ABSTRACT

The Testing and Use of a New Method to Evaluate Chronic Toxicity in *Daphnia Magna*

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The importance of toxicity testing to the protection of people and the environment cannot be overstated, however in order for the data to be of use it must be both relevant to actual conditions of exposure and conducted in a manner that efficiently uses resources. In this research we examined the effect of the exposure of simulated natural waters upon the toxicity of Silver Nanoparticles in order to ascertain if a change in toxicity would be observed compared to lab water exposure and if this toxicity would change over time. In addition, we evaluated new methods to conduct toxicity testing including a new test that would reduce the duration of a standard toxicity test from 21 days to 10 while still testing the same endpoints.

The Testing and Use of a New Method to Evaluate Chronic Toxicity
in *Daphnia Magna*

by

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A Thesis

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Approved by the Thesis Committee

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DEDICATION

To my mother. You were always my biggest source of support. “I love you more”

CHAPTER ONE

A New Method for Assessing Acute Mortality and Reproductive Impacts in the Aquatic Cladoceran *Daphnia magna*.

Abstract

As primary consumers, zooplankton such as *Daphnia magna* are important components of aquatic ecosystems and thus are frequently used in toxicity testing. In fact, they have been designated as an US Environmental Protection Agency (EPA) model organism. The most common standard toxicity testing methods include 24–96 h acute tests that use <24 h old neonates and chronic methods that last 21 to 28 d. Recent research has also indicated the utility of two subchronic tests with durations of 4 d and 10 d. While the shortened duration of these tests is useful for reducing the time required for a toxicity assay, a shorter duration test method could further reduce time and manpower demands while still providing viable mortality and reproduction data. Therefore, we have developed a new method to study reproductive effects as well as the acute mortality screened for in similar duration tests. This method doses 4–5 d old *D. magna* for 4 d, thereby avoiding the difficulties of manpower, resources, and time that are associated with tests of longer duration. The shorter duration test has been compared to standard 48 h acute tests and 10 and 21 d subchronic and chronic tests, and the new method is less sensitive in detecting acute toxicity. Although chronic toxicity is observed, comparisons are difficult to make due to high levels of acute mortality in all tests. Thus, we have suggested further refinements for this new test method.

Introduction

Due to the need for a reliable, rapid, and cost effective reproduction assay for *Daphnia magna*, we have shortened the duration of the test from 10–21 d to 4 d. The aquatic zooplankton *D. magna* is a primary consumer that is an integral part of many freshwater food webs. *D. magna* is a filter feeder: it uses its antenna to create a current in order to filter food particles from the water. However, as filter feeders, *D. magna* are at higher risk of exposure to potentially toxic particulate matter. This vulnerability, combined with their importance to the ecosystem, has rendered them a subject of interest for ecotoxicologists. In fact, since the early 20th century researchers have used *D. magna* to study the toxicity of chemicals in aquatic systems (Warren 1900)**.** The advantages of these organisms include their rapid maturation, fecundity, short life cycle, low cost of colony maintenance, and clonal reproductive strategy that allows for minimal genetic variation and ease of handling compared to similar species. Because these organisms serve as a major food source for fish (Adema 1978), their use in toxicity testing can predict potentially ecologically relevant impacts a toxin may have on aquatic food webs. The US Environmental Protection Agency (EPA) has recognized the scientific value of *D. magna* and has designated them as a model organism (EPA 1986). Moreover, several tests using *D. magna* and closely related organisms are named on the EPA List of Approved Biological Methods for Wastewater and Treatment Sludge located in the Code of Federal Regulations 40§136.3 Table IA. These tests include acute mortality tests for *Ceriodaphnia dubia*, *Daphnia pulex* and *D. magna* (24, 48, or 96 h) as well as longer chronic tests for mortality and reproduction of *Ceriodaphnia dubia* (7 d).

Chronic survival and reproduction tests with *D. magna* typically last 21 d, or approximately half of their expected life span (~40 d at 25 °C), as described in the EPA's *D. magna* life cycle chronic toxicity assay (EPA 2002). While this test can provide very significant information, the 21 d duration can make it difficult and time consuming to conduct. This difficulty can become even more burdensome if a series of tests are needed to compare the toxicity of effluents at different time points. As a result, a researcher may need to conduct multiple tests concurrently, each with a different starting point. Thus, the maximum number of tests conducted is often dictated by manpower limitations rather than best scientific design. The EPA has also published a 10 d method using *D. magna* neonates (EPA 1994). This method, like the 21 d method, was not included in the List of Approved Biological Methods of Wastewater and Sewage Sludge, but it remains in use (Dzialowski et al. 2006, Dobbins et al. 2009). It is a static renewal test with water changes every 48 h. Survival, growth, and reproduction are included as endpoints. Although this reduced duration has reduced some of the time constraints associated with the 21 d assay, some difficulties persist, even with the 10 d assay. Nonetheless, both assays continue to be utilized by researchers.

Several methods are used with *D. magna*, and each has its strengths, weaknesses, and timeframe (Figure 1). One of the most common methods is a 48 h acute toxicity test using <24 h old neonates. These individuals are exposed to a stressor, usually without the presence of food, and mortality is measured at the conclusion of the experiment. This shorter duration method is cost effective and time efficient. However, its results are only applicable to neonates that may or may not have a greater sensitivity to the stressor than organisms at different life stages, and no reproductive data or growth information can be

gathered. Furthermore, while removing food from the test prevents interaction between the food and the substance being tested, it adds to the organism an additional stressor, lack of food, and is likely to impact its response.

A second common test is the standard EPA chronic toxicity assay. This is a 21 d reproduction test utilizing <24 h old neonates at the beginning of the experiment. This test provides detailed information on reproduction and is well established and accepted by the scientific community. Yet due to the 21 d time frame, man-hours and costs are higher than the acute assay, and these costs can limit the number of trials possible over a given time. As such, this time frame may limit research efficiency and drain manpower as well as funding.

A third choice is the EPA 10 d chronic *D. magna* toxicity assay. This test also measures growth, reproduction, and mortality utilizing <24 h old neonates. The length of this assay was reduced from 21 to 10 d while keeping the rest of the parameters of the test similar the 21 d assay. Indeed, the 10 d experiment length may still be too long for some research needs; researchers could easily complete two acute assays over that period.

While the EPA has published several test methods using *D. magna* and closely related species, many researchers still consider these test limitations to be problematic. In fact, to determine if a 7 d *D. magna* test was feasible, Winner (1988) compared the sensitivity of a 7 d test using 4 d old *D. magna* to the standard EPA 7 d *Ceriodaphnia dubia* test. Using organisms exposed to sodium pentachlorophenate and cadmium, researchers measured body length, reproduction, and survival. Their findings have suggested that if indices of toxicity are carefully selected, the 7 d test can estimate concentrations of no effect (NOEC) as low as those provided by a 21 d assay (Winner et al. 1988). Other

researchers have also tested alternative methods to shorten the standard *D. magna* and *C. dubia* assays (Masters et al. 1991, Oris et al. 1991).

To incorporate growth into the shorter acute EPA assays, Lazorchak et al. (2008) have developed a 4 d test, also using neonates, that is capable of measuring growth and mortality while maintaining a sensitivity similar to that of longer tests. They provided food and measured growth at the end of the experiment. Their test has produced consistent results and reproducibility, which make it useful for detecting toxicity. However, because reproduction could not be observed with this test, it is insufficient for some research goals as certain materials influence reproduction at concentrations lower than those that affect mortality.

Contemporary test methods require significant manpower and time in order to be effective in cases where multiple comparisons of complex exposure media are required. Our research intends to develop a 4 d static-renewal method using reproduction and survival as endpoints. This new assay would reduce the difficulties associated with longer duration assays while retaining the use of reproduction and survival as endpoints, both of which are critical in screening for potential toxicity associated with chemicals of emerging concern that have unknown modes of action. Our study raised *D. magna* neonates in 500 mL containers from the point at which they were less than 24 h old until they were 4–5 d old. The test followed the parameters developed by Dzialowski et al. (2006), with the exception of two changes: the age of the organisms used and the test duration. This test duration reduction allowed for twice as many tests to be conducted over the same duration of time and with less expenditure of manpower and financial resources. We only measured the reproductive output of each individual's first brood since the short test duration did not

permit that all individuals could reliably produce a second brood independent of the exposure. However, the use of only the first brood could have added uncertainty into otherwise statistically significant results.

In the present study, the sensitivity of the proposed method is compared to that of the <48 h acute assay and the 10 and 21 d subchronic assays, using NaCl, $AgNO_3$, and silver nanoparticles (AgNP) as reference toxicants. These tests are the most commonly used assays in the industry for the same species. Mortality and reproduction have been recorded throughout the duration of the experiment. At the conclusion of the experiment, the results of the test were compared to standard methods in order to assess the test's sensitivity and validate its potential utility.

Materials and Methods

Culture Methods

D. magna were obtained from established lab cultures at Baylor University's Ecotoxicology and Aquatic Research Laboratory. Cultures were maintained as described by Kolkmeier and Brooks (2013), with modifications as follows. The *D. magna* cultures used in these experiments were transitioned from EPA hard water to EPA moderately hard water (MHW) (EPA 2002), which allowed the culture to acclimate to the softer waters they would be exposed to in another experiment. The cultures were maintained in glass 500-mL beakers filled with MHW at a density no greater than 15 individuals per beaker. Less than 24 h old neonates were removed from the main culture and raised in the same manner until they were 4–5 d old, at which point they were used for the assay. The cultures were fed daily a liquid mixture of the algal species *Pseudokirchneriella subcapitata* and *Ceriophyll*

sp. grass extract, and they were renewed with fresh MHW once every 48 h. The cultures were maintained in a climate controlled incubator at 25 ± 1 ºC with a 16:8 h light to dark photoperiod.

Synthesis of AgNP (Gum Arabic-Coated Ag Nanoparticles)

The nanoparticles for this experiment were provided by the Center for Environmental Implications of Nanotechnology (CEINT). The nanoparticle synthesis methods, which were first described by Yang et al. (2011), are as follows: First, 271 mL of reverse osmosis deionized water, 9 mL of 10g/L gum arabic, and 9 mL of 0.1 M $AgNO_3$ were added to an Erlenmeyer flask, and the solution was stirred for 5 min; simultaneously, 11 mL of 0.08 M ice-cold sodium borohydride was added and stirred for an additional 10 min; multiple batches were combined, and the nanoparticles were purified and concentrated by dialysis (Optiflux F200NR Fresenius Polysulfone Dialyzer, Fresenius Medical Care); lastly, the suspension was diluted with water and concentrated two additional times in order to obtain the final product (Yang et al. 2011).

Laboratory Exposures

48 h Acute test. Less than 24 h old neonates were removed from the main culture and placed individually into polystyrene cups containing 25 mL of dosing solution. The main culture was fed 2 h before dosing began, and dosed individuals received no food after that point. Five individuals were dosed at each concentration, and there were 6 concentrations of each toxicant and a control. Individuals were observed daily. Any carapaces that were shed were removed. The cups were covered with a sheet of clear plexiglass (Polymethyl methacrylate) and incubated at 25 ºC with a light to dark cycle of

16:8 h. Toxicants were tested using a six concentration dilution series (X0.5). The high concentration for each reference toxicant was 15.2 µg/L Ag for the AgNP and $AgNO_3$ and 8 mg/L for NaCl. At the end of the experiment, final mortality was recorded and the remaining individuals sacrificed.

10 and 21 d Sub-chronic Tests

Less than 24 h old neonates were individually placed in polystyrene cups containing 25 mL of dosing solution in combination with 0.6 mL of the food that was used to feed the culture. The cups were covered with a sheet of clear plastic and incubated at 25 ºC with a light to dark cycle of 16:8 h. Each day, individuals were fed and the number of neonates produced by each individual birthed were recorded. Every 48 h, static renewal water changes were conducted using the same concentrations of wetland water and MHW with which they were dosed. These renewals were achieved by adding to new cups 25 mL of dosed water, together with food, and manually transferring each individual. The toxicants were tested using a 6-concentration dilution series (X.05). The high concentration for each referent toxicant was 30.4 and 15.2 µg/L Ag for AgNP and $AgNO_3$, respectively, while 8 mg/L was used for NaCl. Reproduction and mortality were recorded daily, although only the offspring of surviving adults were used in the analysis. After 10 d, final reproduction and mortality were recorded for the 10 d test. Then, the test continued for 11 d, after which point the final reproduction and mortality were recorded for the 21 d test.

4 d Sub-chronic test. Less than 24 h old neonates were removed from the main culture and raised until they were 4–5 d old. Then, they were used for the assay. The test was conducted in the same manner as the 10 d sub-chronic test with the following modifications: The neonates were 4–5 d old at the beginning of the test, and the test

concluded after 4 d of exposure. In addition, the highest doses for $AgNO_3$ and AgNP were increased 15.2 and 60.9 µg/L Ag, respectively, after organisms failed to exhibit an acute response to lower concentrations.

Results

Mortality

48 h Acute test. Mortality was recorded daily throughout the duration of the experiment. Organisms exposed to Ag in these test conditions were more sensitive than those exposed in sub-chronic tests, as 20% mortality was observed at the 0.95 µg/L concentration for $AgNO_3$ and AgNP. The 20% mortality continued at the 1.9 µg/L concentration for AgNP, and complete mortality was observed at the same concentration for $AgNO_3$. Complete mortality was observed for $AgNO_3$- and AgNP-dosed individuals after the 1.9 µg/L concentration (Figure 1.2).

Individuals exposed to NaCl also displayed a sharp increase in toxicity between concentrations. A 20% mortality was observed at the 4 g/L NaCl concentration, and 100% mortality was observed at the 8 g/L concentration.

10 and 21 d Sub-chronic test. Mortality was recorded daily for the duration of the experiments. For $AgNO_3$, during the 21 d test at least one death occurred in each of the concentrations, including the control. Mortality increased to 100% at the 7.6 µg/L concentration. This change resulted in an LC50 of 3.94 µg/L, with a 95% confidence interval between 2.25 to 9.155 µg/L. Almost all of the majority of deaths occurred during the 10 d trial, although one occurred on d 12 at the 0.95 µg/L concentration. The calculated

LC50 for the 10 and 21 d $AgNO_3$ exposure was 4.17 µg/L, with a 95% confidence interval of 2.57 to 8.64 µg/L **(**Figure 1.3).

