ NPs had a zeta potential of -15.4 mV which shifted to -5.37 mV after addition to MHW. PSBs had a zeta potential of -41.2 mV, indicating that

<table>
<thead>
<tr>
<th>Nanoparticle Core Size (nm)</th>
<th>Zeta potential in stock solution (mV)</th>
<th>Zeta potential in MHW (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 nm AuNP</td>
<td>-41.3</td>
<td>-19.6</td>
</tr>
<tr>
<td>18 nm AuNP</td>
<td>-38.3</td>
<td>-17.8</td>
</tr>
<tr>
<td>50 nm AuNP</td>
<td>-40</td>
<td>-17.2</td>
</tr>
<tr>
<td>4 nm PtNP</td>
<td>-57.5</td>
<td>-29.8</td>
</tr>
<tr>
<td>8 nm AgNP</td>
<td>-64.5</td>
<td>-9.7</td>
</tr>
<tr>
<td>8 nm Fe$_3$O$_4$ NP</td>
<td>-15.4</td>
<td>-5.37</td>
</tr>
<tr>
<td>24 nm PSB</td>
<td>-41.2</td>
<td>-14.8</td>
</tr>
</tbody>
</table>
the stock solution is very stable. In MHW, zeta potential of the beads in MHW was also closer to zero (-14.8 mV), which could indicate instability when exposed to this medium. This is expected since PSBs are stabilized by a negatively charged carboxylate surface coating. Measurement of zeta potential in bacterial media was conducted with AuNPs, however it was inconclusive, as AYE is a complex medium that contains numerous proteins. Zeta potential of AYE was measured without AuNPs using the same standard operation procedure as if the particles were present. Results showed a zeta potential of -13 mV. When AuNPs were introduced into the media, the zeta potential ranged from -15 mV to -17 mV. This is not uncommon, as this phenomenon has been observed before (1). Proteins can have various surface charges, and even predominately negatively charged proteins have been found to bind strongly to negatively charged NPs (52). Therefore negatively charged proteins bind to the NPs resulting in a negative surface charge, which produces a zeta potential similar to the value of the media alone without NPs. The zeta potential of the proteins, not the NPs, is most likely being measured in this case.
Bio-volume and roughness of Legionella biofilms after exposure to NPs shows changes in biofilm morphology in a size, concentration, and core chemistry dependent manner

Previous results showed qualitatively that biofilm bio-volume was decreased after exposure to 4 and 18 nm citrate capped AuNPs, but not 50 nm AuNPs (55). In order to quantify this analysis, the program COMSTAT (22) was used to quantify biofilm parameters. In concert with qualitative findings, quantitative analysis demonstrated a significant reduction in biofilm bio-volume (Figure 2a), as well as a significant increase in roughness after exposure to 0.7 µg/L 4 and 18 nm AuNPs (Figure 2b). This was not observed with 50 nm AuNPs (Figure 2a-b) or higher concentrations of 18 nm AuNPs (Figure 2c-d). Fluorescent confocal images show representative images of control and NP exposed biofilms (Figure 2e-j).
Figure 2. COMSTAT analysis of biofilms after exposure to 0.7 µg/L 4 and 18 nm AuNPs shows a decrease in biofilm bio-volume (a) and an increase in roughness coefficient (b). This was not observed with larger 50 nm AuNPs (a, b) or 100 µg/L 18 nm AuNPs (c,d). Fluorescent confocal images show control biofilm (e) and loss of biofilm after exposure to 0.7 µg/L 4 and 18 nm AuNPs (f, g) but not 100 µg/L 18 nm AuNPs (h) or larger 50 nm AuNPs (i).
In addition to gold, low concentrations (1 µg/L) of 4 nm citrate capped PtNPs also showed decreased biofilm coverage (Figure 3d). When analyzed using COMSTAT, a significant decrease in biofilm bio-volume was observed (Figure 3a). Roughness coefficients of 1 µg/L PtNP exposed biofilms were significantly higher than controls (Figure 3b). No effect on bio-volume or roughness was observed when biofilms were exposed to 100 µg/L PtNPs (Figure 3a-b, 3e). To determine whether these effects were due to core chemistry or the citrate coating, citrate was tested as an additional control. There was no difference in bio-volume or roughness coefficient observed after citrate addition to biofilms (data not shown), suggesting that the effect is due to the core chemistry of the particles and not the surface coating. Interestingly, when 8 nm citrate

Figure 3. COMSTAT analysis of biofilms after exposure to 1 µg/L 4 nm PtNPs shows a decrease in biofilm bio-volume (a) and an increase in roughness coefficient (b). Fluorescent confocal images show control biofilm (c), and loss of biofilm after exposure to 1 µg/L 4 nm PtNPs (d) but not 100 µg/L 4 nm PtNPs (e).
capped AgNPs were analyzed for effect on the biofilm, they exhibited no significant effect on bio-volume or roughness of *L. pneumophila* biofilms (Figure 4a-b, d-e). Studies have shown that the antimicrobial effect of AgNPs is due primarily to Ag+ ions, not to AgNPs (33). But, *Legionella* is also known to be fairly resistant to Ag+ ions in the biofilm form (61), so it was not unexpected that the particles produced no effect.

