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Effects of Direct and Dietary Exposure to Silver Nanoparticles on a Tritrophic System

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Abstract

This experiment examined the ecological impacts of silver nanoparticle pollution. The production and application of silver nanoparticles have increased dramatically due to their antibacterial properties and the nanoparticles are found in many consumer textiles and plastics. During the production, use, and disposal of these products, nanoparticles can be released into aquatic ecosystems. The impact of this pollution is relatively unknown. This study used a tritrophic system or three-level food chain to understand how organisms on different trophic levels are affected by direct exposure to the nanoparticles through the surrounding environment and indirect exposure of nanoparticles through diet. We expected that both direct and dietary exposure to silver nanoparticles would result in increased mortality and mobility in our system. We expected that the lower trophic levels, *Chlamydomonas* and *Daphnia*, will be the most affected by direct exposure to the nanoparticles and the highest trophic level, *Danio*, to be most affected by dietary exposure.

Keywords: Silver Nanoparticles, Nanoparticle Pollution, Tritrophic System

1. Introduction

Nanotechnology is a rapidly developing field with annual revenues expected to reach one trillion dollars annually [11]. Silver is the most common material used to make nanoparticles and has a widespread application [5]. These nanoparticles can be integrated into a variety of consumer products ranging from cosmetics and food packaging to medical textiles and plastics[12, 6, 9]. The primary reason for the widespread application of silver nanoparticles is that the particles have biocidal or toxic properties[19, 12, 17]. Silver is an effective biocidal agent against viruses and bacteria which can cause infections and diseases in humans [19, 4, 12]. These antimicrobial properties have made silver nanoparticles a convenient and useful material to integrate into consumer products for sterilization purposes[5].

The increased production and application of these silver nanoparticles may pose a risk to the natural environment as nanoparticles are a possible toxic pollutant. Studies of silver nanoparticle-integrated textiles have shown that the particles can leach out of these products at measurable levels[9, 2]. These studies indicate that these nanoparticles are being leached out of the products and into our wastewater systems and can then be released into local waterways. Estimates of silver pollution in wastewater suggest that at least 15% of the silver being introduced into the environment comes from nanoparticles and that this percentage will increase with the growing production and application of these materials[4].

After silver nanoparticles are released into local waterways, there is a risk of the nanoparticles causing toxic effects in the aquatic ecosystems. Toxic effects of silver nanoparticles have been observed not only in bacteria and viruses, but also in different species of algae, zooplankton, and fish [3, 20, 10, 18, 15]. There is little information available about how the toxic effects of silver nanoparticles are altered by abiotic factors, such as pH and temperature, in

natural waterways. Preliminary studies using microcosms have found that the particles can remain suspended in the water-column and be taken up by organisms under simulated estuarine environmental conditions[7].

The limited data available on the levels of nanoparticle pollution and on how the nanoparticles interact with their natural environment makes the ecological impacts of these nanoparticles relatively unknown and the subject of many current studies. In this study the ecological impacts of silver nanoparticles were assessed using a freshwater tritrophic system or food-chain which consisted of an alga, *Chlamydomonas reinhardtii*, zooplankton, *Daphnia magna*, and a small fish, *Danio rerio* (zebrafish). For the study, each trophic level of the system was directly exposed to nanoparticles suspended in their medium and the higher trophic levels, *Daphnia* and *Danio*, were exposed to nanoparticles through eating contaminated prey. The expectations of the study were to determine whether all levels of the trophic system were affected by direct exposure to nanoparticles and whether there was a bioaccumulation or transfer between trophic levels of the particles.

2. Methods and Materials

2.1 Experimental Organisms

Both *Chlamydomonas* and *Daphnia* were purchased from Carolina Biological Supply (Burlington, NC). Adult zebrafish were purchased from a local pet store (Critter Corner, New Castle, PA). The *Chlamydomonas* were cultured in Alga-Gro® (Carolina Biological Supply, Burlington, NC) under a grow table and the *Daphnia* and zebrafish were cultured in local spring water (Leesburg Falls, PA). The *Daphnia* were fed both *Chlamydomonas* and a commercial invertebrate food (Wards Natural Science, Rochester, NY) and the zebrafish were fed *Daphnia*. All organisms were kept under a nine hour day-night cycle.

