EFFECTS OF LAYER DOUBLE HYDROXIDE NANOCHELANTS ON THE TOXICITY OF COPPER TO *Daphnia magna*

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Nanoparticles may affect secondary pollutants such as copper. Layer Double Hydroxides (LDH) are synthetically produced nanoparticles that adsorb copper via cation exchange. Pretreatment of copper test solutions with LDH nanoparticles followed by filtration removal of LDH nanoparticles demonstrated the smallest LDH aggregates removed the most copper toxicity. This was due to increased surface area for cation exchange relative to larger particle aggregates.

Co-exposure tests of copper chloride and clay were run to determine if smaller clay particles increased copper uptake by *D. magna*. Coexposure treatments had lower LC50 values compared to the filtration tests, likely as a result of additive toxicity. LDH nanoclays do reduce copper toxicity in *Daphnia magna* and may serve as a remediation tool.
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A nanoparticle is a particle having at least one dimension under 100 nm (Klaine et al., 2008). Naturally occurring nanoparticles in the environment result from volcanic eruptions, soot, and physiochemical weathering (Handy et al., 2008). Anthropogenic sources also produce them as byproducts through the burning of fossil fuels and wood (Grazyna et al., 2009). Nanoparticles possess many unique properties leading to their increased use including mechanical, catalytic, and optical properties. Many different compound classes are now produced such as: carbonaceous, metal oxides, semiconductor materials (including quantum dots), and zero-valent materials such as iron, silver, and gold nanoparticles (Klaine et al., 2008). These nanomaterials can be found in sporting goods, stain-resistant clothing, sunscreens, and cosmetics. They are also used for medical purposes including imaging and drug delivery (Nel et al., 2006). Increased manufacturing and use of nanoparticles will likely lead to increased environmental deposition including into aquatic environments.

Nanoparticles in air or water are commonly referred to as colloids (Klaine et al., 2008). Colloids can be defined as small particles (generally < 1um) that are not affected by gravity (Gustafsson and Gschwend, 1997). They are important in aqueous environments because of their ability to affect transport and bioavailability of nutrients or pollutants (Ju-Nam and Lead, 2008). While there is an abundance of naturally occurring colloids, the potential effects of manufactured nanoparticles on the environment are relatively unknown (Ju-Nam and Lead, 2008).
Effects on Organisms

There are several examples of toxic interactions between nanoparticles and enzymes/proteins. A study of the nematode Caenorhabditis elegans demonstrated an increased expression of the gene cyp35a2 (xenobiotic metabolism enzymes involved in the fat storage pathways) in the presence of 15 nm and 45 nm sizes of CeO$_2$ and 7 nm particles of TiO$_2$ (Roh et al., 2010). In the marine mussel Mytilus galloprovincialis nanoparticle TiO$_2$, SiO$_2$, and fullerenes activated and perturbed the immune system through the p38MAPK (stress-activated antibodies) (Canesi et al., 2009). Fullerenes had an in vivo impact on fathead minnows by upregulating P450 as a result of exposure (Zhu et al., 2006). The small size of the particles allows them to penetrate cell membranes and lodge in mitochondria and other organelles, leading to deposits in organs and/or inflammatory responses (Nel et al., 2006). In mammals, nanoparticles may bind to acetylcholineesterase (AChE) (Wang et al., 2009). AChE is a widely distributed enzyme in the brain and blood and effects the function of the nervous system (Wang et al., 2009). Interference with AChE can cause nervous system failure leading to respiratory problems and even death (Wang et al., 2009). In a 2009 in-vitro study single walled and multi-walled carbon nanotubes were shown to have the most effect on electric eel AChE activity (reduction) out of eight nanoparticles studied. Copper and carbon-carbon coated copper came in next resulting in a 40% and 45% reduction in AChE activity, respectively (Wang et al., 2009). It has been hypothesized that DNA repair, which is on the nanolevel, can be disrupted by the presence of nanoparticles (Balbus et al., 2007).