![Figure 4](image_url)

**Figure 4.** COMSTAT analysis of biofilms after exposure to 1 and 100 µg/L 8 nm AgNPs shows no statistical difference in bio-volume (a) or roughness coefficient (b) compared to controls. Fluorescent confocal images show control biofilm (c), and biofilms after exposure to 1 µg/L 8 nm AgNPs (d) and 100 µg/L 8 nm AgNPs (e).
Surprisingly, Fe$_3$O$_4$ NPs demonstrated a significant reduction in biofilm bio-volume and increase in roughness coefficient at both 1 and 100 µg/L (Figure 5a-b, d-e). The effect of 24 nm PSBs was also examined at concentrations equivalent to 0.7 µg/L 18 nm AuNPs (due to similarities in size). No significant difference in bio-volume or roughness coefficient was observed compared to controls (Figure 6a-b, c-d).

Figure 5. COMSTAT analysis of biofilms after exposure to 1 and 100 µg/L 8 nm Fe$_3$O$_4$ NPs shows a decrease in biofilm bio-volume (a) and an increase in roughness coefficient (b). Fluorescent confocal images show control biofilm (c), and loss of biofilm after exposure to 1 µg/L 8 nm Fe$_3$O$_4$ NPs (d) and 100 µg/L 8 nm Fe$_3$O$_4$ NPs (e).
Figure 6. COMSTAT analysis of biofilms after exposure to $1.2 \times 10^7$ polystyrene beads/ml (equivalent of 100 µg/L 18nm AuNPs) shows no statistical difference in biofilm bio-volume (a) or roughness coefficients (b) compared to controls. Fluorescent confocal images show control biofilm (c) and biofilm after exposure to $1.2 \times 10^7$ PSBs/ml (d).
Viability of cells released from biofilm surface is similar between controls and treatments

The observed decrease in bio-volume after exposure to Au and Pt NPs led to the hypothesis that cells were being released from the biofilm. Consequently, the supernatant was collected to determine the number of viable cells being released from the biofilm after NP exposure. Data obtained on biofilms exposed to 4 nm, 18 nm, and 50 nm AuNPs compared to the control demonstrates a linear trend of cells being released from the biofilm as size decreases (Figure 7a). However, this trend is not significant, suggesting that similar numbers of viable cells are released from the biofilm after NP exposure. Treatment with 1 µg/L 4 nm PtNPs demonstrated that there was a linear trend of viable cells being released from the biofilm as NP concentration decreases (Figure 7b). But, no significant difference was observed between treatments and control. The control in both of these graphs is the baseline for the normal dispersal event that occurs in biofilms. This method only quantifies the number of viable cells released from the biofilm, and there are most likely dead or non-viable cells being released as well.
Figure 7. Colony forming units on biofilm supernatant after exposure to Au and Pt NPs. With a decrease in AuNP size, an increase in the number of viable cells released from the biofilm is seen (a) and with a decrease in PtNP concentration, an increase in number of viable cells being released off of the biofilm is observed (b).
Viability of biofilm cells after exposure to PtNPs is similar between controls and exposures

To determine whether cell viability in the biofilm was being affected by NP exposure, CFU counts were conducted after exposure to 1 and 100 µg/L 4 nm PtNPs. Experimental data demonstrates that there was no significant difference in cell viability between controls or NP exposed biofilms (Figure 8). This result suggests that the NPs are not affecting the viability of the cells in the biofilm form, but instead destabilizing the biofilm matrix.

Figure 8. Viability of biofilm cells after exposure to 1 and 100 µg/L 4 nm PtNPs show similar viability between treatments and controls.
NP exposure to planktonic L. pneumophila cells shows no effect on biomass, pigment production, or cell viability