2.2 Nanoparticle Suspension

A silver nanoparticle powder (99.95% purity, 20-30 nm spherical particles) was purchased from Skyspring Nanomaterials (Houston, TX). To break up any agglomerations within the nanopowder, the powder was sonicated for five minutes using a dismembrator (Fisher Scientific Sonic Dismembrator 500, Pittsburgh, PA). The nanopowder was than suspended in 2 mM sodium citrate, and ultra-sonication (Fisher Scientific Solid State Ultrasonic FS-9, Pittsburgh, PA) for one hour was used to deaggregate the suspension. After ultra sonication, the suspension was filtered with a 0.45µm nylon membrane filter (Whatman (filter paper), Piscataway, NJ; Millipore (filter holder), Billerica, MA). To determine the concentration, the suspension was analyzed using atomic absorption spectroscopy (Varian SPECTRAA 220FS, Santa Clara, CA) with a calibration curve between 1 and 10 ppm, prepared with a silver standard (Inorganic Ventures, Christiansburg, VA) diluted with 2% nitric acid.

2.3 Direct Exposure Study

To examine the effects of direct exposure to silver nanoparticles on our tritrophic system, the individual trophic levels were placed directly in spring water that contained a suspension of the particles. The study used four concentrations of nanoparticles (0.25, 0.50, 0.75, and 1.0 ppm) based off of levels of particles found leached from consumer products[9, 2]. For *Chlamydomonas*, the effects of direct exposure were assessed by monitoring population size over time. The algae were cultured in a 100 mL suspension, with five replicates per concentration, and the population size was monitored over four hours using a hemocytometer. For *Daphnia*, individual adult daphnids were placed in wells with 2.5 mL of a nanoparticle suspension and their survival was monitored over a one-week period. *Daphnia* were fed *Chlamydomonas* during the exposure. The zebrafish were placed in individual bowls with 200 mL of a suspension and their survival was monitored over a two week period. Zebrafish were fed *Daphnia* during the exposure. For both *Daphnia* and zebrafish there were 30 replicates per concentration. Controls for each trophic level were placed under identical conditions in spring water that did not contain any silver.

2.4 Dietary Exposure Study

To determine whether the nanoparticles can be bioaccumulated or transferred between trophic levels, the higher trophic levels were fed contaminated prey items. The prey, *Chlamydomonas* and *Daphnia*, were directly exposed to nanoparticle suspensions at four concentrations (0.25, 0.50, 0.75, and 1.0 ppm) for 24 hours. They were removed

from the suspension and rinsed to remove any excess particles on their exterior. The contaminated *Chlamydomonas* were fed to *Daphnia* during a seven day period, during which the survival of the *Daphnia* was monitored. The contaminated *Daphnia* were fed to zebrafish during a two week period, during which the survival of the zebrafish was monitored.

2.5 Statistical Analysis

A two-way analysis of variance (ANOVA) and a multiple comparisons test were used to analyze the effects of concentration and time on population size in *Chlamydomonas*. To determine if there was a significant difference in survival in the *Daphnia* and *Danio* in our direct and dietary exposure studies, a repeated measures ANOVA was used. To determine which treatments and which days were significantly different, if any, Tukey's multiple comparisons test was used.

3. Results

Population size of our primary producer, *Chlamydomonas*, declined after direct exposure to silver nanoparticles (d.f. = 4, F = 30.33, $p < 0.001$; Fig. 1). The time during the exposure did not have a significant effect on the population size of the cultures (d.f.= 3, F = 0.18, $p = 0.91$). Population sizes in all of the exposures were significantly lower than the control, except for our lowest concentration of nanoparticles (0.25 ppm). The population size in the 0.25 ppm concentration was significantly higher than in the 0.50 and 0.75 ppm concentrations.