Gold nanoparticles are being developed as an alternative treatment to cancer (Arnida et al., 2011). Poly ethylene glycol coated nanospheres and nanorods were injected intravenously into mice with ovarian tumors (Arnida et al., 2011). Just changing the shape and charge of the
particles made a significant difference on how many particles accumulated in the tumor versus the liver and spleen, with nearly 15% of the rods making their way to the tumor (Arnida 2011). The spheres mostly accumulated in the liver and spleen indicating that a simple change in a particle’s shape may alter which cells or organs are affected.

Individuals as well as populations are susceptible to the biological effects of nanoparticles. This may be especially true for aquatic organisms because the aquatic environment acts as a sink for many contaminants (Scown 2010). TiO₂ has been shown to cause significant *D. magna* mortality at 5 mg/L (Kim et al., 2010). TiO₂ nanoparticles have been observed clinging to the outside of *D. magna*, possibly compounding stress to the organisms (Kim et al., 2010). Aquatic organisms can be exposed to nanoparticles through ingestion (Cuker 1993, Roberts et al., 2007). Roberts et al. demonstrated that daphnia indiscriminately consume carbon nanomaterials if they are present in the water, possibly leading to internal exposure. The single walled nanotubes in this study were also observed on the external surface of the *D. magna* in numbers that limited their mobility (Roberts et al., 2007).

In aquatic organisms, the potential for biological impacts may be determined based on the particle’s size, structure, chemical composition, shape and aggregation, as well as aquatic factors such as pH, hardness, and organic matter (Nel at al., 2006, Handy et al., 2008). Sea water may allow for more aggregation than freshwater due to the additional ionic charges from the salt that lead to more particle collisions, and thus, more aggregation (Handy et al., 2008). pH can have a similar effect depending on the surface charge of the particles in question (Handy et al., 2008 and Alloy et al., 2011). Chemical contamination may facilitate nanoparticles to behave in unexpected ways. In a 2010 study involving fullerenes and two pesticides, fullerenes decreased acute toxicity of the pesticide tribufos, but increased acute toxicity of bifenthrin (Brausch et al.,
It is not understood why the toxicity patterns differed (Brausch et al., 2010). Short-term exposure to fullerenes can protect the gut tract of daphnia to damage from UV and PAH toxicity, while long term fullerene exposure has been shown to cause damage on the cellular level to the same area (Yang and Oris, 2010). This is relevant because despite knowing a nanoparticle’s characteristics and potential impact on aquatic organisms, the interactions that take place with other compounds in the aqueous environment may cause unknown organism or population effects.

Organic matter may allow for greater particle dispersion (Handy et al., 2008). Particularly, negatively charged humic or fulvic acid may bind to positively charged nanoparticles, keeping them suspended (Handy et al., 2008). A recent study with multi-walled nanotubes (MWNT) relayed changes in pH changed the number of NOM particles that could bind to MWNT, changing the aggregate size of the MWNT particles (Alloy 2011). Aggregation may in turn affect how long a particle stays in solution or whether it settles out (Handy et al., 2008). This can dictate the biological effects. If particles aggregate and settle quickly, they may have less effect on the pelagic organisms and greater impact on benthic organisms.

Organisms may also alter the structure of the nanomaterials themselves (Roberts et al., 2007). When *D. magna* were exposed to lysophosphatidylcholine (LPC) coated single-walled nanotubes the LPC coating was partially digested by the *D. magna* allowing for aggregation of the particles post digestion (Roberts et al., 2007). In one study *Daphnia hyaline* reduced the number of >600 nm sized inorganic particles in water taken from Lake Brientz, Switzerland, without reducing the overall amount of particles (Fiella et al., 2007). It was theorized that the daphnia’s digestive system might have been breaking aggregates into smaller particles (Fiella et al., 2007). This is
important because the properties of nanomaterials upon entering an aquatic environment may be altered by the environment or organisms in the environment.

Metals in the Environment

Forty percent of hazardous waste in the United States is a combination of organic and metal compounds (Sandrin and Maier, 2003). Metals can increase the oxidative stress to organisms and inhibit enzymes (Sandrin and Maier, 2003). Metals in the environment may also react with organic matter, clay minerals, and even organic pollutants, which all affect their bioavailability (Sandrin and Maier, 2003).