Stojak et al. (55) previously showed that when 4, 18 and 50 nm AuNPs were added at t=0 hr, no effect on biomass, pigment production, or cell viability was observed. At concentrations ranging from 1 to 10,000 µg/L, PtNPs also had no significant effect on biomass, pigment production, or cell viability (Figure 9a-c). No effect of AgNPs or Fe₃O₄ NPs on any of the measured endpoints was observed at 1, 100, or 1000 µg/L (Figure 10a-c and 11a-c). PSBs were tested at 1.7 x 10⁹ beads/ml, an equivalent number of beads/ml to 100 µg/L 18nm AuNPs, and no effect on biomass, pigment production, or cell viability was seen (Figure 12a-c).
Figure 9. PtNP interaction with planktonic *L. pneumophila*. Cells were exposed to 1, 100, and 10,000 µg/L 4 nm PtNPs for 78 hours show no significant difference in biomass (A), pigment production (B), or cell viability (C).
Figure 10. AgNP interaction with planktonic *L. pneumophila*. Cells were exposed to 1, 100, and 1,000 µg/L 8 nm AgNPs for 78 hours show no significant difference in biomass (A), pigment production (B), or cell viability (C).
Figure 11. Iron oxide NP interaction with planktonic *L. pneumophila*. Cells were exposed to 1, 100, and 1,000 µg/L 8 nm Fe$_3$O$_4$ NPs for 78 hours show no significant difference in biomass (A), pigment production (B), or cell viability (C).
Figure 12. Polystyrene bead interaction with planktonic *L. pneumophila*. Cells were exposed to $1.7 \times 10^9$ PSBs/ml (equivalent of 100 µg/L 18 nm AuNPs) for 78 hours show no significant difference in biomass (A), pigment production (B), or cell viability (C).
18nm AuNPs elicit similar effects on *L. pneumophila* Philadelphia 1 and *L. pneumophila* JR32 strains

*L. pneumophila* Philadelphia 1 and JR32 wild type biofilms were compared before and after exposure to 1 µg/L 18nm AuNPs to examine whether similar effects on biofilm morphology were observed. As previously stated in Stojak et. al. (55), when *L. pneumophila* Philadelphia 1 biofilms were exposed to a concentration of 0.7 µg/L 18 nm AuNPs, a significant reduction in biofilm bio-volume and significant increase in roughness coefficient were observed. The same result was obtained when biofilms were exposed to 1 µg/L 18 nm AuNPs (Figure 13a). In comparing the two wild type strains, *L. pneumophila* Philadelphia 1 formed control biofilms with 46.7% more bio-volume than the JR32 strain (Figure 13a). Although the JR32 control contained less bio-volume than the Philadelphia 1 control, the 18 nm AuNP exposed biofilms exhibited loss of bio-volume in both strains. The change in bio-volume after NP exposure in the JR32 strain was not however, statistically significant. The higher roughness coefficient of the control biofilms with the JR32 wild type strain suggests that the biofilm is more unstable to begin with, and that the NPs are less effective because of the difference in the initial biofilm structure.
NPs alter interaction between amoebae and L. pneumophila biofilms, but not between amoebae and planktonic L. pneumophila

Previous assays in our lab demonstrated that AuNPs and PSBs do not affect amoebae viability after a 48 hour exposure. Therefore, infection assays with A. polyphaga and L. pneumophila were conducted to determine the effect of AuNP exposure of bacteria on uptake and replication of bacteria within a host cell. Assays were conducted using both planktonic and biofilm bacteria.

Stationary phase, planktonic L. pneumophila was added to amoebae cultures either alone, simultaneously with NPs or after being incubated overnight with NPs. Overnight incubation results in adsorption and uptake of AuNPs by the bacteria (55). There were no significant differences in the uptake or replication ability of the bacteria
within amoebae after association with NPs (Figure 14a-b). In all samples, bacteria were taken up equally well by amoebae regardless of NP exposure. Bacterial numbers increased from an initial uptake of $10^5$ bacteria/ml to $10^6$-$10^7$ bacteria/ml after the 48 hour incubation.

In biofilm interaction assays, grazing capability of the amoebae and amoebae survival were measured to determine if NP induced morphology changes (55) in the biofilms could alter host-pathogen interaction. COMSTAT measurements were used to determine the surface area of each biofilm after incubation with amoebae. A reduction in surface area would signify grazing by amoebae. As expected, in control biofilms,
amoebae were able to graze the *L. pneumophila* biofilms significantly reducing the surface area by 62.1% compared to the non-grazed control biofilms (Figure 15a-c). However, there was no significant difference in the surface area of 18 or 4 nm AuNP exposed biofilms incubated with amoebae and the respective AuNP exposed biofilms without amoebae (Figure 15a and b, 12.4% and 20.3% reductions, respectively) demonstrating decreased grazing on the biofilm by the amoebae. Similar to controls, there was a significant decrease in biofilm surface area of PSB exposed biofilms incubated with amoebae compared to the corresponding PSB exposed biofilms without amoebae (Figure 15c).