For AgNP, one death was observed at the 15.2 µg/L concentration, and 100% morality was observed at the 30.4 µg/L. Thus, the calculated LC50 values are the same for the 10 and 21 d assay with an LC50 of 16.63 µg/L Ag. No confidence interval was calculated because the nature of the data resulted in an impractically large result (Figure 1.4). NaCl mortality followed a similar pattern to the AgNP mortality results. One death was observed in the control group, but mortality was not further observed until the 4 g/L concentration was reached, at which point mortality was 100%. Similar to $AgNO_3$, all AgNP mortality occurred within the first 10 days of the test. This resulted in an LC50 of 2.743 g/L, with a 95% confidence interval of 1.86 to 4.77 µg/L (Figure 1.5).

Modified 4 d test. For $AgNO_3$ and AgNP, mortality was only observed at the 100% concentration (15.2 and 60.9 µg/L Ag, respectively). However, mortality was complete at these concentrations. This resulted in LC50s of 10.72 and 42.88 µg/L Ag, respectively (Figures 1.6 and 1.7). Since mortality was not observed at more than one concentration, 95% confidence intervals were not calculated. NaCl mortality was gradual, as demonstrated in Figure 1.8, such that the calculated LC50 was 3.66 with a 95% confidence interval of 2.18 to 7.12 g/L NaCl.

Reproduction

10 and 21 d Sub-chronic test. During the 10 and 21 d sub-chronic tests, no statistically significant decrease in reproduction was observed in $AgNO_3$, AgNP, or NaCl.

Furthermore, no significant differences were observed between the results for the 10 d test and the 21 d test (Figures 1.9–1.11).

Modified 4 d test. During the modified 4 d test, no statistically significant decreases in reproduction were observed for $AgNO_3$, AgNP, or NaCl (Figures 1.12–1.14). A trend towards lower reproduction was observed in 4 g/L NaCl. However, because the sample size was reduced by mortality in this concentration, we did not have enough surviving individuals to test the significance of the decline.

Discussion

The concentrations at which we observed acute toxicity in lab water dosed with gum arabic nanoparticles were not as elevated relative to $AgNO_3$ as researchers have noted for AgNPs. However, this variance is not unprecedented. Newton et al. (2013) have reported an LC50 for $AgNO_3$ of 1.06 µg total Ag/L with a 95% confidence interval of 0.85-1.31. The LC50 for gum arabic NPs was 3.16 µg total Ag/L (2.71-3.68), although that was in MHW. The addition of Suwannee River dissolved organic carbon increased the LC50s of $AgNO_3$ and AgNP to 4.85 µg total Ag/L (4.35-5.41) and 3.48 µg total Ag/L (3.02-4.00), respectively. In this case, acute toxicity estimates after the addition of organic material were similar, and the AgNPs were more toxic (Newton et al. 2013). This may have resulted from interactions between the organic materials and Ag when present in the form of AgNP versus $AgNO_3$. As such, further research into these interactions could indicate how differences among interactions of organic material with $AgNO_3$ and AgNP influence toxicity.

A comparison among the acute toxicity results of the modified 4 d test and those of the 10 and 21 d tests revealed that 4 d organisms experienced toxicity at approximately twice the Ag concentration of the 10 and 21 d tests. This difference is attributed to the age difference among the organisms. While the 10 and 21 d tests use individuals that are less than 24 h old, the modified 4 d test requires individuals that are 4–5 d old. Although researchers have demonstrated that *D. magna* have differing sensitivities to metal toxicity based upon age—growing more sensitive as they develop, they have also noted that this trend stops after the organisms reach 48 h for Se and As and 96 h for Cu and Zn. After these time points, increasing age has been observed to decrease metal sensitivity (Hoang and Klaine 2007). Because Ag toxicity to *D. magna* appears to be a result of disturbing ionoregulatory imbalance, the different life stages of the organism may also correspond to its capacities to survive such stress. Moreover, as the organism shifts resources away from growth and towards reproduction, energy reserves available for stress response may differ.

It was difficult to compare the chronic toxicity tests as acute toxicity resulted in mortality before decreases in reproduction could be observed. Yet this difficulty has been prevalent in research using *D. magna* exposed to Ag. For example, Blinvoa et al. (2013) were unable to calculate an EC50 value when dosing *D. magna* with AgNP because the tested organisms died before a 50% decrease in reproduction was observed. Other researchers who encountered this issue have suggested that acute mortality tests may be more useful than longer chronic tests (Nebeker et. al 1983). Due to these findings, and despite the fact that reproductive impact could not be determined, we do not consider this to be a flaw of the test since the standard 10 and 21 d assays also failed to detect reproductive impact. Further testing should use materials that exert greater reproductive

stress upon *D. magna* as a worthwhile next step. This would allow researchers to more accurately define the tests validity as a reproductive assay.

While our test may be less sensitive than the 10 and 21 d tests, given the predictability of differences in acute toxicity, we suggest that the results are comparable. Future research should calculate age adjustment factors based upon a materials mechanism of action in order to use results from the modified 4 d test to estimate those of the longer, more difficult 10 and 21 d assays so that the results can be compared to results from these longer assays.

Conclusion

The goal of this study was to examine the effectiveness of a new 4 d *D. magna* reproductive assay. Chronic toxicity could not be compared given that the tests demonstrated significant acute toxicity before reproductive impacts were observed and that we only measured one brood per individual. This low acute to chronic toxicity ratio is believed to be a function of the close chronic to acute toxicity ratios for Ag, AgNP, and NaCl rather than a flaw in the tests. Other researchers have observed that these thresholds are close for Ag (Bianchini and Wood 2008, Naddy et al. 2011). Therefore, the difference in toxicity thresholds may be negligible.

Future research could increase the test sensitivity by ensuring that at least two broods per individual are recorded. Although several individuals had a second brood during our test, second broods were not included in the analysis since the test duration was too short to allow all individuals the chance to release a second brood. By adding 2 d to the test, thereby extending its duration from 4 d to 6 d, each individual should have sufficient time to release a second brood without interfering with time constraints. That is, the test

should still be able to be completed in 60% of the time needed for the 10 d chronic test and 29% of the time needed for the 21 d assay. Despite the challenges we observed with gathering reproductive data, we believe reproductive impacts are important to consider even for cases in which close chronic and acute toxicity thresholds are expected due to its position as one of the cornerstones of toxicity analysis

Figures

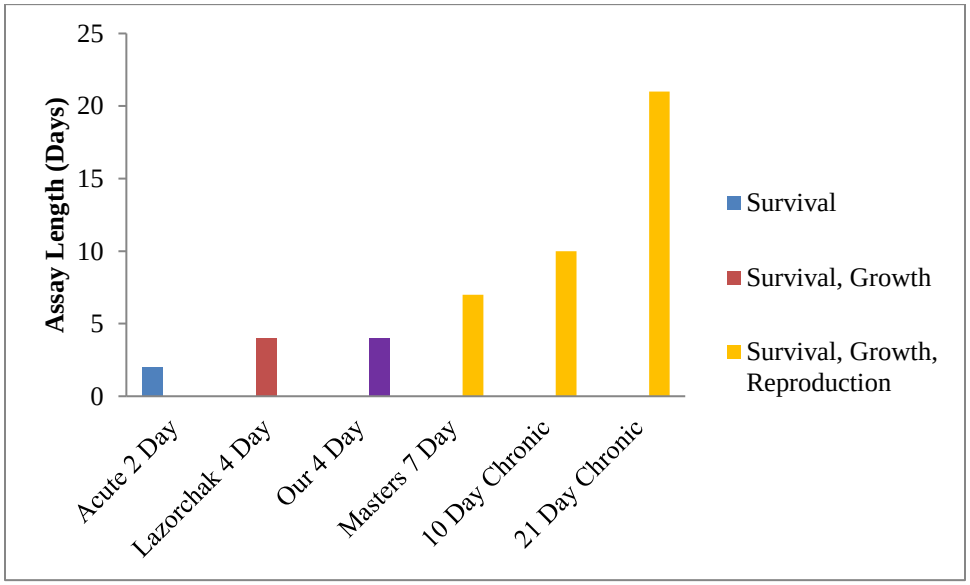

Figure 1.1: A selection of Common *Daphnia magna* assays, their durations, and their endpoints.

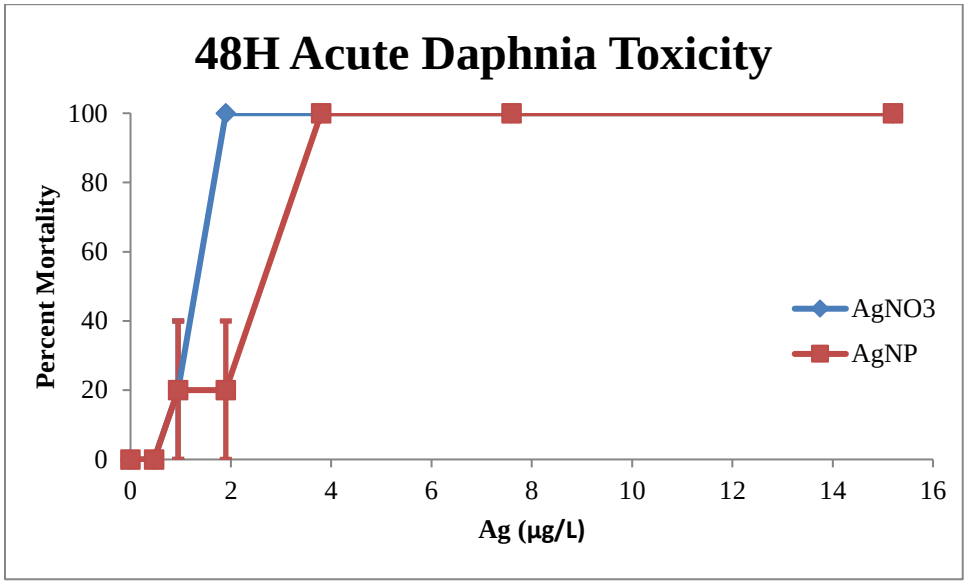

Figure 1.2:Comparison of acute toxicity of $AgNO_3$ and AgNP to *Daphnia magna* using standard 48H neonate assay

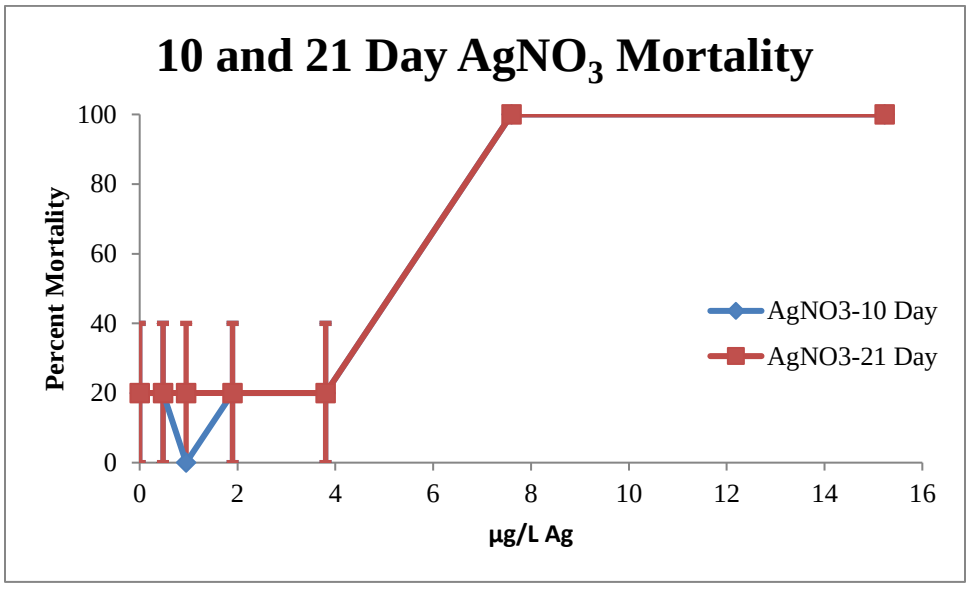

Figure 1.3 Comparison of 10 and 21 Day test *Daphnia magna* mortality with $AgNO_3$

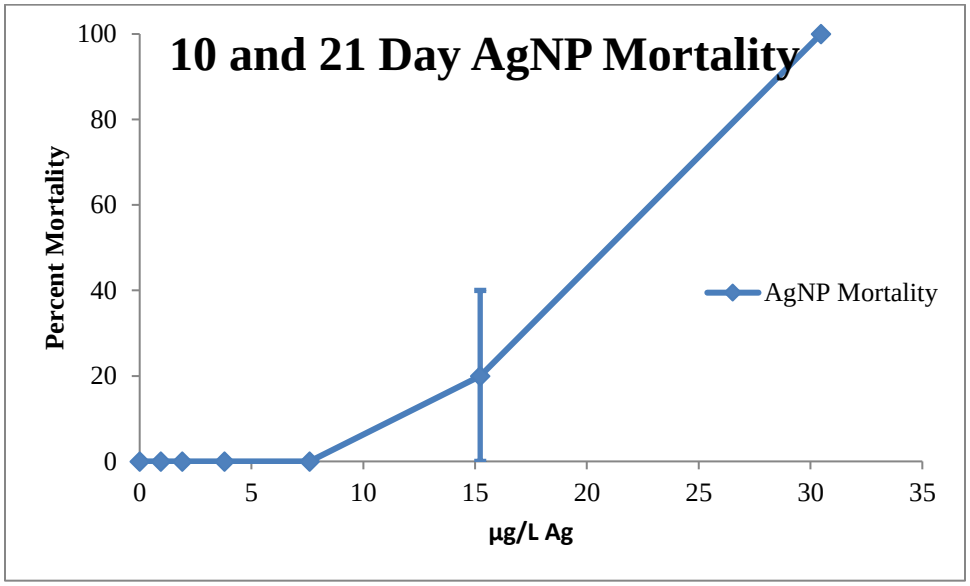

Figure 1.4: Mortality of *Daphnia magna* exposed to AgNP during 10 and 21 day tests. The mortality was the same for both tests with all deaths occurring within the first 10 days.