Copper is a common pollutant in water from urban and agricultural runoff. As an essential element, copper assists with metabolic activities and acts as a catalyst for cellular respiration. Enzymes containing copper include cytochrome c oxidase and lysl oxidase (initiates cross-linking of collagen through oxidation of other enzymes). Besides oxygen metabolism these enzymes allow for electron transport via copper redox reactions (Francis and Harrison, 1988). The same properties that allow for redox reactions can allow ROS (reactive oxygen species) to form. ROS has a damaging, oxidative effect on cells, which is promoted when excess copper leads to excess reactions (Minghetti et al., 2009). Another mechanism of copper toxicity is through the displacement of zinc in proteins (Minghetti et al., 2009). Copper replaces the zinc receptor disallowing zinc enzymes (involved in many metabolic activities) to bind (Francis and Harrison, 1988). Cu-ATPases are proteins, found in all eukaryotes, that have evolved to deal with copper transport and help prevent detrimental reactions (Minghetti et al., 2009). Their functions are to transfer copper from the carrier to the receptor in the cupro-enzymes and also to regulate copper homeostasis (Minghetti et al., 2009). Too much copper exposure can cause these homeostatic systems to fail. In fish, copper toxicity occurs when copper/gill interactions allow
for an electrolyte imbalance in the sodium channels to occur (Gheorghiu et al., 2010). This disrupts the internal ion balance and leads to death (Gheorghiu et al., 2010).

The EPA’s Quality Criteria for Water 1986 – Copper manual states that invertebrates are more sensitive than fish to copper toxicity. *D. magna* and *C. dubia* were in the top six for most sensitive species when species mean acute values were calculated (EPA, 2007). Daphnids had a genus mean acute value of 4.05 ug/L of dissolved copper. Acute toxicity decreases with increased hardness, alkalinity, and total organic carbon. *Americamysis bahia*, commonly studied in laboratories, has a chronic LOEC of 77 ug/L. EPA limits are based on water hardness. For example, at a hardness of 100, the four-day average copper level should not exceed 12 µg/L. Increased hardness is able to diminish copper and other metal’s bioavailability because cations, such as calcium, sodium, and magnesium, compete with the metal for uptake by the organism (Clifford and McGeer, 2008). In a zinc toxicity study with *Daphnia magna*, calcium was found to have the greatest toxicity reducing effect, followed by magnesium, and lastly sodium (Clifford and McGeer, 2008).

Natural organic matter (NOM) is a mixture formed from decomposed plants, animals, and microorganisms (Gheorghiu et al., 2010). NOM can reduce a metal’s bioavailability by binding to it and forming metal-NOM complexes (Gheorghiu et al., 2010). Copper tends to have strong covalent bonds to organic matter ligands found in NOM (Usman et al., 2005). Organic matter, such as humic substances, has negative charges that allow for the positively charged copper ions to coagulate on them (Usman et al., 2005). It is important when studying copper toxicity to take into account additional factors such as hardness, pH, and DOC (dissolved organic carbon), that could affect the test outcome because of the effect on bioavailability (Ryan et al., 2009).
Nanoparticle Clays for Decontamination

Nanoparticles are already being utilized for the decontamination and removal of chemicals including lead and bisphenol A (Grazyna et al., 2009). Nanomaterials are useful in water purification for reducing chemical toxicants, bacteria, and viruses (Grazyna et al., 2009). Silver is known to have antibacterial properties and Fe₃O₄, with a mesoporous silica coating, has the capacity for waste remediation (Grazyna et al., 2009). Another possibility for bioremediation comes through the proposed use of nanoparticle clay to remove metals from water (Usman et al., 2005).