With the decrease in grazing ability observed after amoebae interaction with 4 and 18 nm AuNP exposed biofilms, we wanted to determine if this alteration was due to changes in the virulence ability of the bacteria due to NP exposure. Amoebae survival is indicative of the infectious ability of the bacteria, in that if bacteria are taken up by the amoebae, this normally results in the lysis of the amoebae, thereby decreasing amoebae numbers. In control biofilms, amoebae numbers decreased 79.7% from the initial number added. However, amoebae numbers decreased only 11.2% after interaction with 18 nm AuNP exposed biofilms (Figure 16). Biofilms exposed to 4 nm AuNPs and 24 nm PSBs showed numbers of amoebae after interaction that were not significantly different from controls (Figure 16), suggesting that the amoebae that did take up bacteria resulted in normal host-pathogen processes. These results imply that core chemistry and NP size play a role in how NPs alter the host-pathogen interaction.
Figure 15. Amoebae interaction with *L. pneumophila* biofilms – grazing analysis. *L. pneumophila* biofilm surface area after *A. polyphaga* grazing on biofilms that were previously exposed to a) 18 nm AuNPs, b) 4 nm AuNPs, or c) PSBs. Asterisks indicate significant differences from respective controls (treatment without *A. polyphaga*). Apoly = *A. polyphaga*, AuNP = gold nanoparticle, PSB = polystyrene bead
Figure 16. Amoebae interaction with *L. pneumophila* biofilms – amoebae survival. *A. polyphaga* survival after incubation on control or NP exposed *Legionella* biofilms. Asterisk indicates significant difference from control. AuNP = gold nanoparticle, PSB = polystyrene bead
**DISCUSSION**

The demand for detailed studies on NP interactions in aquatic systems is increasing exponentially because of the unknown environmental risks created by the increase in products and applications involving nanomaterial. Biofilms play integral roles in aquatic ecosystems and alteration of normal functions at this level can be detrimental to whole ecosystems. At the base of food chains, NP contamination may alter trophic level interactions through direct and indirect interactions leading to community composition changes. Therefore, it is of utmost importance to understand these interactions in order to predict ecosystem effects affecting health of aquatic organisms.

Advances in the field of nanotechnology have provided us with the tools to determine the size and stability of NPs. Characterization of stock solutions is relatively simple, though detection limits bind our ability to characterize NPs exactly as they are used in experiments. The NPs used in these studies were well dispersed and stable in their stock solutions, as demonstrated by TEM, DLS, and zeta potential data (Figure 1, Tables 2 and 3). However, understanding how the media used in biological systems affects the stability is more difficult. MHW, the exposure media for all biofilm experiments, contains various salts, which changes the ionic charge and the electrostatic forces of the particles in the solution through interaction with the particles. In high ionic strength media, the energy barrier becomes lower and NP aggregation occurs, causing the NPs to be less stable in solution and the zeta potential to be closer to zero (27). This interaction causes van der Waals forces to become more dominant (35). When NPs are stabilized by citrate, a surface coating that relies on ionic interactions, this increase in zeta potential
occurs with all core chemistries (Au, Pt, and Ag). However, the PEG surface chemistry increases the stability of NPs. The PEG coating on the Fe$_3$O$_4$ NPs provides steric stabilization of the NPs, rather than ionic stabilization. Therefore, although the initial zeta potential (-15.4 mV) is close to zero, it does not mean that the particles are unstable. This result is expected because the PEG is a neutral sterically stabilizing surface coating.

When the NPs are added to MHW, the zeta potential shifts even closer to 0; however, this is not a drastic change. DLS of these NPs in both UPW and MHW demonstrated that the average hydrodynamic diameter of these PEG coated Fe$_3$O$_4$ NPs remained the same in both types of media. These data provide further evidence that these particles are sterically stabilized and not affected by an increase in ionic strength like the citrate coated NPs. The PEG surface coating is designed to ensure the NPs remain sterically stabilized in aqueous media (69), so it is not unexpected that the particles do not aggregate when placed into MHW. The use of DLS and zeta potential instruments provides insight into how interparticle interactions are affected by various types of exposure media.

Nevertheless, the NP concentrations used in all experiments were much lower than those used for characterization methods, so it is possible that aggregation in experiments does not occur as quickly as in the characterized solution.