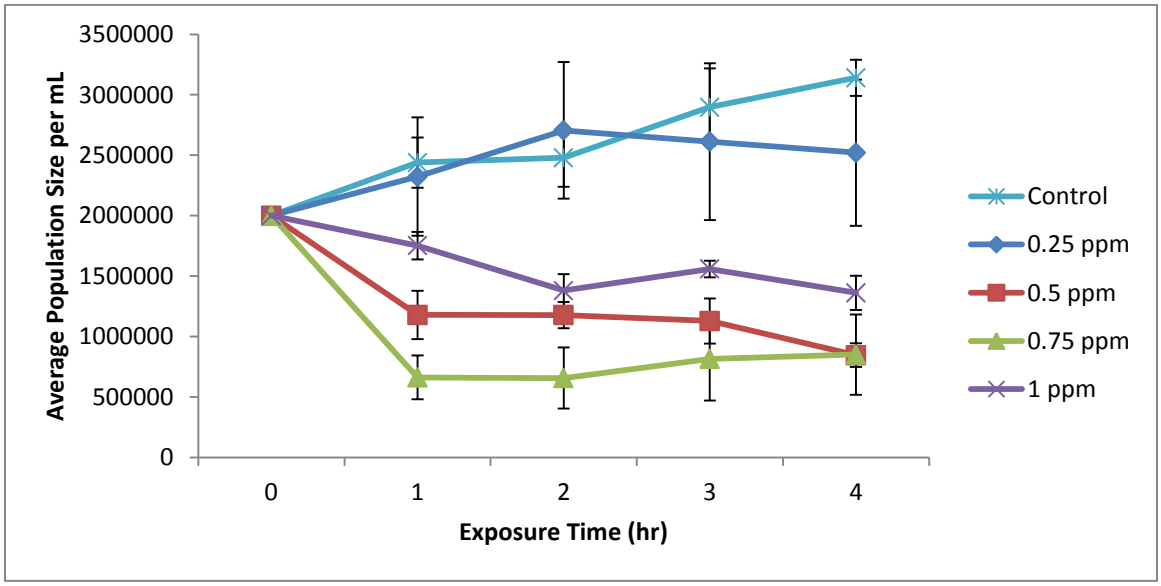

Figure 1: Average population size per mL (± SE) of the *Chlamydomonas* cultures (n=5)

Figure 1. The average population size per mL of the 5 *Chlamydomonas* cultures per concentration over the four hour exposure period. There was not a significant difference in population size between the control and the lowest concentration 0.25 ppm, but all three higher concentrations had significantly lower population size than the control. The biggest decline on population size was seen in the first hour.

The survival of the *Daphnia* declined due to direct nanoparticle exposure with the largest change in survival during the first twenty-four hours, as all four concentrations had higher mortality than the control (d.f. = 4, F = 46.61, $p < 0.001$; Fig. 2). There was also a significant difference between the first day and all the following days of exposure (d.f. = 7, F = 21.40, $p < 0.001$). The survival of *Daphnia* did not differ for those exposed to 0.25 and 0.5 ppm, but both differed significantly from the control (0.25 ppm: T = 6.648, $p < 0.001$; 0.50 ppm: T = 7.080, $p < 0.001$) and the 1.0 ppm treatment (0.25 ppm: T = -6.994, $p < 0.001$; 0.50 ppm: T = -6.562, $p < 0.001$). The 0.75 ppm treatment had significantly higher mortality than the control (T = 7.166, $p < 0.001$) and the 1.0 ppm treatment (T =

7.166, $p < 0.001$) while the 1.0 ppm treatment was only significantly different from the control ($T = 13.64$, $p < 0.001$). There was a significant difference between day 0 and all subsequent days, as mortality occurred quickly (day 1-7: $T \leq -8.19$, $p < 0.001$).