Clay reduces the bioavailability of copper by adsorbing it, and thus reducing its toxicity (Usman et al., 2005). Clay is an important material for ion exchange due to its large surface area, abundance of reactive sites on the surface, and proliferation in surface waters (Sperry and Pierce, 1999). The mechanism by which clays absorb metals is surface cation exchange, which can be dependent on pH (Xu et al., 2007). In a 2007 study, absorption of lead onto bentonite (clay with large surface area and high ion-exchange capabilities) was highly related to pH with the highest adsorption (approximately 80%) occurring at pH 10 (Xu et al., 2007). This is because as pH increases, metal solubility decreases, so more metals bind to the clay (Grazyna et al., 2009). Differences other than size, such as surface structure modifications and surface reactivity can allow nanoparticles to adsorb more than their micro counterparts (Charlet L. et al., 2011). In an arsenic adsorption experiment, 11 nm magnetite adsorbed three times more arsenic than 20 nm magnetite (Charlet L. et al., 2011). One explanation for this could be differences in reactivity between the very small nanoparticles and larger particles of the same material (Charlet L. et al., 2011).
Layer Double Hydroxides (LDH) are anionic clays that have a structure consisting of positively charged layers separated by anions and water molecules (Mousty 2010). They have chemical and physical properties similar to cationic clays (Mousty 2010). They may be hydrophobic or hydrophilic depending on their constituents or the constituents of the environment in which they are found (Besse-Hogan et al., 2009). LDH have demonstrated the ability to absorb a wide range of contaminants, including chromium, selenium, and arsenic through ion-exchange and absorption mechanisms (Xu et al., 2007). LDH clays can even adsorb negatively charged bacteria cells between their positively charged layers leading to increased biodegradation of non-sorbed materials like atrazine (Besse-Hoggan et al., 2009). LDH nanoparticle clays, with their increased surface area, offer even more potential for remediation.

The surface of clay can be changed with organic treatments to render the clay more hydrophobic and more compatible with polymers (Sharma et al., 2010). Changing coatings or ligands in the clay can alter its charge and ability to sorb materials (Zhu 2011). For example, when phosphate ligands were added to Fe-deformed montmorillonite, copper adsorption was enhanced more than when sulfate ligands were added (Zhu 2011).

However, clays themselves may be detrimental to aquatic organisms if they are ingested. The main uptake mechanism for organisms is through feeding (Roberts et al., 2007). Daphnia are non-selective feeders within a certain particle size range (Kirk and Gilbert, 1990). The addition of clay and increased turbidity on zooplankton populations has been shown to have detrimental effects (Cuker 1993). Clay addition can decrease light attenuation, therefore decreasing photosynthesizing algae (food for zooplankton). Adding clay can also alter zooplankton feeding ability (Cuker 1993). Ingestion of clay particles causes a reduction in the amount of phytoplankton that can be ingested by *D. magna* (Kirk and Gilbert, 1990). Robinson
et al. tested three clays: montmorillonite, kaolinite, and a natural clay from South Carolina, with pulsed exposures to D. magna to determine if there was a relationship between these exposures and organism response (Robinson et al., 2010). The authors demonstrated survival and growth of *Daphnia magna* were adversely affected by clays with Na+montmorillonite (the the smallest clay tested) having the greatest effect on survival (Robinson et al., 2010). Coatings added to clay may compound potential toxicity. A 2010 comet assay with ammonium treated montmorillonite had dose-response genotoxic effects while the untreated montmorillonite had no genotoxic effects (Sharma et al., 2010). Even after the clay was filtered out the ammonium compound remained in the solution and caused genotoxic effects on the same level of magnitude as the clay/ammonium compound mix (Sharma et al., 2010).

Clay that has adsorbed metals may pose a greater risk to aquatic life. A microcosm study by Meader et al. demonstrated that algae contaminated with copper resulted in increased transport of copper into the daphnids (Meador et al., 1993). Copper-contaminated clays may also have the same detrimental effect. Small particles have a greater surface area to weight ratio than larger particles, potentially increasing the exposure (Klaine et al., 2008). Nano-particle clay may be able to adsorb even more metal because of its small size and increased surface area. While these clays may adsorb water column Cu and make it less bioavailable, those copper ions may be re-solubolized in the acidic environment of the gut. The goal of this research was to examine if nanoparticle clays could reduce copper toxicity to *Daphnia magna* by reducing copper bioavailability, and to determine the effects of clay particle size and aggregation on copper toxicity. The thesis is organized into four chapters; an overall literature review (1), research chapters detailing the remediation of copper-contaminated water by nanoclays (2) and
the toxicity of copper-contaminated nanoclays (3), followed by an overall conclusions chapter (4).
CHAPTER 2