Quantitative analysis of the morphology changes observed by Stojak et. al. (55) using COMSTAT (22) showed that biofilm bio-volume was significantly reduced when *L. pneumophila* biofilms were exposed to 0.7 µg/L 4 and 18 nm citrate capped AuNPs, likely due to NP mediated destabilization of biofilm layers. This effect was not observed at 100 µg/L 18 nm AuNPs or with any concentration of 50 nm AuNPs. The hypothesis is
that 50 nm AuNPs are too large to integrate within the biofilm matrix, and that more aggregation is observed with the smaller particles at the 100 µg/L concentration causing these NPs to increase in overall size, reducing their ability to interact with the biofilm. Peulen et al. examined the diffusion coefficients of carboxylate coated polymer microspheres (sizes 57, 92, and 135 nm) and silver NPs (approximately 2-10 nm) on Pseudomonas fluorescens biofilms. The authors found that the diffusion coefficient generally decreased with an increase in NP size (46). Nano Ag diffused readily throughout loosely packed biofilms, while the larger polymer microspheres diffused at a slower rate. The diffusion coefficient of these larger microspheres decreased as size increased, though this trend was only observed for particles less than 100 nm (46). A similar size dependent diffusion may be occurring in our system when a higher concentration of NPs is used. MHW likely causes the NPs to aggregate more readily and reach a larger size than the NPs would at a low concentration. Therefore it is thought that this prevents them from diffusing into and integrating within the biofilm matrix. Similar to our previous research with AuNPs, a reduction in biofilm bio-volume after treatment with 4 nm PtNPs (citrate capped) was observed. The similarities in response to Au and Pt NPs suggest a similar mechanism involved in response to metallic NPs. This similarity is significant, as it implies that other stable metallic NPs may act the same way, due to their small size and high surface reactivity. This citrate surface coating is an ionic stabilization, leaving parts of the metallic surface exposed, demonstrating that both core chemistry and surface chemistry may play a role in this interaction. Interestingly, bio-volume of biofilms exposed to 8 nm citrate capped AgNPs was not significantly reduced. Most of
the work done with AgNPs has shown that the NPs exhibit an antimicrobial activity that is primarily due to silver ions (16, 40). The data here then suggest that *L. pneumophila* biofilms are fairly resistant to effects from AgNPs and that core composition likely plays a large role in biofilm destabilization. The AgNPs also have a zeta potential in MHW that is closer to zero when compared to Au and Pt NPs. This makes it probable that the AgNPs may aggregate more readily than Au and Pt NPs and form complexes too large to integrate into the biofilm matrix. *L. pneumophila* have been shown to be more resistant to the effects of silver ions than many other bacterial species (25).

Fe$_3$O$_4$ NPs caused a reduction in biofilm bio-volume at both low and high concentrations. These particles are very stable in MHW and retain the properties of their original stock solutions. The NPs do not aggregate but instead are likely individually interacting with the biofilm matrix. The results from these experiments further support the hypothesis that surface chemistry plays an important role in the stability of NPs in the exposure media, and therefore the state they are in when they interact with the biofilm. With the PEG surface coating these NPs have an average hydrodynamic diameter of 73.11 nm. Although this is fairly large and we did not see a loss in biofilm bio-volume when larger citrate capped 50 nm AuNPs were used (55), the citrate capped NPs are likely aggregating into much larger NPs, while the iron oxide NPs are not. The iron oxide NPs have a greater chance of interacting with the biofilm matrix since they remain as individual NPs in solution and not NP aggregates.

When NPs come in contact with biofilms, the first line of interaction will be the biofilm EPS, not the bacterial surface. Duncan *et. al.* identified an Lcl binding protein in
*L. pneumophila* species that is involved in biofilm formation (12). This protein is excreted as well as being found in the outer membrane (12). *L. pneumophila* Lp02 Δlpg2644 (Lcl) mutants were deficient in biofilm formation and controls formed significantly thicker biofilms than the mutant strain. Complemented mutants showed biofilm formation similar to controls. The authors suggested that the Lcl binding protein is likely important in biofilm adhesion, and that it might also be involved in cell-cell communication within the biofilm (12). As the EPS of *Legionella* has not been characterized, it makes it difficult to hypothesize specific interactions. But, it is known that EPS generally consists of polysaccharides, proteins, and DNA which can all interact with NPs (23, 56). In our biofilms exposed to 0.7 µg/L 4 and 18 nm AuNPs and 1 µg/L 4 nm PtNPs, we seen an increase in roughness coefficient. NPs may interact with EPS and bind to adhesion proteins like Lcl, and induce structural changes in the biofilm that lead to reduced adhesion and dispersal events. The authors also examined the charge interactions between Lcl binding to negatively charged heparin, a sulfated glycosaminoglycan, found as part of the host extracellular matrix. The Lcl protein also has a negative charge through a hydrophilic repeat region exposed on the surface of the protein, therefore it was suggested that electrostatic interactions play a minor role in the binding of Lcl to negatively charged heparin (12). The changes in biofilm morphology observed are thought to occur due to NPs interacting with the EPS, which in many cases has an overall negative charge due to its components. Consequently, the Au and Pt NPs used in our studies are coated with negatively charged citrate. The data provided in Duncan *et al.* suggests that negatively charged molecules can interact with other
negatively charged molecules. This provides support for our citrate coated NPs interacting with negatively charged components of the EPS. However, data has also shown that AuNPs interact with polysaccharides and plasmid DNA (70), suggesting that citrate capped NPs have the potential to bind to these molecules as well.