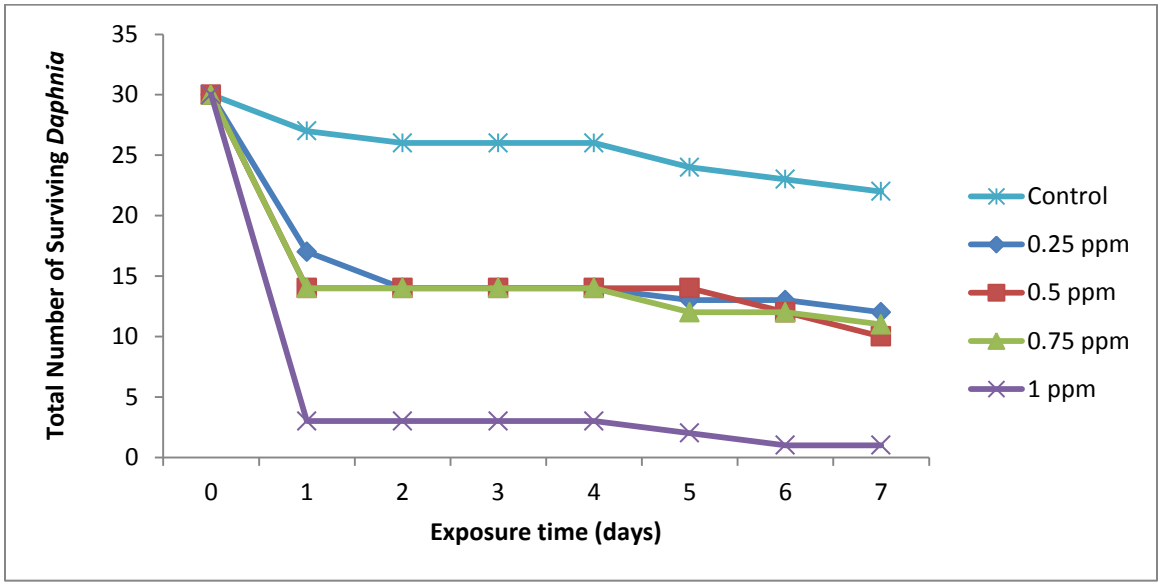

Figure 2: Total number of surviving *Daphnia* (n=30) during the direct exposure of silver nanoparticles

Figure 2. The survival of *Daphnia* during the direct exposure to silver nanoparticles over a one week exposure period. *Daphnia* survival was highest in the control and significantly lower in all of the nanoparticle treatments, with the highest treatment of 1.0 ppm having the lowest survival. The biggest decline in survival was seen during the first day of treatment.

In the *Danio* direct exposure experiment, there was a significant difference in number of individuals surviving between treatments with an overall decline in survival due to nanoparticle exposure (d.f. = 4, $F = 171.4$, $p < 0.001$; Fig. 3) and between days of exposure (d.f. = 14, $F = 8.21$, $p < 0.001$). All four concentrations had reduced survival compared with the control (0.25 ppm: $T = 10.20$, $p < 0.001$; 0.50 ppm: $T = 8.54$, $p < 0.001$; 0.75 ppm: $T = 21.60$, $p < 0.001$; 1.0 ppm: $T = 21.60$, $p < 0.001$) and survival was significantly higher in the 0.25 and 0.5 ppm treatments than in the 0.75 ppm treatment (0.25 ppm: $T = -11.40$, $p < 0.001$; 0.50 ppm: $T = -13.06$, $p < 0.001$) and 1.0 ppm treatment (0.25 ppm: $T = -11.40$, $p < 0.001$; 0.50 ppm: $T = -13.06$, $p < 0.001$). Analyzing survival over time, we found that day 0 was significantly different than all of the other 14 days (days 1-14: $T \leq -6.761$, $p < 0.001$).