PRETREATMENT WITH LDH NANOCLAYS REDUCES COPPER TOXICITY TO Daphnia magna

Literature Review

Copper is a common pollutant in water from urban and agricultural runoff. As an essential element, copper assists with metabolic activities and acts as a catalyst for cellular respiration. Enzymes containing copper include cytochrome c oxidase and lysl oxidase (initiates cross-linking of collagen through oxidation of other enzymes) (Francis and Harrison, 1988). Besides oxygen metabolism these enzymes allow for electron transport via copper redox reactions (Francis and Harrison, 1988). The same properties that allow for redox reactions can allow ROS (reactive oxygen species) to form. ROS has a damaging, oxidative effect on cells, which is promoted when excess copper leads to excess reactions (Minghetti et al., 2009).

Another mechanism of copper toxicity is through the displacement of zinc in proteins (Minghetti et al., 2009). Copper replaces the zinc receptor and prevents zinc enzymes (involved in many metabolic activities) to bind (Francis and Harrison, 1988). Cu-ATPases are proteins, found in all eukaryotes, that have evolved to deal with copper transport and help prevent detrimental reactions (Minghetti et al., 2009). Their functions are to transfer copper from the carrier to the receptor in the cupro-enzymes and also to regulate copper homeostasis (Minghetti et al., 2009).

Too much copper exposure can cause these homeostatic systems to fail. In fish, copper toxicity occurs when copper/gill interactions allow for an electrolyte imbalance in the sodium channels to occur (Gheorghiu et al., 2010). This disrupts the internal ion balance and leads to death (Gheorghiu et al., 2010).
The EPA’s *Quality Criteria for Water 1986 – Copper* manual states that invertebrates are more sensitive than fish to copper toxicity. *D. Magna* and *C. dubia* were in the top six for most sensitive species when species mean acute values were calculated (EPA, 2007). Daphnids had a genus mean acute value of 4.05 ug/L of dissolved copper. Acute toxicity decreases with increased hardness, alkalinity, and total organic carbon. *Americamysis bahia*, commonly studied in laboratories, has a chronic LOEC of 77 ug/L. EPA limits are based on water hardness. For example, at a hardness of 100, the four-day average copper level should not exceed 12 ug/L. Increased hardness is able to diminish copper and other metals bioavailability because cations, such as calcium, sodium, and magnesium, compete with the metal for uptake by the organism (Clifford and McGeer, 2008). In a zinc toxicity study with *Daphnia magna*, calcium was found to have the greatest toxicity reducing effect, followed by magnesium, and lastly sodium (Clifford and McGeer, 2008).

A nanoparticle is a particle having at least one dimension under 100 nm (Klaine et al., 2008). Nanoparticles in air or water are commonly referred to as colloids (Klaine et al., 2008). Colloids can be defined as small particles (generally < 1um) that are not affected by gravity (Gustafsson and Gschwend, 1997). They are important in aqueous environments because of their ability to affect transport and bioavailability of nutrients or pollutants (Ju-Nam and Lead, 2008). While there is an abundance of naturally occurring colloids, the potential effects of manufactured nanoparticles on the environment are relatively unknown (Ju-Nam and Lead, 2008).

Nanoparticles are already being utilized for the decontamination and removal of chemicals including lead and bisphenol A (Grazyna et al., 2009). Nanomaterials are useful in water purification for reducing chemical toxicants, bacteria, and viruses (Grazyna et al., 2009).
Silver is known to have antibacterial properties and Fe₃O₄, with a mesoporous silica coating, has capacity for waste remediation (Grazyna et al., 2009). Another possibility for bioremediation comes through the proposed use of nanoparticle clay to remove metals from water (Usman et al., 2005).