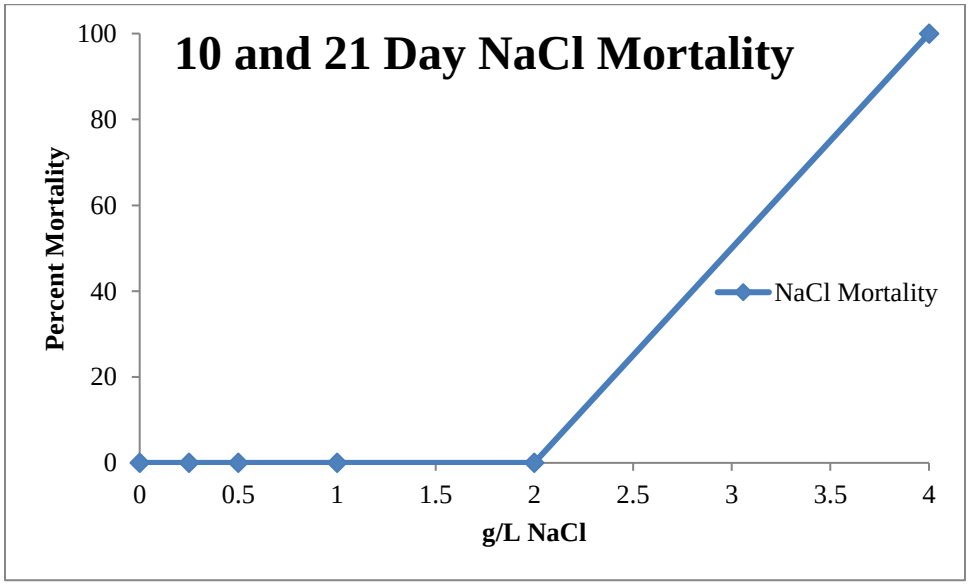

Figure 1.5: Mortality of *Daphnia magna* exposed to NaCl during 10 and 21 day tests. The mortality was the same for both tests with all deaths occurring within the first 10 days.

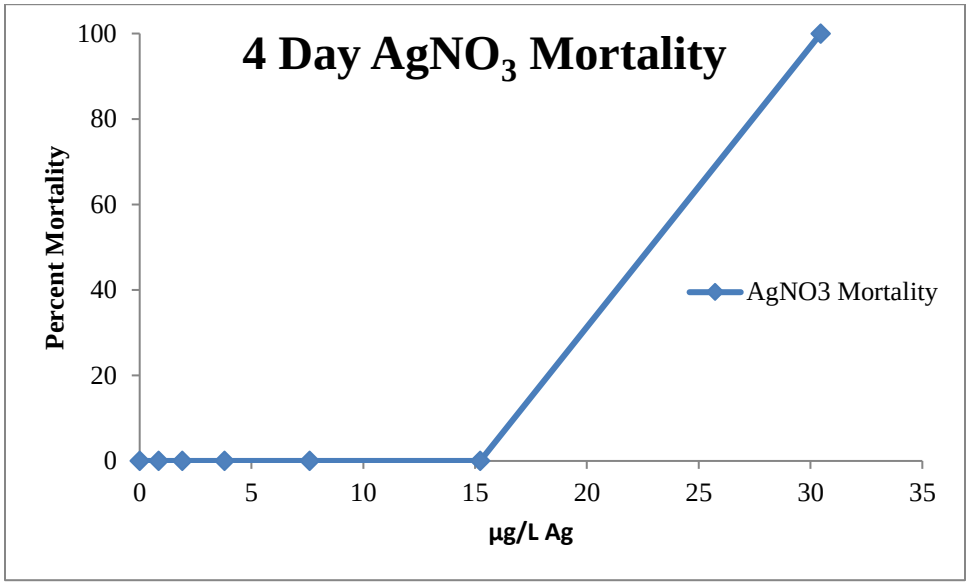

Figure 1.6 *Daphnia magna* mortality when exposed to $AgNO_3$ during 4 day test.

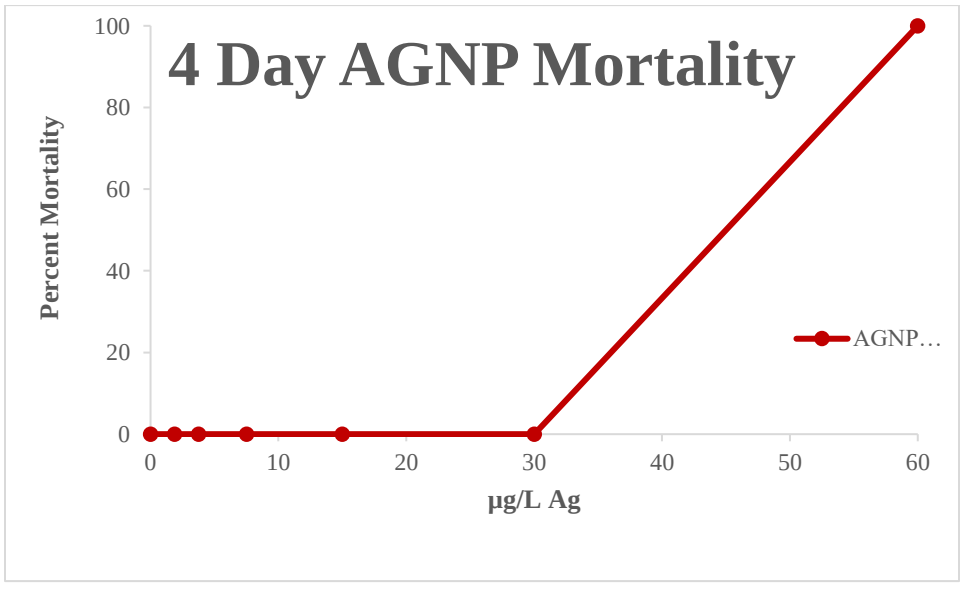

Figure 1.7 *Daphnia magna* mortality when exposed to AgNP during 4 day test.

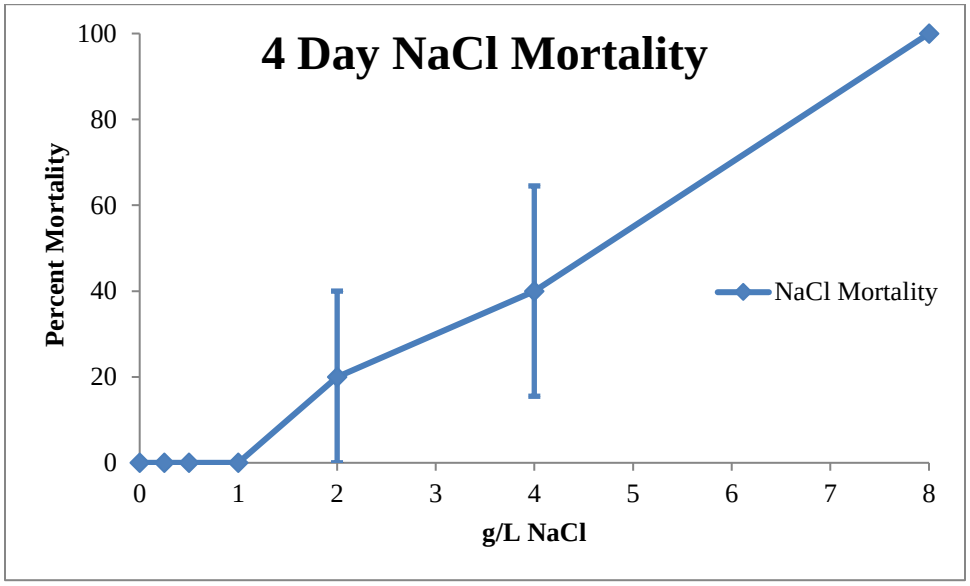

Figure 1.8 *Daphnia magna* mortality when exposed to NaCl during 4 day test.

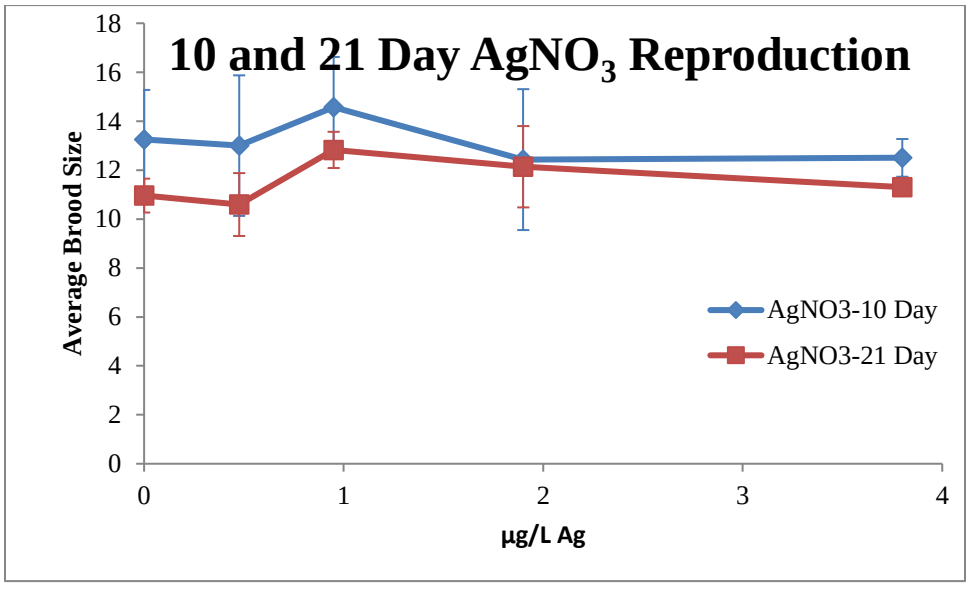

Figure 1.9 Comparison of reproduction measured when *Daphnia magna* are exposed to $AgNO_3$ in a 10 and 21 day reproduction assays.

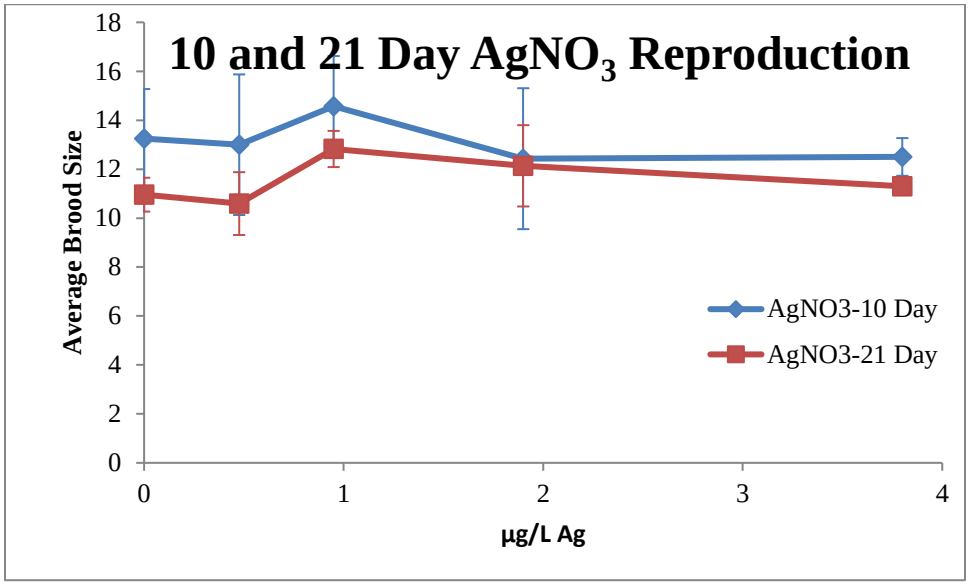

Figure 1.9 Comparison of reproduction measured when *Daphnia magna* are exposed to $AgNO_3$ in a 10 and 21 day reproduction assays.

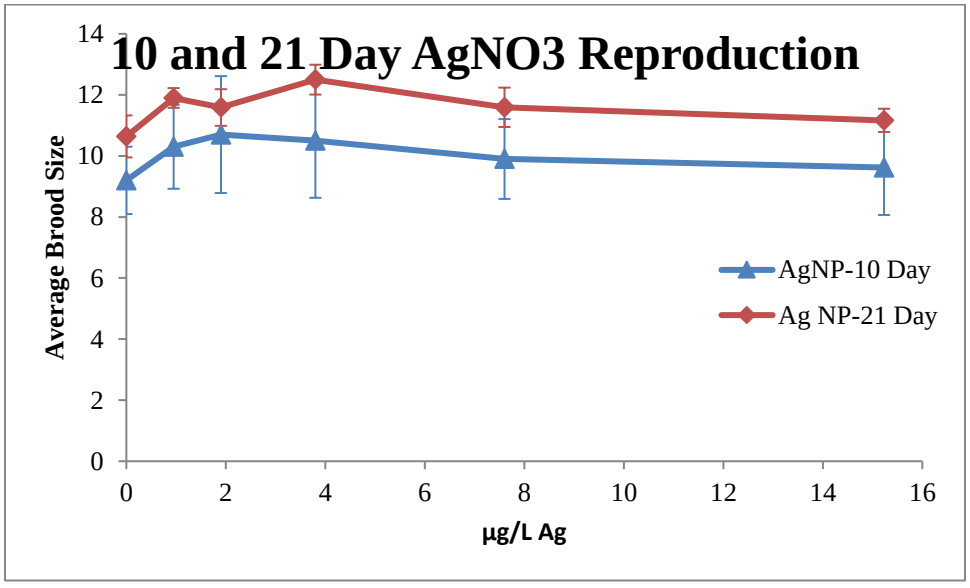

Figure 1.10 Comparison of reproduction measured when *Daphnia magna* are exposed to AgNP in a 10 and 21 day reproduction assays.