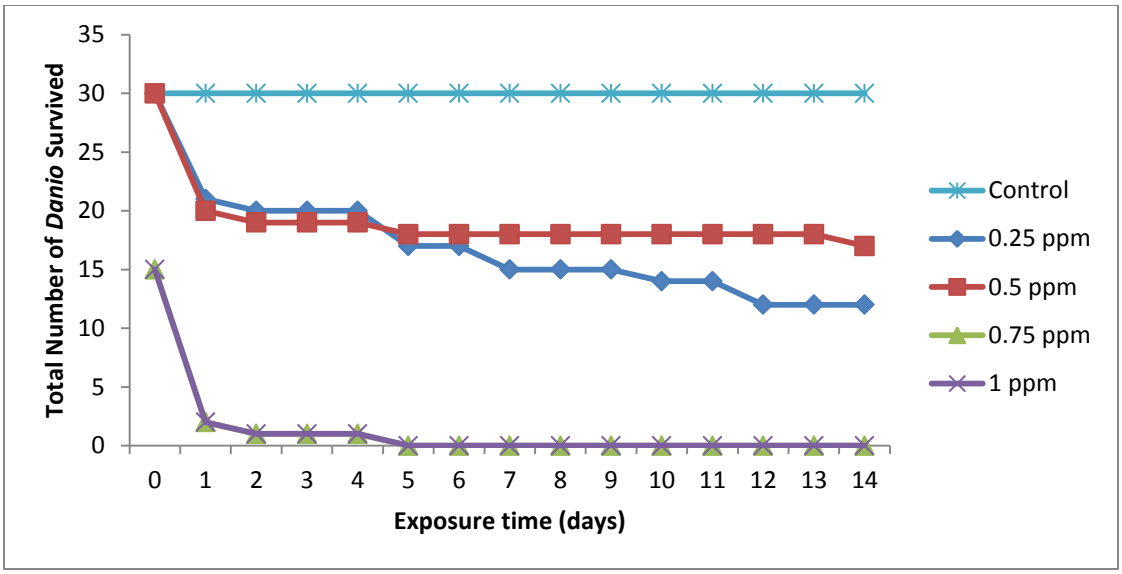

Figure 3: Number of zebrafish that survived during the direct nanoparticle exposure (0.25 and 0.50 ppm: n=30; 0.75 and 1.0 ppm: n=15)

Figure 3. Survival of zebrafish during the direct exposure to silver nanoparticle suspension over a two week period. Survival declined in all treatment groups, with the highest two treatment groups having the lowest survival. Survival significantly declined in all treatment groups during the first day of exposure.

For the *Daphnia* fed contaminated algae, there was a significant decline in daphnid survival between treatments (d.f. = 4, F = 11.35, $p < 0.001$; Fig. 4), but not between days of exposure (d.f. = 7, F = 1.92, $p = 0.11$). Survival in the 0.25 ppm treatment was not significantly different than the control (T = -1.535, $p = 0.55$), but was higher than the 0.5 ppm treatment (T = -5.003, $p < 0.001$) and significantly higher than the other two treatments (0.75 ppm: T = -5.003, $p < 0.001$; 1.0 ppm: T = -5.003, $p < 0.001$). Survival in the two highest concentration treatments were significantly lower than the control (0.75 ppm: T = 3.468, $p < 0.001$; 1.0 ppm: T = 3.468, $p < 0.001$), but did not differ from each other (T = 0.0, $p = 1.0$).

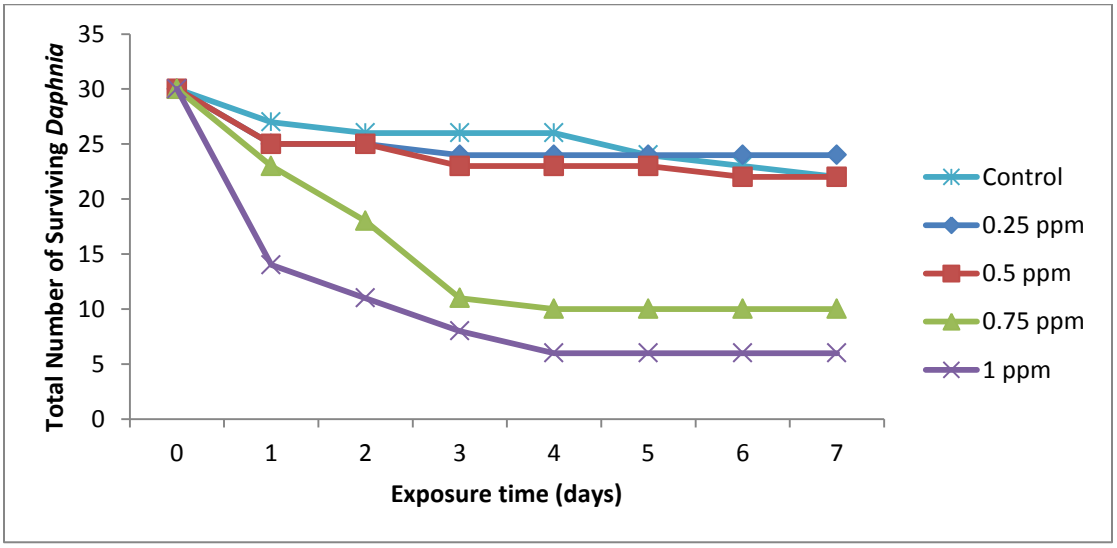

Figure 4: Number of *Daphnia* surviving during the dietary exposure to silver nanoparticles (n=30)

Figure 4. Survival in *Daphnia* while being fed nanoparticle-contaminated *Chlamydomonas* during a one week exposure period. Survival was highest in the control and the two lower concentrations which did not differ significantly. The largest impact on survival was seen during the first day of exposure.