Layer double hydroxides (LDH) are anionic clays that have a structure consisting of positively charged layers separated by anions and water molecules (Mousty 2010). They have chemical and physical properties similar to cationic clays (Mousty 2010). They may be hydrophobic or hydrophilic depending on their constituents or the constituents of the environment in which they are found (Besse-Hogan et al., 2009), and can be formed in a wide range of compositions with different anions sandwiched between the metal cation layers (Hibino 2011). LDH have a demonstrated ability to absorb a wide range of contaminants, including chromium, selenium, and arsenic, through ion-exchange and absorption mechanisms (Xu et al., 2007). LDH clays can even adsorb negatively charged bacteria cells between their positively charged layers leading to increased biodegradation of non-sorbed materials like atrazine (Besse-Hoggan et al., 2009).

LDH nanoparticle clays, with their increased surface area, offer even more potential for remediation. The surface of clay can be changed with organic treatments to render the clay more hydrophobic and more compatible with polymers (Sharma et al., 2010). Changing coatings or ligands in the clay can alter its charge and ability to sorb materials (Zhu 2011). For example, when phosphate ligands were added to Fe-deformed montmorillonite, copper adsorption was enhanced more than when sulfate ligands were added (Zhu 2011). Coating and the anions used in the clay can also raise or lower solubility of the clay. For example, Zn-Al LDH clays have lower solubility than Mg-Al LDH clays, so they are more stable and more durable aqueous
solution (Hibino 2011). It is not known how the solubility will affect toxicity remediation in a solution, or if the different coatings or anions used in the LDH clay will contribute to toxicity themselves.

The goal of this research was to ascertain if nanoparticle clays could reduce copper toxicity to *Daphnia magna* by reducing copper bioavailability and if so, to also determine which clays reduced toxicity the most. To accomplish this goal, the following hypothesis was tested. Nanoclays reduce metals toxicity by reducing bioavailability. Smaller particles will result in greater reduction of copper bioavailability than larger particles due to differences in surface area per unit mass.

**Methods**

Dilution water and control water used for the test was reverse osmosis water that was also filtered through deionization (DI) resin and reconstituted to a pH of 7.8, hardness of 140, and alkalinity of 50 (hard synthetic freshwater, HSFW) (USGS.com). High-density polyethylene containers were used to store all test media. Copper chloride was obtained from Fisher Scientific. Copper chloride dilutions of 0, 25, 50, 100, 200, and 400 ug/L were used for each test. These dilutions will be referred to as copper-contaminated water throughout this paper. Copper chloride stock was 0.05 grams of copper chloride into .5 L DI water. Dilutions were produced by spiking 1 L of reconstituted lab water with an appropriate amount of copper chloride stock. Copper chloride stock was made fresh weekly and stored in a high-density polyethylene 1 L container at 4 °C when not in use. To prepare the 25 ug/L dilution, .25 mls of stock was added and the volume was brought up to 1 L with HSFW in a volumetric flask. The 100 ppb dilution was 1 ml of stock into 1 L of HSFW, etc.
Clays were selected based on hydrophobicity, which affects particle behavior and size in aqueous solutions. The clays used were (in increasing order of hydrophobicity) Na⁺montmorillonite, Zn-Al LDH-Nitrate, Zn-Al LDH-Carbonate, and R-montmorillonite. A Metler AE 240 scale was used to weigh 20.0 and 200.0 mg of each clay. These were added to 2 L of copper-contaminated water so each copper dilution had either 10.0 mg/L or 100.0 mg/L of clay. The solutions were initially hand-shaken for 30 seconds and then allowed to react for two hours, after which time each sample was filtered once through a .45 micron glass fiber filter and filtering apparatus attached to a GAST ® 1/6 horsepower vacuum pump. Adsorption of Cr (VI) can reach equilibrium adsorption with C. bentonite after 2 hours and Mo (VI) can reach equilibrium with bentonite after 1 hour (Atia 2008). The clay particles are <100 nm in thickness but aggregates are >500 nm in diameter. Thus the filter size was sufficient to remove the clay. Solutions were filtered in increasing copper concentrations and the filtering apparatus was acid-washed with 20% HCl between sets of dilutions and rinsed with DI water to prevent cross-contamination.

10 mg/L and 100 mg/L clay blanks were prepared by adding clay in those amounts to 1L of HSFW. All blanks underwent the same treatment as the copper-contaminated water. The purpose of the blanks was to observe if the clay themselves induced toxicity.