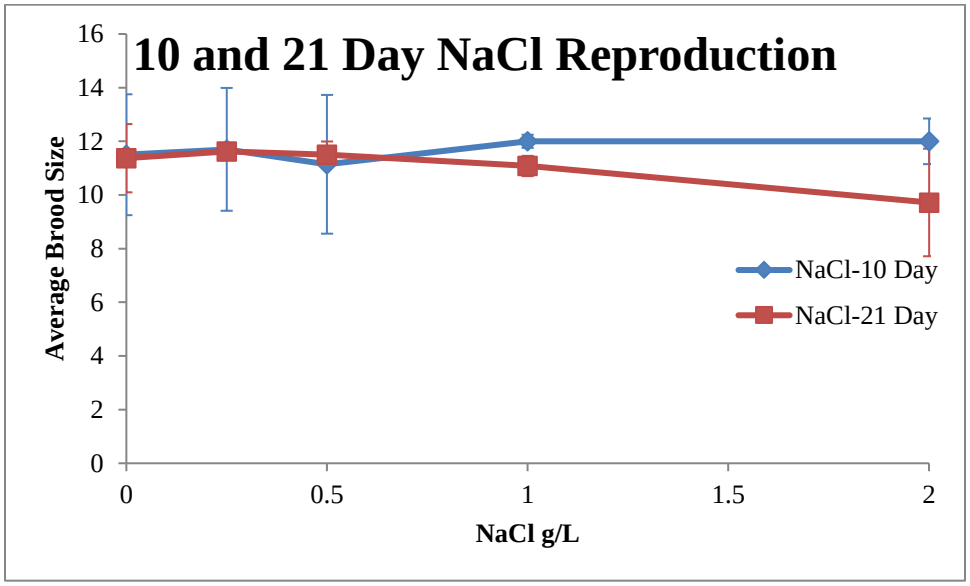

Figure 1.11 Comparison of reproduction measured when *Daphnia magna* are exposed to NaCl in a 10 and 21 day reproduction assays.

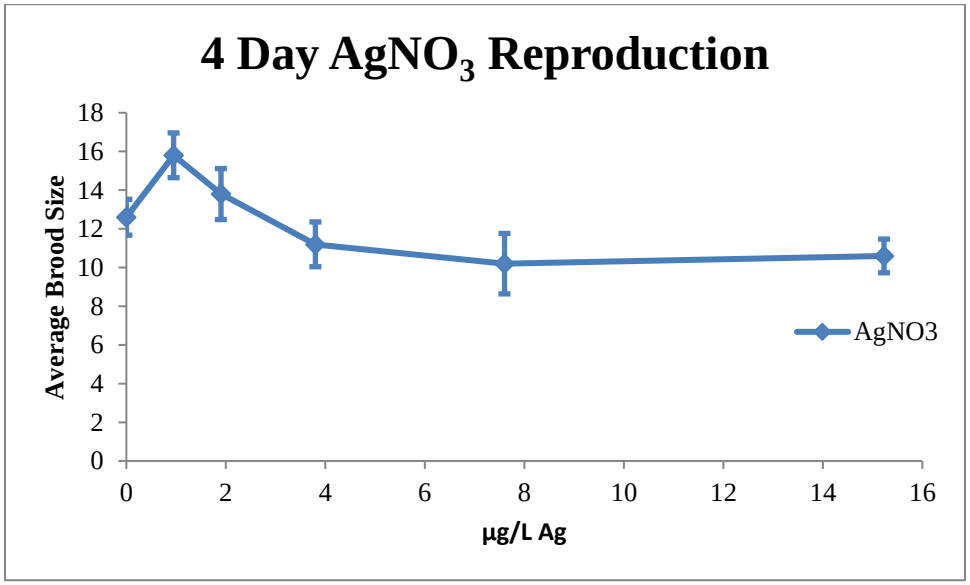

Figure 1.12 *Daphnia magna* reproduction when exposed to $AgNO_3$ during 4 day test.

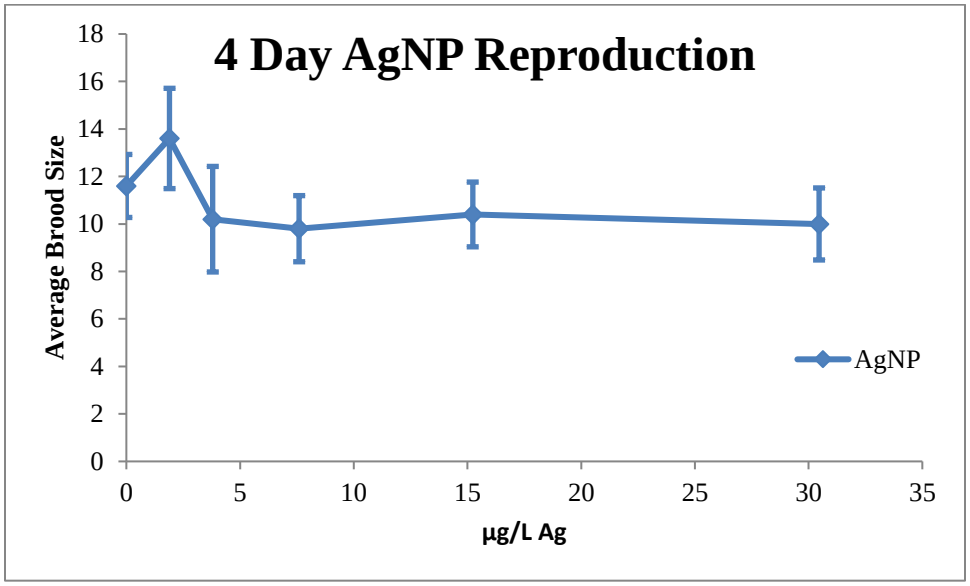

Figure 1.13 *Daphnia magna* reproduction when exposed to AgNP during 4 day test.

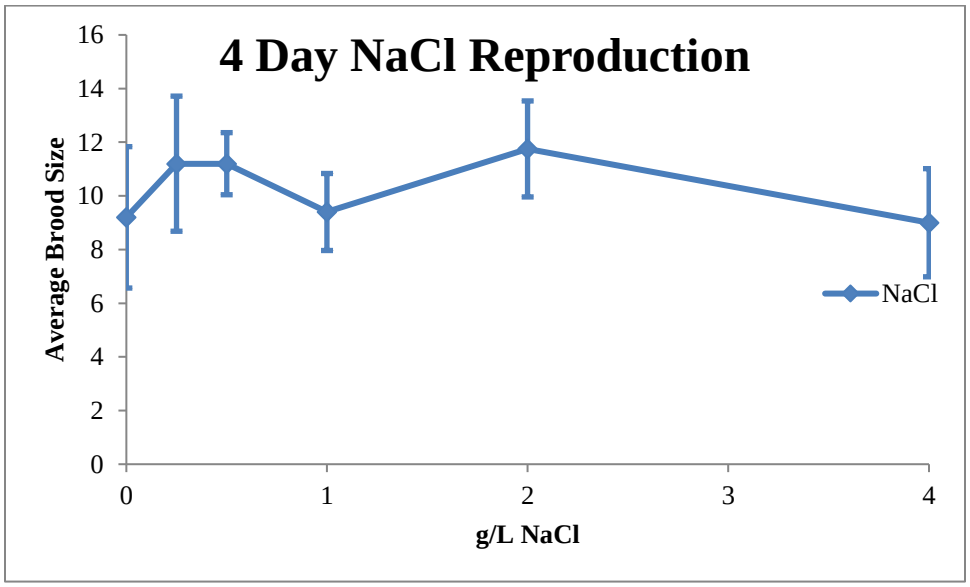

Figure 1.14 *Daphnia magna* reproduction when exposed to NaCl during 4 day test.

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CHAPTER TWO

The Impact of Sulfidation on the Toxicity of Silver Nanoparticles to the Aquatic Cladoceran *Daphnia magna* in Wetland Mesocosms

Abstract

The manufacture and use of nanoparticles, already a billion dollar industry, is expected to grow as engineers continue to develop new products containing the materials. As these products grow in volume and variety, the volume of nanoparticles entering the environment is expected to grow in number. However, these materials pose novel ecological threats that were not previously observed in their core material, and researchers have begun to assess their toxicity in laboratory settings. Yet the relatively simple chemical matrix of artificial water used in their experiments does not replicate the expected conditions of biota when exposed to these materials. Therefore, this study examines the effects of exposure to a toxicant in a more natural and complex chemical matrix. After dosing *Daphnia magna* with mesocosm water containing nanomaterials aged one day, one week, and four weeks after introduction, we measured the mortality, reproduction, and growth in comparison to controls. As expected, the natural waters were protective of *Daphnia magna* exposed to silver nanoparticles at concentrations that exceeded the levels which exert acute toxicity in synthetic lab water. This protective effect increased as the material aged and may have provided complete protection after one week. Thus, further testing that uses other chronic indicators is warranted.

Introduction

Anthropogenic silver nanoparticles (AgNP) are an emerging environmental concern. Of over 1,000 products that claim to contain nanomaterials, approximately 25% claimed to contain silver (Ag) (EPA 2010). AgNP are known to have powerful antimicrobial properties (Li et al. 2008, Lok et al. 2006), and therefore are included in many consumer products that report to have medicinal qualities, including clothing, medicine, personal care items, and even bottled water (Wu et al. 2010, US EPA 2010). Although the use of this material is increasing, the impacts it has on the environment remain unclear (Bystrzejewska-Piotrowska et al. 2009). Without this information, it is difficult to develop risk assessments to determine safe levels of these materials and, if necessary, to implement new regulations.

At different points in their life cycle nanomaterials enter the environment: by their intended use in consumer products, through unintended releases such as spills, or during product degradation (Biswas and Wu 2005). A portion of these nanomaterials are discharged after use into wastewater systems and enter wastewater treatment plants. Some particles that pass through the system are discharged in effluent and enter the environment. Other particles are retained in the wastewater treatment plant. One study has observed this to be the fate of up to 85% of particles that are retained in wastewater treatment sludge (Kaegi et al. 2011). Although sludge is often applied on land, where particles enter the terrestrial environment, if that sludge is eroded by rain or wind, the particles can enter aquatic environments.

Concern about the environmental impacts of AgNP has prompted researchers to investigate its toxicity. In addition to being toxic to microbes, AgNP are extremely toxic

to aquatic invertebrates. This toxicity is a function of dissolved Ag+ (Newton et al. 2013), which causes ionoregulatory disturbance in aquatic invertebrates (Bianchini et al. 2002). *Daphnia magna* are especially sensitive to AgNP. One literature review has noted a median L(E)C50 of *D. Magna* to Ag nanomaterials at 40 µg/L (Kahru and Dubourguier 2010), although other studies have reported toxicity at lower concentrations. Newton et al. determined the LC50 for *D. Magna* as 3.16 µg total Ag/L, while Kim et al. observed behavioral effects at 1.0-1.4 µg/L (Newton et al. 2013, Kim et al. 2010).

More researchers are investigating nanomaterials, but the majority of their work has focused on gaining mechanistic insights by exposing organisms to nanomaterials under laboratory conditions using synthetic water (Colman et al. 2013). Few studies have examined the toxicity of these materials under realistic exposure conditions. Laboratory investigations using synthetic waters can demonstrate the mechanisms of toxicity, but they fail to account for the complexity and diversity of natural waters, which affects toxicity by modifying particles or altering their bioavailability through interactions with components of the water (McLaughlin et al. 2012) such as organic material. It is important to account for the effects of organic material because he research has suggested that organic materials present in natural waters provide a measure of protection against toxicity associated with Ag (Gao 2012). This results from the organic materials coating the nanomaterials and reducing their bioavailability by altering material surface properties (McLaughlin et al. 2012).

To replicate natural systems, many scientists have begun to use mesocosms in their research. Mesocosms are simulations designed by researchers to mimic natural environments and bridge the gap between laboratory systems and real world ecosystems.

They allow scientists to examine and influence environmental factors—including water quality, sediment content, biota diversity, and abundance—in order to mirror specific environments. These systems are more easily replicated than whole ecosystems, and they retain a degree of realism that is impossible to create in laboratory conditions (Odum 1984). By using mesocosms we can create environments in miniature that model real world ecological systems. In these environments, unlike in real world systems, we can add contaminates and otherwise modify the systems without concern for environmental damage.

In aquatic environments, AgNP are generally unstable over the long-term. They transform into Ag_2SNP when they age in natural waters (Lowry et al. 2012), and this transformation occurs in as little as 2 h in anaerobic portions of wastewater treatment plants (Kaegi et al. 2011). This sulfidation results in a marked decrease in Ag toxicity to *D. magna*, reducing acute toxicity by approximately 5.5 fold when environmentally relevant levels of sulfide are present (Bianchini et al. 2002 and 2008). Sulfidation can reduce available Ag+ and thus limit bioavailability, which results in a reduction of AgNP toxicity (Lowry et al. 2012, Choi et al. 2008, Choi et al. 2009, Reinsch et al. 2012).

This study has tested the toxicity of mesocosm waters dosed with AgNP and demonstrated how toxicity changes as the particles age. We have observed the reduction of toxicity in relation to the presence of the complex chemical matrix of natural waters and determined how this toxicity changes with time. We gathered this data by measuring mortality, growth, and reproduction endpoints using mesocosm water samples collected 1 d, 7 d, and 30 d after dosing. To test toxicity, we used the 4 d assay, which was developed and described in Chapter I, in addition to measuring growth parameters. This method was

optimal as it allowed completion of the assay by the end of the week in order to prepare the next samples for receipt. The manhour demands of a more traditional assay would have made it difficult to compare the effects of particles aged 7 d in the mesocosm with those aged 1 d.

A secondary goal of this experiment was to compare how the toxicity of AgNP differs when AgNP is added directly to a mesocosm and when samples of water are collected from the mesocosm and dosed in the lab. Previous research has suggested that the mesocosm waters dosed in the lab differ in toxicity compared to those dosed in the field (Bone et al. 2015). This difference indicates that factors beyond water chemistry may be present in the mesocosms and may alter the toxicity of the particles.