The decline in survival of *Danio* fed silver nanoparticle-contaminated *Daphnia* differed significantly between the treatments (d.f. = 4, F = 158.86; $p < 0.001$; Fig. 5) and between the days of the exposure period (d.f. = 14, F = 9.52; $p < 0.001$). *Danio* survival was greater in the control than in any of the dietary exposures (0.25 ppm: T = 11.59, $p < 0.001$; 0.50 ppm: T = 9.85, $p < 0.001$; 0.75 ppm: T = 20.56, $p < 0.001$; 1.0 ppm: T = 21.93, $p < 0.001$). The two lower concentrations, 0.25 and 0.5 ppm, did not differ significantly from each other (T = 1.74, $p = 0.42$), but had significantly greater survival than the 0.75 ppm (0.25: T = -8.97, $p < 0.001$; 0.5: T = -10.72, $p < 0.001$) and 1.0 ppm treatments (0.25: T = -10.34, $p < 0.001$; 0.5: T = -12.09, $p < 0.001$). Survival was extremely low in the two highest concentration but they did not differ from each other (T = -1.371, $p = 0.65$). Survival was highest on day 0 than on any other day (days 1-14: $T \leq -6.476$; $p < 0.001$).

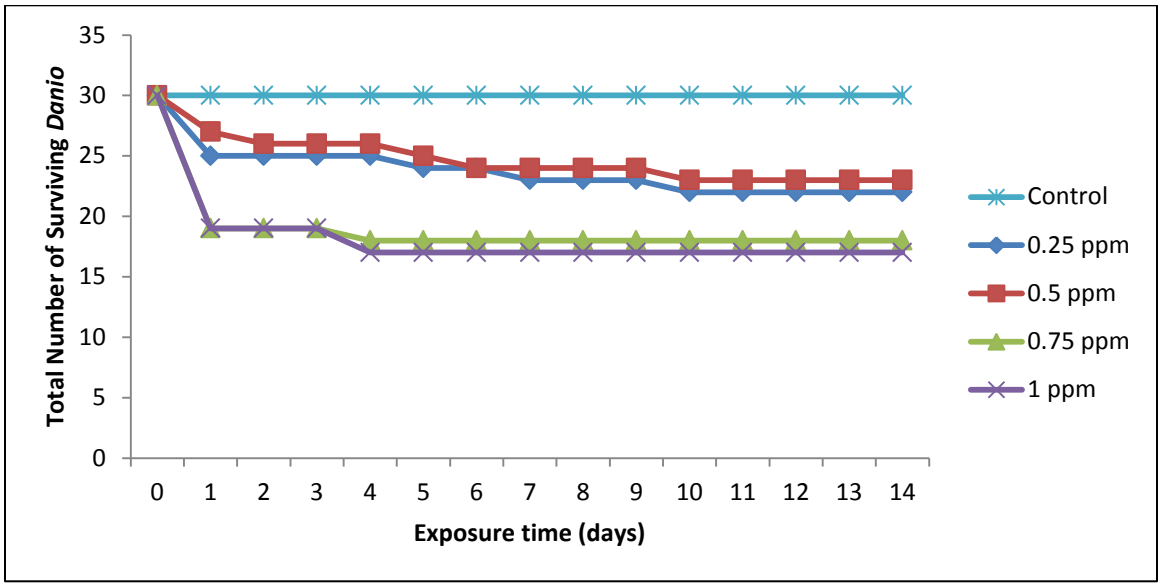

Figure 5: Number of zebrafish surviving (n=30) during the dietary exposure

Figure 5. Zebrafish survival while being fed nanoparticle-contaminated *Daphnia* during a two week exposure period. Survival was highest in the control with lower survival in all of the treatment groups. The largest decline in survival was seen during the first day of exposure.