A *Daphnia magna* acute toxicity test was run using filtered water from all the copper and copper/clay dilutions. A control of HSFW was run with every test. Tests were conducted in a 20⁰ C room with a 16 hours light, 8 hour dark light cycle. Test chambers consisted of 100mL plastic cups containing 40mL of test solution. Each concentration had 5 replicates with 8 organisms each, giving a total of 40 per treatment. *D. magna* were loaded into the tests using a glass pipette and the pipette was changed out between concentrations to prevent cross-
contamination. *D. magna* used in tests were all <24 hours old. Each test chamber was fed a 1mL suspension of the green algae, *Selenastrum capricornutum*, along with YTC daily. Tests ended after 96 hours and survival was recorded daily.

Copper was measured using a SpectrAA 600, Atomic Absorption Spectrometer with Zeeman correction following EPA method number 7010 “Graphite Furnace Atomic Absorption Spectrophotometry”. For copper, the recommended wavelength is 327.4, slit width is 0.5 nm, temp char = 2300. Graphite tubes were replaced after 300 firings and the inert gas used was either argon or helium, depending on what was available.

A calibration curve of 6, 15, 25, and 45 ug/L was used with a copper sulfate solution as the standard. The copper sulfate stock was made by adding 30 mg copper which was 117.87 mg of copper sulfate to 1 L of water. Each day new standard was made by adding 10 uL of stock to 9.99 ml of make-up water. Make-up water was .1% nitric acid diluted with milli-Q water and was used to dilute samples that were run on the AA. Each sample was run twice and all values were recorded in a lab notebook.

Four replicates of five *D. magna* were exposed to each copper/clay concentration for 24 hours for the purpose of measuring their copper content via atomic absorption spectroscopy. The same temperature, light cycle, test cup volume, volume of solution, and feeding regime were followed as used for the bioassays. All daphnia were <24 hours old. Following exposure they were poured over a fine-micron nylon mesh, rinsed with DI water, and tweezed into a centrifuge vial to be digested for AA analysis.

The *D. magna* were digested using the aid of a conventional microwave. Microwave digestion allows for quick decomposition of the sample with little loss of volatile elements (Oliveira 2003). It can also use a small amount of reagent (Oliveira 2003) which was desirable
due to the small sample that required digestion. 100 µL of 70% nitric acid were added to each vial containing *D. magna*. Vials were capped and secured in a microwave proof container, then heated at 50% power for 15 second intervals until all *D. magna* were fully digested. Digestion was determined by visual inspection and was complete when the mixture was a clear, yellow fluid. The *D. magna* digest was run on the AA as a 1:10 ratio of digest to makeup water. This was necessary because the daphnia contained such high levels of copper. 50 µL of digest were pipetted into an AA vial with an epindorf pipette and then 500 µL of makeup water were added to it.

100mL of each copper-contaminated/clay solution was filtered once through a .45 micron glass fiber filter and filtering apparatus attached to a GAST ® 1/6 horsepower vacuum pump. Blanks and control water were filtered in a similar fashion. Filters were cut into 16 pieces with acid-washed scissors and placed in 100 mL copper digestion vessels from Environmental Express. To each vial 5.0 mLs of 70% nitric acid was added. Filters were allowed to sit in acid solution for 48 hours after which time 1.5 mLs was transferred into an appropriately labeled micro-centrifuge vial. Vials were allowed to sit at least 24 hours to allow for settling of filter media. Vial contents were diluted to a 1:10 ratio for the blanks, 25 µL copper chloride, and 50 µL copper chloride. A 1:20 ratio was used for the 100 µL copper chloride, 200 µL copper chloride, and 400 µL copper chloride for AA analysis. For the 1:10 ratio, 50 µL of acidified filtrate was added to 500 µL of make-up water. The 1:20 ratio used 25 µL of acidified filtrate to 50 µL of make-up water.