Materials and Methods

Site Description

The wetland mesocosm facility is located at Duke University's Forest Research Center in the Duke Forest, Durham, North Carolina, USA (Latitude 35° 58' 54" N, Longitude 78° 56' 33" W). The outdoor mesocosms were approximately 3.66 m long, 1.22 m wide, and 0.8 m deep. They contained an approximate 13° slope on the bed (Figure 2.1) and had treated wood frames. The slope allowed researchers to simulate terrestrial and aquatic or semiaquatic environments. The interior lining was made of 0.45 mm geotextile reinforced with polypropylene (Firestone Specialty Products, U.S.). The soil was a blend of "Sandhills" from Lemon Springs, NC, USA and "Clay" and "Topsoil" from Durham, NC, USA. It was provided by Soils and Sand (Durham, NC, USA). The final soil texture was 63.9% sand, 28.3% silt, and 13% clay, with 5% lost on ignition. An onsite well

provided groundwater for the mesocosms. Flora planted in the mesocosms included soft rush (*Juncus effuses*), large-flowered waterweed (*Egeria densa*), waterthread pondweed (*Potamogeton diversifolius*), and duckweed (*Landoltia punctate*). Algae and zooplankton were obtained by collecting 250 ml of unfiltered water from a nearby wetland and adding it to the units. Fauna stocked in each mesocosm included American bullfrog tadpoles *Rana* (*Lithobates*), *catesbeian*, eastern mosquitofish (*Gambusia holbrooki*), and benthic invertebrates. In addition, Cope's tree frog tadpoles (*Hyla chrysoscelis*) self-colonized the mesocosms. Organisms were given 40 to 90 d to acclimate to the mesocosms before dosing began. A more detailed description of the mesocosm setup and design is available in Colman et al. (2018).

Experimental Treatments

Nanoparticle additions began on August 13, 2013. Three mesocosms were assigned to each treatment group as well as the control. Three groups were press additions, including AgNP (24 µg/L) $n = 3$, sulfidized silver nanomaterials (Ag_2SNP) (24 µg/L) $n = 3$, and gold nanomaterials (AuNP) (50 µg/L) $n = 3$. Moreover, three mesocosms received a one-time pulse addition of 450 mg AgNP (initial concentration, ~1.25 mg Ag/L). Ag_2SNP were added to the mesocosm to simulate aged nanomaterials. AuNP were added to serve as a particle size control in order to ensure that the Ag in the nanomaterial, not its size, was the cause of any observed toxic effects. Water samples were collected from the control and the pulse-treated mesocosms at 1 d, 1 week, and 1 month post treatment. Water samples were collected from the Ag_2SNP and AuNP press-treated wetlands 1 month post treatment.

Culture Methods

D. magna were obtained from established lab cultures at Baylor University's Ecotoxicology and Aquatic Research Laboratory. Cultures were maintained as described by Kolkmeier and Brooks (2013), with modifications as described. The *D. magna* cultures used in these experiments were transitioned from EPA hard water to EPA moderately hard water (MHW) (EPA 2002) in order to acclimate the culture to the softer waters they would be exposed to in another experiment. Cultures were maintained in glass 500-mL beakers filled with MHW at a density no greater than 15 individuals per beaker. Less than 24 h old neonates were removed from the main culture and raised in the same manner until they were 4–5 d old. At that point, they were used for the assay. Cultures were fed daily a liquid mixture of the algal species *Pseudokirchneriella subcapitata* and *Ceriophyll sp*. grass extract. They were renewed with fresh MHW once every 48 h. Cultures were maintained in a climate controlled incubator at 25 ± 1 ºC with a 16:8 light to dark photoperiod.

Particle Synthesis

Synthesis of AgNP (gum arabic-coated Ag nanoparticles). Nanoparticles were provided for this experiment by the Center for Environmental Implications of Nanotechnology (CEINT). The nanoparticle synthesis methods were first described by Yang et al. (2011), as follows: First, 271 mL of reverse osmosis deionized water, 9 mL of 10g/L gum arabic, and 9 mL of 0.1 M $AgNO_3$ were added to an Erlenmeyer flask, and the solution was stirred for 5 min; simultaneously, 11 mL of 0.08 M ice-cold sodium borohydride was added and stirred for an additional 10 min; multiple batches were combined, and the nanoparticles were purified and concentrated by dialysis (Optiflux

F200NR Fresenius Polysulfone Dialyzer, Fresenius Medical Care); lastly, the suspension was diluted with water and concentrated two additional times in order to obtain the final product (Yang et al. 2011).

Synthesis of Ag_2SNP (GA-coated Ag_2S nanoparticles). Ag_2SNP particles were prepared using a modified published procedure (Djokovic et al. 2009). First, AgNP nanoparticles were synthesized as described above. Then, 5 mL of 0.18M thioacetamide was added to the unpurified AgNP, and the suspension was covered and stirred for 24 h. Multiple batches were combined, and the nanoparticles were purified and concentrated by dialysis (Optiflux F200NR Fresenius Polysulfone Dialyzer, Fresenius Medical Care). The suspension was diluted with water and concentrated two additional times in order to obtain the final product.

Synthesis of AuNP (gum arabic-coated Au nanoparticles) In an Erlenmeyer flask, 271 mL of water, 9 mL of 10 g/L gum arabic, and 9 mL of 0.1 M hydrogen tetrachloroaurate trihydrate were combined and stirred for 5 min. Then, 11 mL of 0.08 M ice-cold sodium borohydride was added, and the solution was stirred for an additional 10 min. Several batches were combined, and the nanoparticles were concentrated and purified by dialysis (Optiflux F200NR Fresenius Polysulfone Dialyzer, Fresenius Medical Care). The suspension was diluted with water and concentrated two additional times in order to obtain the final product.

Mesocosm Exposure

After the nanoparticles were added to the wetland mesocosm, the wetland water/nanoparticle mixture was collected at 1 d, 1 week, and 4 weeks post-treatment and was shipped, with freezer packs, to Baylor University. Prior to the sample shipping, less than 24 h old neonates were removed from the main culture and raised until they were approximately 4–5 d old, at which point they were used for the assay. *D. magna* adults were exposed to concentrations of 100%, 75%, 50%, 25%, 12.5%, and 6.25% wetland water, diluted with synthetic MHW. Each adult was individually placed in a polystyrene cup containing 25 ml of wetland/MHW mixture and with 0.6 ml of the food used to feed the culture. The cups were covered with a sheet of clear plexiglass (Polymethyl methacrylate) and incubated at 25 ºC with a light to dark cycle of 16:8 h. Individuals were fed, and the number of neonates produced by each individual was recorded daily. Water changes were conducted after 48 h, using the same concentrations of wetland/MHW with which the individuals were initially dosed. Four days after the experiment began, the *D. magna* were removed, cleaned by immersion in deionized water, dabbed dry using Kimwipes, and oven-dried for future growth and uptake analysis. Finally, survival, reproduction, and growth were assessed as an indicator of toxicity to the mesocosm water/nanomaterial mixture.

Lab Spike

Duke University provided the control mesocosm water and a sample of the AgNP that was used to dose the mesocosms. The average Ag concentration in pulse-dosed mesocosms was measured at approximately 437.8 µg/L. The waters spiked in the lab received AgNP sufficient to match this concentration. Then, the controls and the dosed

waters were wrapped in foil for 48 h in order to replicate the effects of shipping for the mesocosm dosed water. Afterward, the water was stirred with stir bars to ensure that any settled material was equally redistributed throughout the samples. From this point forward, the conditions and procedures used to dose *D. magna* with these waters were identical to the ones that used mesocosm-spiked water.

ICP-MS

In preparation for ICP-MS analysis, previously dried and weighed *D. magna* were combined into single samples based upon the concentration of dosed wetland water to which they were exposed. Samples were digested using a 3:2:1.5 mixture of 65% nitric acid (TraceUltra grade, Sigma Aldrich) and 35% hydrogen peroxide (Reagent grade, Sigma Aldrich), in addition to 37% hydrochloric acid (TraceUltra grade, Sigma Aldrich). First, nitric acid and peroxide were added to the sample and heated in capped, acid-washed, 50 mL borosilicate glass culture tubes for 60 min at 90 °C in an AIM500-C block digestion system (SEAL Analytical Inc., Mequon, W, USA). An additional aliquot of hydrochloric acid was added to the digestate and heated to 90 °C for an additional 60 min. Cooled digests were diluted to 4% HNO_3, 2% H_2O_2, and 1% HCl, with ultrapure water (18.2 MΩ) and were allowed to settle overnight. Aliquots of the supernatant were further diluted with a diluent of 4% HNO_3, 2% H_2O_2, and 1% HCl in ultrapure water, as needed. Elemental Ag and Au concentrations were determined in the digested samples using inductively coupled plasma mass spectrometry (ICP-MS) at Baylor University on an Elan 9000 ICP-MS (Perkin Elmer, Waltham, MA, USA). Detection limits for Ag and Au were 0.0067 µg/L and 0.0158 µg/L, respectively. Analytical runs included duplicate samples, reagent blanks, spike recovery samples, and inter-calibration or cross-calibration verification samples. Ag

spike recovery averaged 101.8 +/- 2.8%, n = 4. The mean relative percent difference between duplicate samples was 1.47% +/- 1.88%, n = 4.

Results

Uptake

IC-PMS was used to measure Ag concentration in all concentrations of dosed d 1 survivors as well as the 100% concentrations of dosed d 1 and d 30 survivors. Ag concentrations in *D. magna* were similar between populations at the 6.25% and 12.5% concentrations. However, at the 35% concentration and higher, a pattern was observed in which Wetland 18 individuals contained more than twice as much Ag as Wetland 1 individuals at the same concentration. The Ag concentrations of Wetland 20 individuals closely matched the average concentrations of Wetland 18 and Wetland 1 individuals. Moreover, d 1 Ag concentrations for *D. magna* exposed to the three pulse-dosed wetlands increased with dose until they reached their maximum concentration at the 50% concentration. The means of Wetlands 1, 18, and 20 equaled 216.64, 549.65, 401.94 µg/kg Ag, respectively. After that point, concentrations declined at 75%, with mean concentrations for Wetlands 1, 18, and 20 equaling 206.07, 516.07, and 326.13 µg/Kg respectively. As the 100% concentration was based on one survivor from Wetland 18, it was not sufficient to make an observation; however, it was included in order to present a complete dataset (Figure 2.2). Compared to concentrations measured in d 1, Ag body burdens measured from d 7 survivors dropped to an average of 67.6 mg/kg. By d 30, these concentrations declined to 10.2 mg/kg (Figure 3.3).

Mortality

Mortality was recorded daily for the duration of the experiment. While mortality was observed from all three time points when individuals were exposed to nanomaterials, statistically significant mortality was only observed in individuals exposed to d 1 mesocosm water (Figures 2.4–2.6). This mortality was only significantly different from the controls after the 75% concentration was reached (Student's t-test, $p = 0.0015$), with the LC50 at a concentration of 72.81% wetland water. This observed mortality translates to an LC50 of 318.76 µg/L AgNP.

Individuals that were dosed in lab-spiked mesocosm water did not experience any mortality until they reached 75% concentration. However, mortality was high (80%) and significantly different from the controls (Student's t-test, $p = 0.0001$). The LC50 was at a concentration of 65.99% lab-spiked water, which translates to an LC50 of 268.95 µg/L AgNP. No individual survived 100% concentration (Figure 2.7).

Reproduction

Reproduction was measured by counting the number of live neonates for each individual and was recorded daily. Per EPA guidelines, only the neonates of individuals that survived the experiment were used to calculate toxicity thresholds (EPA 1996). In organisms exposed to d 1 mesocosm water, reproduction declined at each concentration. However, this reduction only became statistically significant when compared to controls at the 50% concentration (Student's t-test $p = .001$). At this concentration, the nominal Ag concentration was 218.9 µg/L. Reproduction reported at the 100% concentration resulted from a single surviving individual and was not included in the statistical analysis (Figure

2.8). The calculated EC 50 for reproduction was 59.27% wetland water, which translates to an EC50 of 259.48 µg/L AgNP.

No statistically significant differences were observed in individuals dosed with 7 d or 30 d mesocosm water when compared to their respective controls (Figures 2.9 and 2.10).

Unlike individuals exposed to mesocosm water dosed in the field, those exposed to mesocosm water dosed in the lab did not experience reproductive impacts that were significantly different from controls until the 75% concentration (Student's t-test p = 0.00001) (Figure 2.11).

Growth

Average growth was measured by calculating the dry mass of individuals at the end of the experiment, after they were preserved. Individuals exposed to d 1 pulse-dosed mesocosm water did not present significant differences in mass compared to controls until the 50% concentration was reached (Student's t-test p = .0028). Although the mass would have increased between 75% and 100%, because the mass of the 100% concentration was that of one surviving individual, it has not been statistically analyzed in comparison to controls (Figure 2.12).

The average growth of individuals exposed to d 7 mesocosm water was close to that of the controls, as no statistically significant differences were observed at any concentration (Figure 2.13).

The growth of individuals exposed to d 30 pulse-dosed mesocosm water was consistently greater than that of controls at all concentrations. However, this difference was only significant at the 6.25%, 75%, and 100% concentrations (Figure 2.14)

Discussion

Mesocosm Uptake

Several patterns were observed during uptake data analysis. First, Ag concentrations in *D. magna* were comparable between each wetland at the 6.25% and 12.5% concentrations. However, at the 25% concentration and above, wetland-specific patterns in Ag body burdens were observed. More specifically, individuals exposed to waters from Wetland 18 had twice the Ag body burden as individuals from Wetland 1. Individuals from Wetland 20 had a body burden that was similar to the mean of these two mesocosms. Part of this uptake pattern is explained by an examination of the Ag concentrations measured in each wetland. Ag levels measured in the water samples were 365.4, 505.8, and 442.2 µg/L for Wetlands 1, 18, and 20 respectively. This results in an average of 437.8 µg/L. However, the concentrations in water are insufficient to explain these differences. While Ag water concentrations in Wetland 1 were approximately 72% of Wetland 18, body burden levels in Wetland 1 were 30–39% of Wetland 18's levels, starting at the 25% concentration.