The reduction in bio-volume and increase in roughness coefficient in *L. pneumophila* biofilms implies that cells are likely being dispersed from the biofilm. Loss of bio-volume led us to examine whether the cells being dispersed from the biofilm were viable or not. Viability of cells released from biofilms exposed to AuNPs and PtNPs was determined by supernatant collection of cells after exposure. Although not significant, the highest number of viable cells was dispersed from the biofilm after exposure to smaller AuNPs and lower concentrations of similar sized PtNPs. This data supports the reduction in bio-volume observed, and demonstrates that at least some of the dispersed cells are still viable. However, more work needs to be done to determine whether these cells are capable of colonizing new surfaces or infecting host cells.

Natural and anthropogenic sources may alter cell viability within biofilms. The EPS of biofilms is known to protect the cells within the biofilm (20). Sheng *et. al.* used original wastewater biofilms and wastewater biofilms with loosely associated EPS removed to examine the effects of 200 mg/L AgNPs on viability (51). Biofilms without loosely associated EPS had similar biofilm viability compared to original controls. When exposed to 200 mg/L AgNPs, no effect on the amount of viable cultivable heterotrophic bacteria was observed in the original control biofilms. But, when wastewater biofilms without loosely associated EPS were exposed to 200 mg/L AgNPs, significantly reduced
viability was observed. In addition, the AgNPs were more toxic to fast-growing bacteria than slow-growing bacteria, as an initial 1.6 log reduction is observed in the first 4 hours of exposure. The total number of viable cells recovers over 24 hours resulting in a total log reduction that is less than 1. It should be noted that the authors also saw that 200 mg/L Ag+ was more toxic than the AgNPs, suggesting that the release of Ag ions might play an important role in the toxicity observed. It is likely that the EPS acts as a ligand to bind any silver ions that are being released from the AgNPs, which would reduce their toxicity to biofilm cells. To determine whether the cells in biofilms exposed to 4 nm PtNPs were still viable, biofilms were collected after exposure and cell viability was examined. It was determined that there was no significant difference in biofilm cell viability with or without NPs at concentrations of 1 or 100 µg/L. The EPS may be providing some protection from the NPs, but it is more likely that the NPs are primarily dispersing dead cells from the biofilm. Webb et. al. demonstrated that cell lysis and death occurs inside of microcolonies in 10 day old Pseudomonas biofilms (63). Subpopulations of live cells were continuously observed around microcolonies where dead cells were observed. This cell death in biofilms is thought to be an important in the differentiation and dispersal of subpopulations in biofilms that is significant for cell to cell adhesion and biofilm stability (3, 63). If L. pneumophila is losing dead cells, which are a vital part of the biofilm, then this may result in future problems, ultimately causing a reduction in biofilm growth and stability.

In the environment, though bacteria are most commonly found in the biofilm form, it is still important to examine whether NPs can interact directly with planktonic
cells to help us understand how NPs might interact with individual bacterial cells. Studies have demonstrated antimicrobial activity of NPs to bacteria (44, 67). Pelletier et. al. demonstrated antimicrobial activity of cerium oxide NPs against E. coli and B. subtilis. NP inhibition of growth was different for both organisms, and there was no true relationship of inhibition based on NP size. But, when NPs did inhibit the growth of both organisms, it was in a concentration dependent manner (44). In our planktonic assays L. pneumophila was exposed to PtNPs, AgNPs, Fe₃O₄ NPs and PSBs, and no significant effect on biomass, pigment production, or cell viability was observed at concentrations ranging from 1 µg/L-10 mg/L. The concentrations used in Pelletier, et. al. ranged from 50-150 mg/L which is much higher than the concentrations used in our planktonic assays. Though the authors used a type of NP that was not used in our studies, concentration also plays a role in the effects observed and may explain why inhibition was observed in their experiments. Similar to our studies, Zhou et.al. looked at the interaction of citrate capped AuNPs with E. coli. At 10 µg/ml no effect on E. coli growth was observed, however at concentrations of 0.1, 1, and 5 µg/ml E. coli growth was significantly inhibited (71). Our concentrations are comparable to the ones used in this study, and the NPs used were 20-30 nm, which is similar to our 18 nm AuNPs. This suggests that L. pneumophila may be more resistant to AuNPs than E. coli, and this concept is similar to what other studies have showed with AgNPs (25). Our data suggests that NPs tested in our experiments do not have an immediate toxic effect on L. pneumophila planktonic cells.