4. Discussion

The study found that organisms in our tritrophic system were affected by both direct and dietary exposure to silver nanoparticles. The direct exposure to silver nanoparticles resulted in decreased population size to the base of the food chain, *Chlamydomonas*, decreased survival in the primary consumer, *Daphnia*, and decreased survival in the secondary consumer, *Danio*. The dietary exposure study showed that survival for both of the consumers, *Daphnia* and *Danio*, were lower when they consumed contaminated prey. The study also found that direct exposure had a greater negative effect than the dietary exposure on our organisms. The impact of nanoparticle exposure was dependent on both concentration of particles and time of the exposure. The two lowest concentrations had similar effects as the control, while the higher concentrations had greater toxic responses in both studies. For most of the direct exposure experiments, the greatest effect of the nanoparticle exposure on the organisms occurred during the first day, while in the dietary experiments it took longer to see a response to nanoparticle exposure.

The study provides evidence that freshwater ecosystems can be greatly affected by silver nanoparticle pollution. The food chain is an important system in any ecosystem and when it is disrupted there can be drastic effects for the entire system. In this study, all three levels of the food chain were affected and consumers were directly affected by

bioaccumulation of the particles. Since the concentrations used in the exposure were based off of actual experimental data on the amount of nanoparticles released into wastewater, the data has important implications for understanding nanoparticle pollution.

Other experimental studies report similar results. *Daphnia magna* have been shown to take up nanoparticles both directly from their environment and from contaminated *Chlamydomonas* prey[21]. Their bioaccumulation study conflicted with this study, in that they had greater uptake of nanoparticles in *Daphnia* fed prey exposed to lower concentrations, while in this study there was a negative relationship between concentration and survival. The time dependency of the experiment was also observed in other research with nanoparticles. Zhao and Wang [21] found that *Daphnia magna* took up more nanoparticles over time and actually saw a stronger positive relationship in experiments with higher nanoparticle concentrations.

The results showed that all three of the test organisms suffered negative effects from nanoparticle exposure, both from direct and dietary exposure. The exact mechanism behind these toxic responses is not well understood in aquatic organisms. Several physiological mechanisms have been suggested. Several studies, which used zebrafish as model organisms, indicated that oxidative stress and apoptosis may be the results of nanoparticle exposure [6, 1]. These processes may be the mechanisms behind the reduced survival seen in the study organisms due to nanoparticle exposure, but future research is needed to make that determination.

If nanoparticle exposure can induce serious and often fatal toxicological responses in a variety of organisms, what is the risk to humans? Most of the products that contain silver nanoparticles are intended for general consumer use, often with close contact to the skin. There are very few studies available that have assessed risks of nanoparticle exposure and possible poisoning to humans, but the few studies available suggest that there is a measurable risk. Silver has long been associated with certain health conditions including argyria (a bluish skin discoloration), kidney and liver damage, and irritation[16]. The small size of these materials may enable them to be easily absorbed into the human body which increases the risk of adverse health effects. Nanoparticles have been shown to penetrate human skin, an alarming discovery as skin contact with nanoparticles is prevalent with the use of such products as clothing, bandages, and cosmetics[13]. Another possible risk to humans is that nanoparticles may be able to permeate cells and cross important defense barriers in the body. Some nanoparticles are capable of crossing the blood-brain barrier, an important physiological barrier to the brain[14].

In addition to the general risk to each organism, the study found that exposure affected the entire trophic system. This indicates that nanoparticle pollution can have dramatic impacts on aquatic ecosystems by altering relationships and behaviors within a food web. Impairment or mortality in an organism due to a pollutant can result in dramatic shifts in species abundance and community composition[8]. Impacts on one trophic level can have drastic, and possibly devastating, effects on other trophic levels. For example reduction in survival of an intermediate predator species, for instance *Daphnia*, may result in unsustainable population growth in its prey species and can also limit food availability for its predators.

The study has indicated that silver nanoparticles can have important ecological impacts resulting from its negative effects on every level of the food chain. These effects were both seen in both direct and dietary exposure pathways, indicating that silver nanoparticles can affect organisms through both bioconcentration (direct uptake from environment) and bioaccumulation. More studies are needed to understand the extent of nanoparticle pollution, particularly here in the United States where little nanomaterial research is available and on the fate of these particles in the natural environment. Future toxicity studies should focus on understanding the actual mechanisms of the toxicity to organisms, as well as how the nanoparticles are stored within that organism. In zebrafish, for example, studies are needed to understand if the zebrafish are storing nanoparticles in their bodies and if there is biomagnification of the particles.

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