Water chemistry may explain a portion of this difference. Factors such as the ionic strength (Yang et al. 2013), pH (Liu and Hurt 2010), and organic material content (McLaughlin and Bonzongo 2012) of the water can affect nanoparticle toxicity by altering 1) the stability of the particles in solution, 2) the release of Ag^{+}, or 3) the bioavailability or bioreactivity of the particles. Variations in these factors between wetlands can result in changes in the uptake of the particles. Due to differences in Ag concentration, the water chemistry may have affected the stability of the particles. However, at the time of the experiment these parameters were similar between wetlands, and the small differences may

not have accounted for the observed difference in body burden. One chemical parameter that explains the difference is variation in levels of reactive sulfide. Bianchini et al. have observed that while reactive sulfide provides a protective effect against Ag toxicity, individuals exposed to AgNP and reactive sulfides have higher body burdens than individuals exposed only to Ag (Bianchini et al. 2005). They have suggested that the increase results from higher levels of ingestion of Ag after it has been bound into a ligand. Thus, variations in mesocosm sulfide levels may account for the differences in uptake between wetlands. While resulting in increased body burdens, sulfide still protects against Ag toxicity. This variance in sulfide concentration may explain the similarity in toxicity among wetlands at the same concentration, despite variances in body burden.

If this pattern was only a result of differing concentrations in mesocosm water, it would appear at each concentration. However, it did not occur at the 6.25% or 12.5% concentrations. At these levels, the body burden in each mesocosm was similar, which can be attributed to the organisms' ability to depurate Ag quickly enough to reduce any significant variation in available Ag between the wetlands.

Due to the small size of the organisms, we pooled the samples for analysis. This prevented a more detailed statistical comparison of the wetlands. However, measurements of Ag in the collected water samples demonstrate a pattern in the measured uptake of d 1 individuals. While the Ag concentration increased until the 50% level was reached, after that point, measured Ag concentration leveled off with a slight decreasing trend. The low levels of Ag observed at 100% were measured from a single surviving individual, which may have been an outlier. To our knowledge, no other study has been conducted on *D. magna* using these methods. As such, it is impossible to compare uptake levels with those

measured in other studies. Nevertheless, other researchers have suggested that the presence of humic substances reduces Ag uptake (Glover and Wood 2004).

Mesocosm Exposure Toxicity

In d 1 waters we observed significant differences starting at the 75% concentration in mortality between mesocosm control and nanoparticle-dosed water, resulting in a 46.7% mortality rate in dosed individuals. The nominal concentration of Ag concentration in this water was estimated at 328.1 µg/L. As the median LC50 value of *D. magna* to nanoparticles was reported at 40 µg/L (Kahru and Dubourguier 2010), this represents a drastic increase in survival when individuals were exposed in the wetland water compared when they were exposed in lab water.

In addition, no significant acute toxicity was observed when organisms were exposed to pulse-dosed mesocosm water aged 1 week and 4 weeks. Although some mortality was observed, no statistically significant differences were observed between controls and dosed individuals at any concentration. The observed mortality appeared incidental and random, with no pattern from which a trend could be observed. Therefore, we have categorized these deaths as random variation and have not attributed them to toxicity from mesocosm water. The same reasoning applies to the press AgNP-, Ag_2SNP-, and AuNP-dosed individuals. In this case, some incidental toxicity was observed, but it was not statistically significant and demonstrated no pattern that would suggest a trend.

Reproductive and growth toxicity was also observed in d 1 individuals with significant differences between mesocosm control and nanoparticle doses water starting at the 50% concentration (p = .001 and .0028 for reproduction and growth, respectively). This translates into an Ag concentration of 218.9 µg/L, which is higher than the concentrations

at which reproductive and growth toxicity have been observed in other studies. As such, the mesocosm water had a protective effect.

This drop in toxicity likely results from the decrease in Ag concentrations in the water column, the presence of organic material, and the AgNP sulfidation. Measurements of Ag concentrations in water samples have demonstrated a precipitous drop from the average high of 385.96 µg/L in the d 1 50% concentration to 67.62 and 10.19 µg/L in the 100% concentrations of Weeks 1 and 4, respectively. This represents a decline Ag greater than 82% and 97%, respectively. These concentrations are below the level that was associated with toxicity in d 1 organisms. However, other protective effects from the water chemistry matrix may further explain the drop in toxicity.

During d 1 exposures we also observed a trend towards increased reproduction in pulse-dosed individuals exposed to the 6.25% concentration wetland water compared to controls. Although this trend was not statistically significant, the difference resulted in a 40% increase in reproduction. This outcome was not unexpected; other researchers have noted similar increases in reproduction when Ag exposed individuals are compared to controls, and increases in weight have been noted as well (Glover and Wood 2004).

Organic material in water has been suggested to reduce the toxicity of AgNP (McLaughlin and Bonzongo 2012). More specifically, researchers have observed that AgNP in natural water with a low ionic strength/dissolved organic carbon ratio had an LC50 of 221 ppb. However, AgNP in natural water with a high ionic strength/dissolved organic carbon ratio had an LC50 of 0.433 ppb. This variance in toxicity highlights the importance of understanding water chemistry when making AgNP toxicity assessments (Mclaughlin and Bonzongo 2012).

Gao et al. have studied the adsorption of humic acid onto powered AgNP at neutral pH and the effects of adsorption on dispersion stability, Ag dissolution, and aquatic toxicity of the AgNP suspensions. The same amount of AgNP was mixed with different concentrations of humic acid in order to model these parameters. They observed that when the total organic carbon content of the solution was less than 10 mg/L, suspended Ag content in the system increased. This increase was likely due to increased dispersion. However, total Ag content decreased with concentrations of total organic carbon greater than 10 mg/L. As such, they proposed that this was due to an increase in nanoparticle agglomeration and settling. When *D. magna* were exposed to the AgNP, a linear decrease in toxicity was observed with increasing total organic carbon. While Gao et al. have not outlined a precise mechanism for this decrease in toxicity, they have suggested that it results from the organic material serving as a free radical scavenger or from a function of the complexion of Ag+ reducing biological availability (Gao et al. 2012).

In addition, sulfidation affects the reduction of toxicity. As previously explained, the sulfidation of AgNP results in a marked decrease in toxicity (Bianchini et al. 2002 and 2008). This trend has been observed when comparing the toxicity of sulfidized Ag nanomaterials to pristine nanomaterials, duckweed (*Lemna minuta*) and killifish (*Fundulus heteroclitus*) embryos. Although 3% of the Ag nanomaterials were sulfidized, the EC and LC0 values for these two species increased by an order of magnitude (Levard et al. 2013). Recent research has suggested that the sulfidation of AgNP in wastewater treatment plants occurs rapidly, starting at 2 h (Kaegi et al. 2011) and with complete sulfidation occurring within 7 d (Kent et al. 2014). One study has investigated the rate of sulfidation of AgNP in mesocosm wetlands, however, it was conducted over an 18-month period. The researchers

observed extensive but incomplete sulfidation of AgNP, and the rate that they calculated is lower than what others have determined from laboratory conditions (Lowry et al. 2012). In complex environments, such as those simulated by mesocosms, it is possible for multiple factors to combine and leverage an effect on toxicity. In the case of AgNP, we believe it is likely that the aging, sulfidation and decreased concentration of the particles in mesocosms water may have all influenced toxicity. These factors would help explain the decrease of acute and chronic toxicity observed between d 1 results and later results.

Lab-spiked vs. Field-spiked

Individuals exposed to mesocosm water spiked with nanoparticles in the lab did not experience mortality until the 75% concentration. However, at that concentration mortality was at 80%. While these results indicate a greater trend towards mortality than the field water at the same concentration (46.7%), the lab-spiked mesocosm water was not statistically different than the field-spiked water. Furthermore, the LC 50s were comparable (65.99% for lab-spiked vs. 72.81% for field-spiked).

In terms of reproduction, the lab-spiked water was less toxic than field-spiked water. Although at the 50% concentration significant differences were observed between the control and field-spiked individuals, at the 75% concentration lab-spiked individuals were only significantly different from the controls. As previously mentioned, the fluctuating levels of reproduction in the controls make calculating LC50s impractical. This result contradicts results from earlier unpublished work and suggests that for chronic toxicity, waters spiked in the field are more toxic than those spiked in the lab.

This toxicity difference may be attributed to the waters used for the experiment. While the chemistry of all mesocosms was similar, subtle differences may have granted

certain wetlands greater protection against nanoparticle toxicity than others. Nanoparticle toxicity is easily affected by the matrix in which the particles are exposed to organisms. However, our analysis of the ionic strength, pH, and organic material content of the mesocosm suggests that these parameters are similar and thus, the chemical composition of these parameters is unlikely to account for differences in toxicity. Therefore, different parameters in the chemistry, or some other factors that cannot be identified, may be result in differences in toxicity.

Control reproduction variability could also affect comparative toxicity in field vs lab spiked individuals. In terms of control reproduction natural variation resulted in a field-spiked average control reproduction of 12.66 ± 2.73 at 50% concentration. The average in the same concentration of control water for the lab-spiked experiment was 7.6 ± 0.50. Despite this difference, a student's t-test comparing dosed individuals indicated no significant difference in reproduction between these tests. The controls, on the other hand, were significantly different between the field and lab experiments (p = .006). Thus, a large degree of this difference results from the reproduction values in MHW, as the difference in those concentrations was the only one that was statistically significantly (p = .0015). When the MHW values are removed from the analysis, a statistically significant difference is no longer present between the controls. The low level of reproduction in the MHW controls likely results from 4 of the 15 individuals failing to produce offspring by the end of the test.

Conclusion

The goal of this study was to examine the toxicity of AgNP when exposed to *D. magna* in natural waters. Our study of the toxicity of AgNP-dosed mesocosm water has

revealed significant acute toxicity during d 1. In addition, despite failing to determine reproductive impacts when testing AgNP in lab water, significant reproductive impacts were observed with d 1 mesocosm water. Acute and chronic toxicity disappeared by d 7. Measurements of AgNP in the dosing water have demonstrated a decline in Ag concentrations between d 1 and d 7. We suggest that this decline, in addition to the particles' aging, are responsible for the toxicity drop.

The concentration of AgNP at which toxicity was observed in mesocosm water was close to an order of magnitude more than the concentrations that other researchers have reported for dosing in lab water. This result confirms the conclusions of other researchers: organic material has a significant impact on AgNP toxicity. Our research indicates that when dosed in natural water matrices, AgNP toxicity is drastically reduced. This finding is important in order to understand the potential hazards of AgNP exposure to aquatic invertebrates in likely exposure scenarios since silver has been observed to be a problematic material in aquatic ecosystems. In addition, by understanding the true toxicity that organisms may be expected to face when exposed to materials in realistic exposure scenarios, researchers can more accurately calculate levels of pollutants that could be considered dangerous to the environment. For some pollutants, the effect of natural waters is likely to be less significant than others or could have somewhat unpredictable effects on toxicity. Thus it is important that further research be conducted to study the effect of natural waters not only on AgNP, but other toxicants as well.

Figures

Figure 2.1 Duke Mesocosm Facility

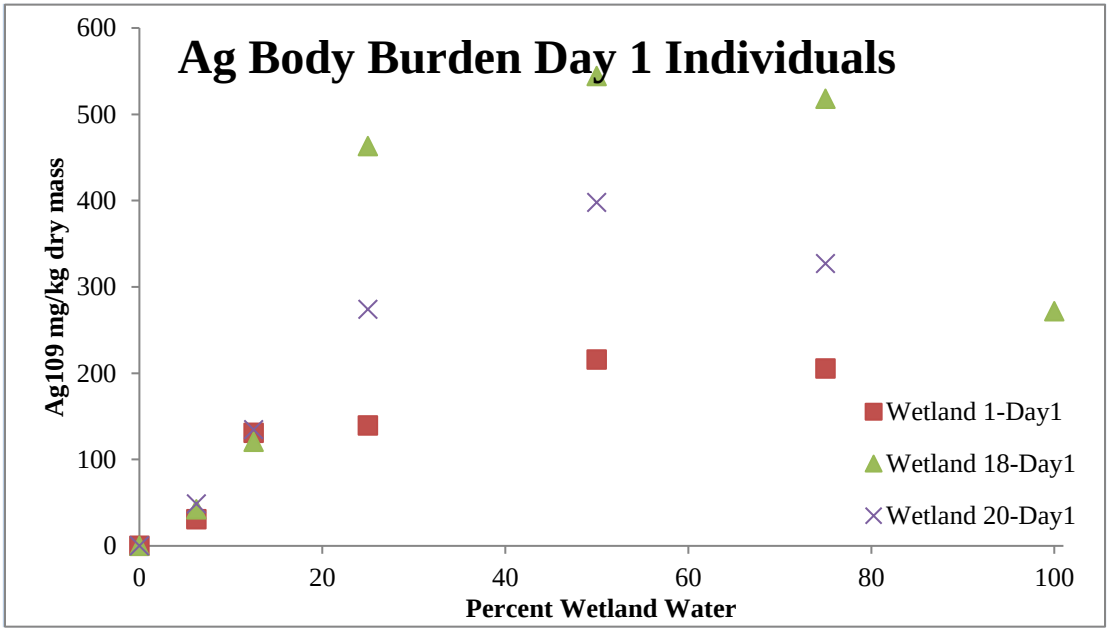

Figure 2.2 Ag uptake in day 1 individuals by wetland.