These data suggest that sublethal NP concentrations can have significant effects on biofilm morphology, and that these direct effects could alter the stability of the
biofilm. In the case of an accidental release of NPs into the environment, our data suggests that low concentrations will still affect biofilms. Other aquatic organisms are more tolerant of NPs and detrimental effects are only observed at higher concentrations. *Daphnia magna* did not show any mortality to C\textsubscript{60} fullerenes stirred in water until exposed to a concentration of at least 1 mg/L for 5 days (72). Even so, mortality never reached more than 50\% even at concentrations up to 35 mg/L. Zebrafish adults exposed to AgNPs and had an LC\textsubscript{50} of 7.07 mg/L (21), which is much higher than the concentrations used in our studies. Though the NPs may not directly affect higher organisms at these low concentrations, these biofilm communities comprise the base of the food chain. Disruption of the biofilm could lead to decreases in grazer populations if the food supply was dispersed. The effects of NP exposure on important ecological trophic interactions have not yet been well explored. These interactions are important to understand, as the escalated use of NPs in everyday products increases their risk of entering the environment, and it is essential to understand the implications this contamination may have on microbial communities in aquatic systems. Stojak *et al.* demonstrated that exposure of *L. pneumophila* biofilms to AuNPs can lead to destabilization and morphological changes in biofilms and result in biofilm dispersal events (55). *L. pneumophila* uses host cells such as amoebae for replication and survival in the environment. The data presented here demonstrates that changes in biofilm morphology also lead to altered interaction between *L. pneumophila* biofilms and their environmental amoebae hosts.
Toxicants such as heavy metals can alter biofilm morphology. One study examined the effect of cadmium (II), copper (II), lead (II), zinc (II), aluminum (III), chromium (III), glutaraldehyde, and phenol on marine biofilm morphology using concentrations ranging from 10-50 mg/L (16). Biofilms clustered into patches when exposed to all of these toxicants at the above concentrations. This response was suggested to be a defense mechanism to the toxicant. Clustering decreases the total surface area of the biofilm exposed to the surface and protects the bacteria from exposure. The authors noted that in previous work at lower concentrations of chromium this effect was not observed, so the effect could be concentration dependent but also specific to each individual toxicant. Bacteria in biofilms are also known to alter gene expression in response to specific environmental signals, resulting in changed biofilm morphology. This changed morphology can be in response to protozoa presence resulting in reduced grazing ability of the protozoa and increased survival of the biofilm in the environment. Biofilms of *P. aeruginosa* alter their morphology in the presence of the surface feeding flagellate *Rhynchomonas nasuta* (36). Matz *et. al.* examined early biofilm formation when *P. aeruginosa* and *R. nasuta* were added simultaneously and subsequently allowed to form biofilms over the course of 3 days. The authors found that while control biofilms were flat, thin, and uniform, biofilms in the presence of grazers formed microcolonies (36). The total percent surface coverage in the grazed biofilms was less than 1%, while non-grazed control biofilms had 100% surface coverage. *Vibrio cholerae* also alters its biofilm phenotype in response to protozoan grazing, which protects the bacteria from grazing predators in the environment. The rugose colony variant of *V. cholerae* produces
an exopolysaccharide that enables the bacteria to form biofilms while the smooth phenotype is biofilm deficient (68). When *V. cholerae* is exposed to a unicellular flagellate grazer (*Cafeteria roenbergensis*), the biofilms formed exhibit a rugose rather than smooth phenotype (37). This phase selection suggests that the switch to the rugose phenotype, which produces a grazing-resistant biofilm, is a survival mechanism of *V. cholerae*. The presence of specific phenotype selection in biofilm bacteria suggests that altering gene expression in response to ecological signals is a conserved phenomenon that may play a role in the biofilm response to NPs that we observe in our system.

When the *L. pneumophila*-amoebae interaction was examined, as expected, there was a significant decrease in biofilm surface area when *A. polyphaga* grazed on control biofilms, demonstrating that *A. polyphaga* can acquire *L. pneumophila* from biofilms. However, *A. polyphaga* was unable to efficiently obtain bacteria from a biofilm after it was exposed to 4 or 18 nm AuNPs. We hypothesize that the significantly altered morphology due to NP exposures makes it more difficult for the amoebae to graze. In a study by Weitere *et. al.*, findings showed that microcolonies, such as those seen in our *L. pneumophila* biofilms, embedded into the exopolysaccharide matrix of the biofilm provided resistance mechanisms to protozoan grazing (64).

Although the morphology changes are the most likely reason behind the reduced grazing, it should be noted that Weitere *et. al.* saw this protozoan response primarily in flagellates and grazing by *Acanthamoeba* on the *Pseudomonas* biofilms was not significantly changed (64). However, while amoebae can feed on *Pseudomonas* (9), *Pseudomonas* species do not replicate within amoebae as do *L. pneumophila*, suggesting
that the specific interaction between *A. polyphaga* and *L. pneumophila* may also be playing a role. This idea is supported by a biofilm study done by Queck *et al.*, where *Serratia marcescens* biofilms grown under batch conditions formed biofilms with microcolonies (47). This type of biofilm was not protected against grazing by the surface feeder, *A. polyphaga*. On the other hand, filamentous biofilms formed under flow conditions provided protection against *A. polyphaga* grazing. The authors suggest the cell chains in the filamentous biofilms are too large to be incorporated into the *A. polyphaga* food vacuole. The NP induced morphology change in *L. pneumophila* biofilms produced similar microcolonies at the surface of the biofilm, though the microcolonies were slightly smaller than the ones formed by *S. marcescens*. But, the result we observed was different than that observed with *S. marcescens*, as we saw a reduction in grazing ability by protozoa. This suggests that the *L. pneumophila*-*A. polyphaga* interaction is specific.