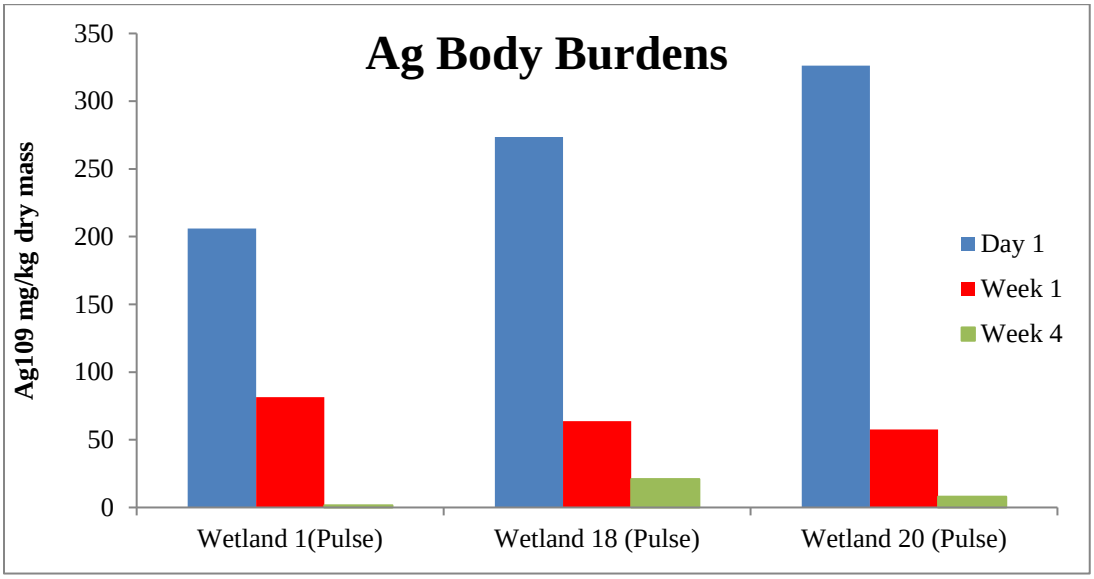

Figure 2.3 Maximum measured Ag uptake in each wetland at each time point

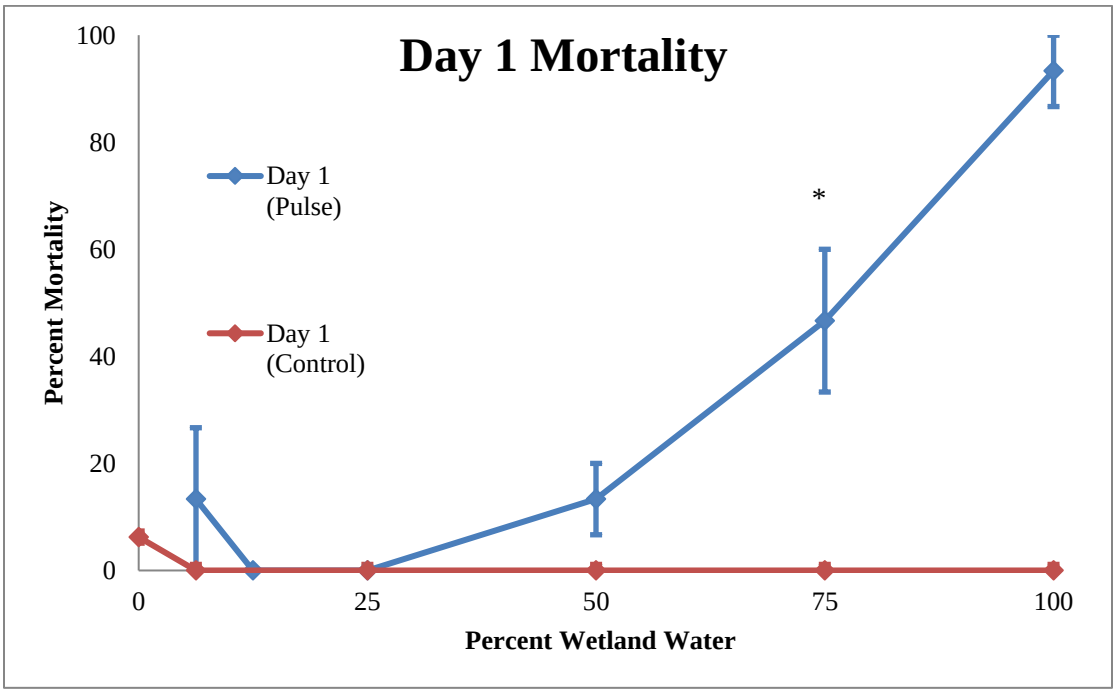

Figure 2.4 Comparison of mortality in Day 1 pulse and control dosed *Daphnia Magna.*

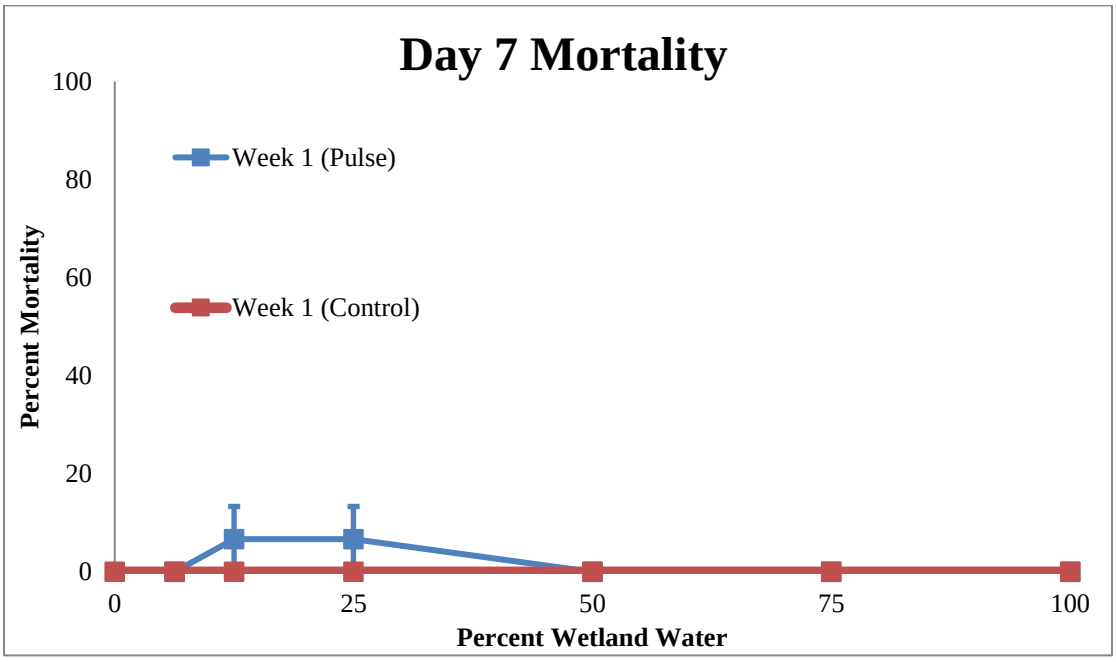

Figure 2.5 Comparison of mortality in Day 7 pulse and control dosed *Daphnia Magna.*

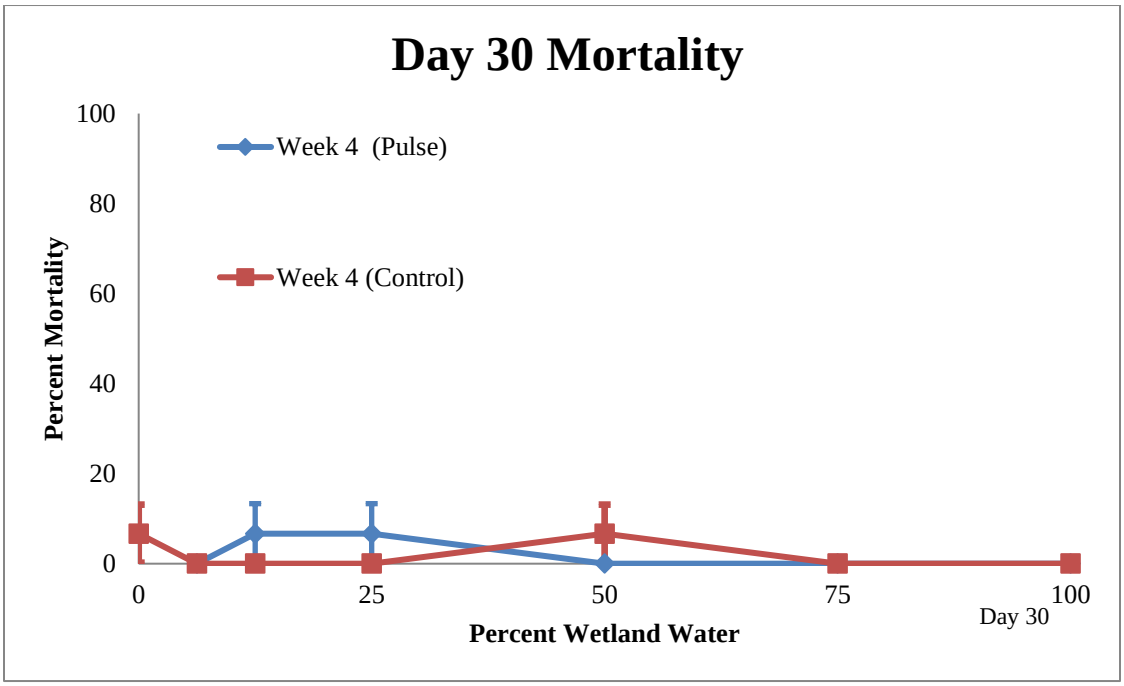

Figure 2.6 Comparison of mortality in Day 30 pulse and control dosed *Daphnia Magna.*

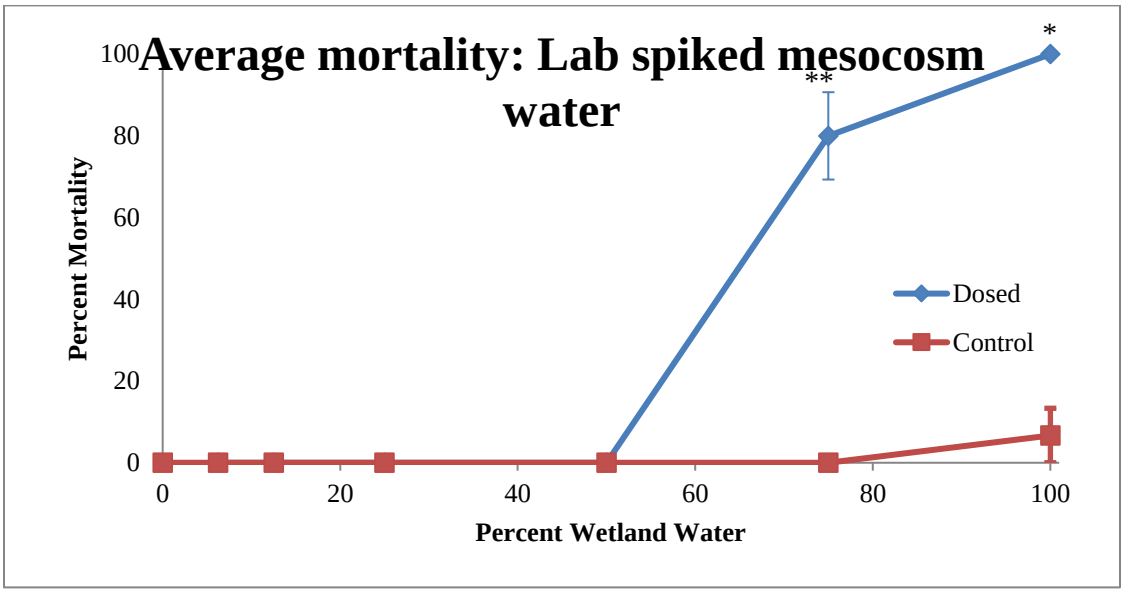

Figure 2.7 Mortality of Lab Spiked Mesocosm Water

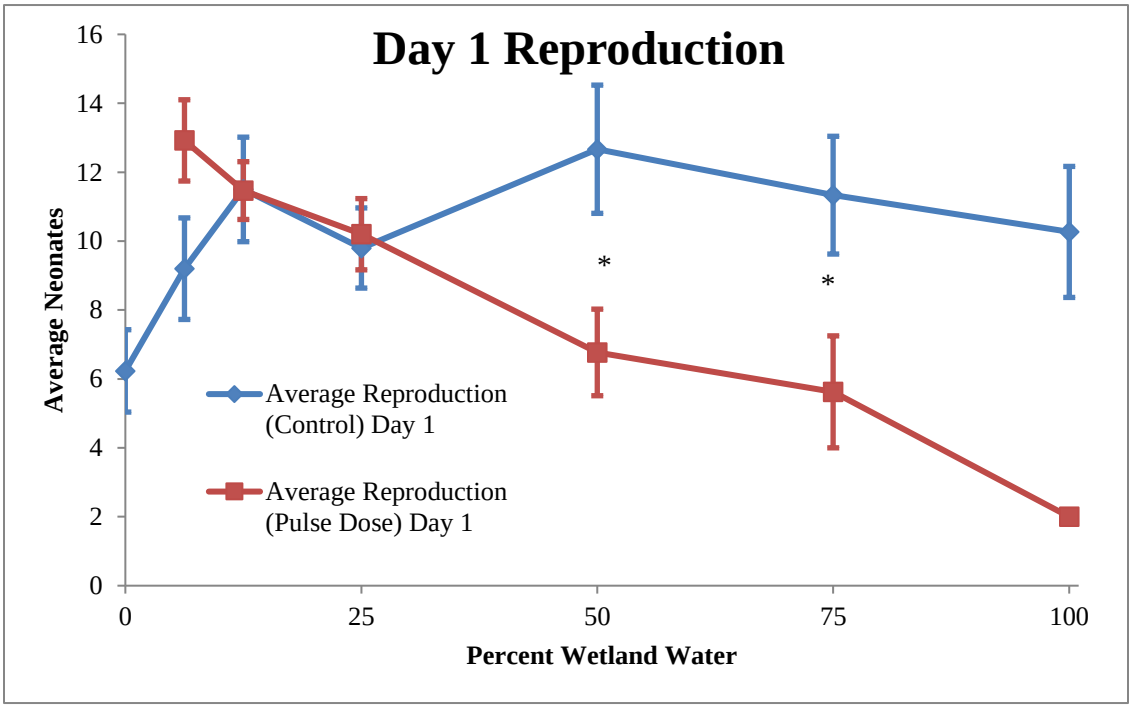

Figure 2.8 Reproduction of Lab Spiked Mesocosm Water

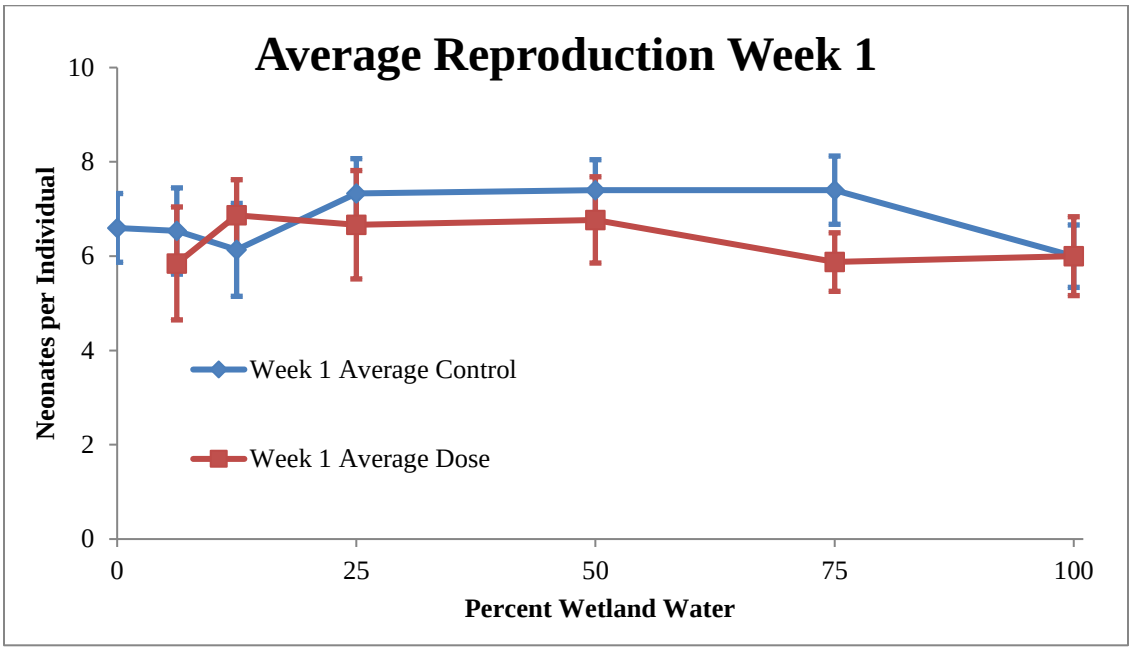

Figure 2.9 Comparison of reproduction in day 7 pulse and control dosed *Daphnia Magna*

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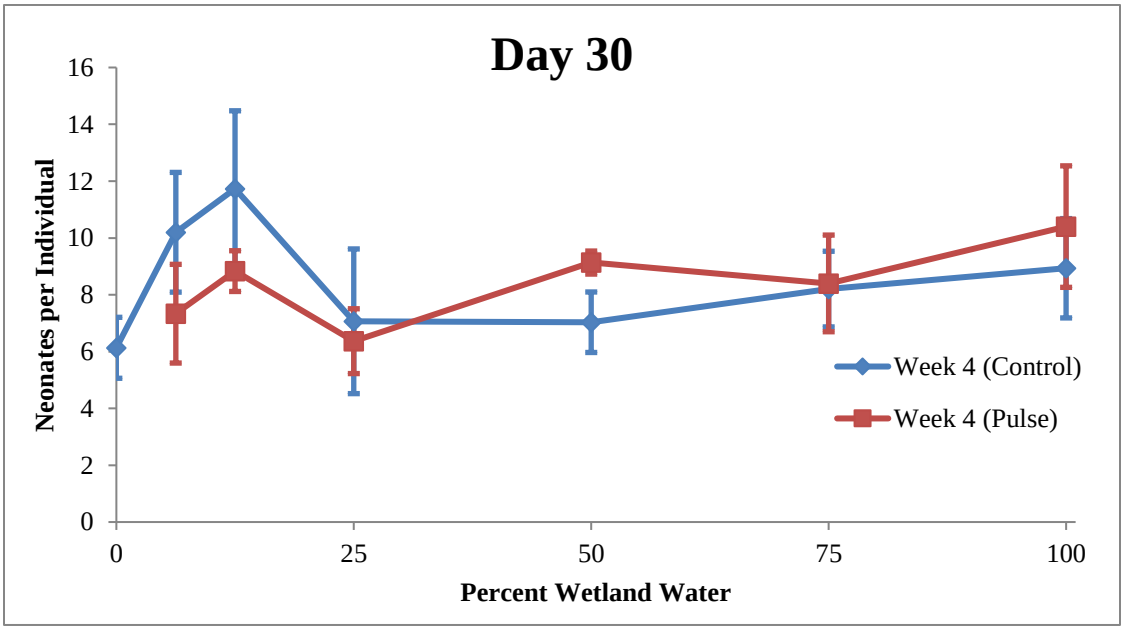

Figure 2.10 Comparison of reproduction in day 30 pulse and control dosed *Daphnia Magna*.

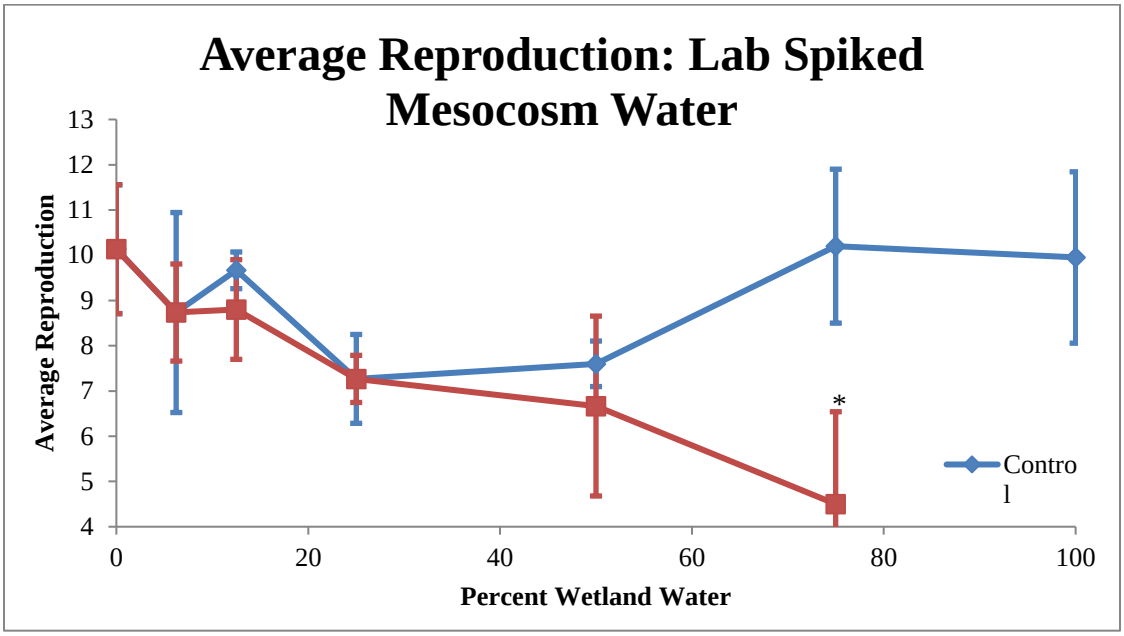

Figure 2.11 Comparison of average brood size in individuals dosed in lab spike mesocosms water and controls.

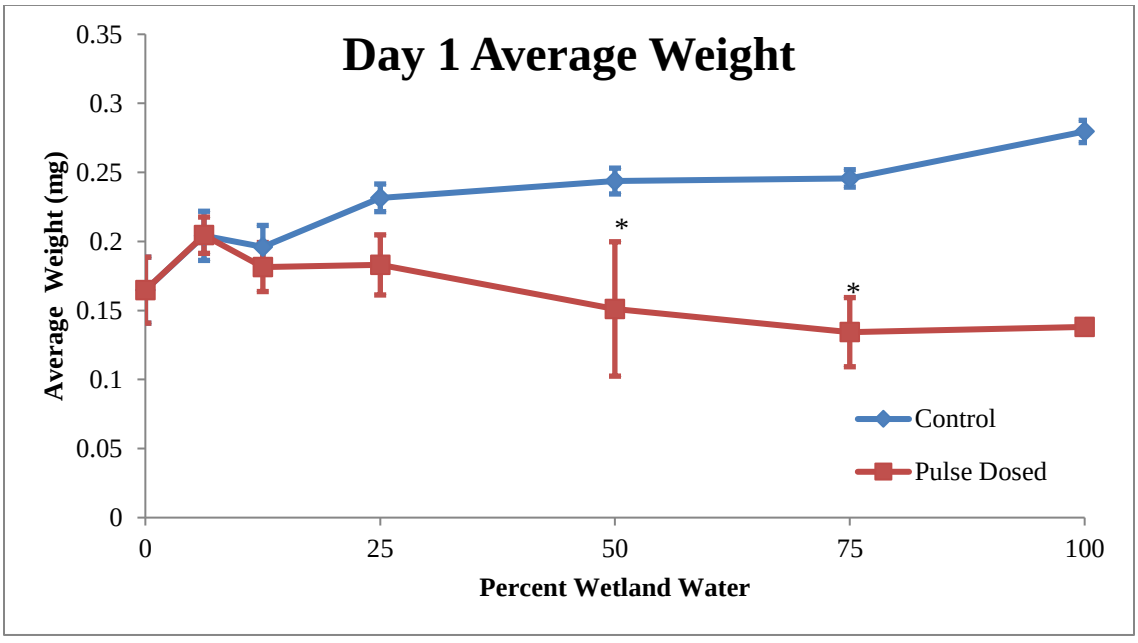

Figure 2.12 Comparison of weight in Day 1 pulse and control dosed *Daphnia Magna*

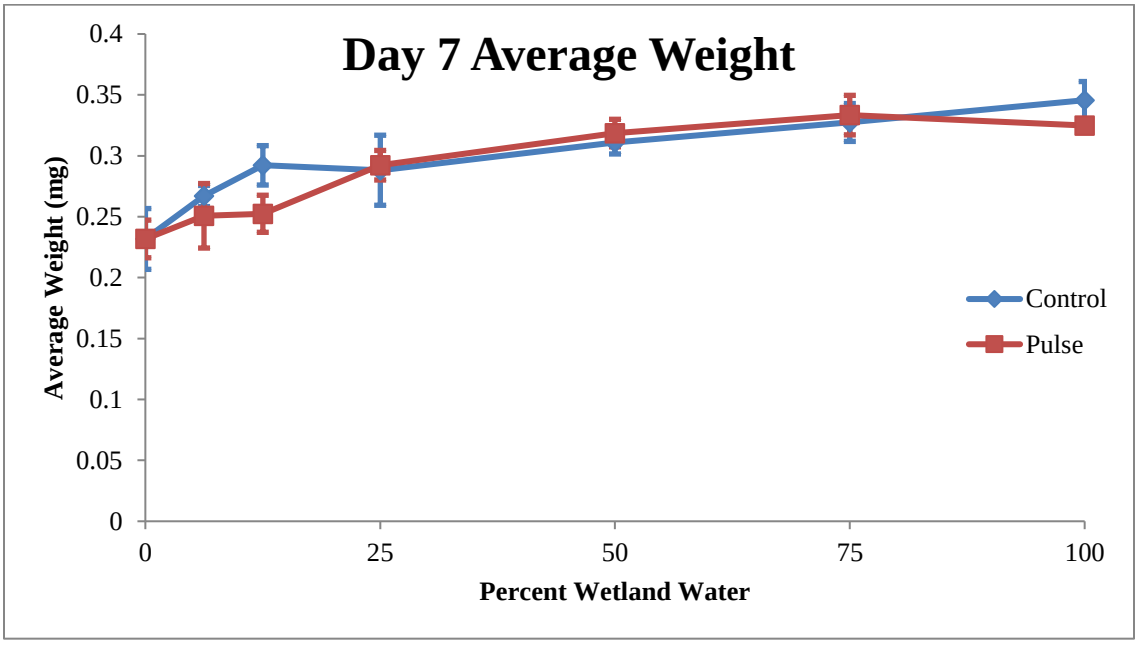

Figure 2.13 Comparison of weight in Day 7 pulse and control dosed *Daphnia Magna*

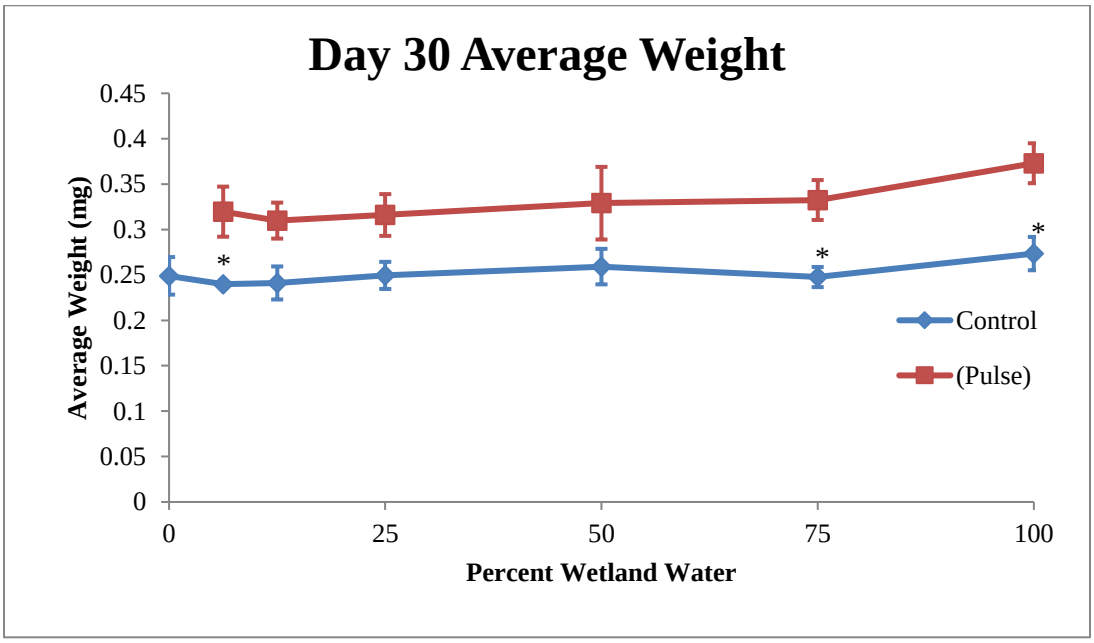

Figure 2.14 Comparison of weight in Day 30 pulse and control dosed *Daphnia Magna*

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