We have demonstrated that 4 and 18 nm AuNPs induce biofilm dispersal (55). Although the 4 nm AuNPs induce biofilm dispersal (55), less bio-volume is lost than after exposure to the 18 nm AuNPs (Figure 2, bio-volume of control=1.07, 4nmAu=0.66, 18nmAu=0.62). Characterization of 4 nm AuNPs in MHW using DLS showed immediate aggregation of the NPs which increases over time. This aggregation in MHW probably increases the overall particle size when they are initially added, causing these NPs to interact differently within the biofilm. The smaller size of 4 nm AuNPs provides a greater surface area ratio which leads to different binding and aggregative abilities. However, 18 nm AuNPs are initially more stable and do not aggregate immediately, and less aggregation is observed over time. This slight difference in bio-volume is most likely due
to the increased aggregation of the 4 nm AuNPs in the water column. As seen in Stojak et al. (55), 50 nm AuNPs could not induce biofilm dispersal. The 4 nm AuNP aggregation in MHW can easily reach similar sizes. This concept can be used to help explain the decrease in *A. polyphaga* death after interaction with biofilms exposed to 18 nm AuNPs that was not observed when *A. polyphaga* grazed on 4 nm AuNP exposed biofilms. Although the morphology change induced by the 4 nm AuNPs significantly reduced grazing, there was still more grazing on the surface of these biofilms than on the 18 nm AuNP exposed biofilms, corresponding to more amoebae loss in the 4 nm exposed biofilm samples. The 24 nm PSBs have a similar size to 18 nm AuNPs but did not alter biofilm morphology or the host-pathogen interaction, nor were they taken up by the bacteria, advocating the idea that core composition also likely plays a role in these interactions as well. Though the chemical composition of the surface chemistries of these two NPs differed slightly, the citrate and carboxyl groups both have a negative surface charge in the exposure media, which is a significant characteristic that affects the interaction of these NPs with biological systems.

Initial analysis of NP interaction with *A. polyphaga* alone in growth media showed that the NPs do not affect the viability of amoebae (data not shown). It is known that the surface properties of NPs change considerably after coming in contact with biological media (1). This lack of effect could be due to the interaction of NPs with components of the media, preventing NP interaction with the amoebae. Therefore, we wanted to determine whether NP exposure could alter the interaction of planktonic *L. pneumophila* with *A. polyphaga*. Growth of *Legionella* in planktonic culture and in
amoebae is characterized by two different phases – the replicative (avirulent) and transmissive (virulent) phases. Transmission traits enable the bacteria to infect amoebae, where they can thereby switch to the replicative phase to promote replication inside of amoebae, eventually promoting lysis of the host cell. In the overnight culture, *L. pneumophila* is exposed to the particles during the replicative (avirulent) phase. When the particles and *L. pneumophila* are added to the amoebae simultaneously, the bacteria are in the transmissive (virulent) phase. Uptake of *Legionella* by amoebae is thought to be a receptor-mediated event that occurs when the bacteria are in the transmissive phase (62). In addition, it is known based on previous studies (55) that internalization of 4 and 18 nm AuNPs occurs in an overnight culture. We hypothesized that AuNPs binding to the surface ligands involved in amoebae recognition of the bacteria could prevent uptake. We also hypothesized that internalization of NPs in the overnight culture would result in AuNP binding to molecules involved in the expression of transmission traits, and would alter the replication ability of the bacteria. But, it was determined that neither uptake nor replication ability of planktonic *L. pneumophila* in *A. polyphaga* were altered by NP exposure. This suggests that NPs do not affect amoebae recognition of bacteria and that internalization of NPs does not alter virulence factors of the bacteria, as the bacteria are able to replicate normally within the amoebae host. However, the most likely interaction of amoebae and *Legionella* in the environment is at the level of the biofilm where there are significant alterations.

Environmentally, biofilms are a common food source for various protozoa and benthic invertebrates in the environment and play a critical role in aquatic ecosystems.
The disruption of microbial communities by natural or anthropogenic sources can alter aquatic ecosystems, but disruption by NPs has not been well explored. This research suggests that sublethal NP concentrations can have significant effects on microbial communities. The NP induced changes in morphology of the biofilm is most likely resulting in the changes seen in amoebae-bacteria interaction. However, it is possible that other genetically driven changes are also occurring in response to the NPs that also affect protozoa interaction and biofilm stability. It is vital to examine the impact that anthropogenic induced disruption of microbial biofilms may have on critical host-bacteria interactions, as it has the potential to disrupt normal ecosystem functioning.
WORKS CITED