Approximately 6 mls of every filtered copper/clay aqueous sample was stored in a freezer until use in a polyethylene, capped test tube. Nine hundred ninety micro liters of filtered 0 µL, 25 µL, and 50 µL concentrations were added to each AA cuvet along with 10 µL of 70% acid.
Concentrations of 100 µL, 200 µL, and 400 µL had to be more dilute to be within range for the AA. The 100 µL was diluted 50% with 1% HNO₃, so 500 µL of 100 µL solution was added to 500 µL of make-up water in the AA cuvet. The 200 µL was diluted by adding 250 µL of 200 µL solution to 750 µL of make-up water and the 400 µL was diluted by adding 125 µL of sample to 875 µL of make-up water. All samples had a pH of <2.0 before being placed on the AA.

Clay solutions in concentrations of 10 mg/L and 100 mg/L were prepared using hard synthetic freshwater. Each clay solution was hand shaken for 30 seconds and allowed to react for at least 2 hours. Particle characterization was carried out by electron microscopy at Clemson University.

NOECs and LOECs were determined using Toxstat Version 3.4 (University of Wyoming). EPA’s Probit or Trimmed-Spearman Karber Method (when appropriate) were used to determine LC50s and confidence limits. Regression analysis was run using Excel 2007 to measure the relationship between mean percent survival and copper body burden in daphnia. SAS Learning Addition 4.1 was used for all other statistical analysis including ANOVA, Tukey Multiple Range Test, and Student-Newman-Keuls test. A p value of <0.05 indicates a significant response with an alpha level of .05 and 95% confidence limits.

Results

Clay digest analyzed for copper concentrations via the AA demonstrated that clay treatment (0 mg/L, 10 mg/L, and 100 mg/L) had a significant effect on the amount of copper present (GLM p < 0.0001) Copper chloride concentration had an effect on copper levels as well (GLM p < 0.0001). There were significant differences in copper concentrations found due to the interaction of copper chloride concentrations and clay amount (GLM p = 0.0105). The 0 mg/L treatment filter digest had slightly increasing copper levels with increasing copper chloride.
concentrations, but the addition of clay, especially the 100 mg/L treatment, allowed much more copper to be retained on the filter and clay digest (Fig. 1).

![Graph showing copper concentration means ± 1 standard deviation from Na⁺montmorillonite clay.](image)

**FIG. 1.** Copper concentration means ± 1 standard deviation from Na⁺montmorillonite clay.

Na⁺montmorillonite reduced copper concentrations in the solutions in a concentration dependent manner (Fig. 2). There was a highly significant difference between the 100 mg/L clay treatment and the 0 and 10 mg/L clay treatment (GLM $p < 0.0001$). Copper chloride concentration had a highly significant effect on the amount of copper present in solution (GLM $p < 0.0001$). The interaction between clay treatments and copper chloride concentration was significant (GLM $p = 0.0247$).
Although the clay bound up a significant amount of copper, there was still copper present in the solutions, especially in the higher copper chloride concentrations. This is reflected in daphnia body burdens of copper and daphnia survival. Regression analysis comparing daphnia body burden of copper in each concentration of 100 mg/L Na\textsuperscript{+} montmorillonite clay addition with mean percent survival at each concentration demonstrated there was a definite relationship between the two ($p = 0.034, R^2 = 0.714$). As body burden of copper increased, mean percent survival decreased (Fig. 3).
Pre-treating copper-contaminated water with 10 mg/L Na⁺ montmorillonite did not improve daphnia survival over the 0 mg/L treatment, but pre-treatment with 100 mg/L Na⁺ montmorillonite did improve survival over the 0 mg/L clay treatment (GLM $p < 0.0001$) (Fig. 4 and 5). This is especially apparent in the 100 µg/L copper chloride concentration where the 0 mg/L treatment survival was 15%. The 100 mg/L Na⁺ montmorillonite treatment increased survival to 100%. In the 200 µg/L concentration survival increased by almost 50% with the addition of 100 mg/L Na⁺ montmorillonite, and even at the 400 µg/L concentration there was still 35% survival following treatment with 100 mg/L Na⁺ montmorillonite (Fig. 4). Copper chloride concentration played a significant role in the survival of the organisms (GLM $p<0.0001$). The interaction between copper chloride concentration and clay treatment was also highly significant (GLM $p < 0.0001$).
FIG. 4. Mean survival (as percent control) ± 1 standard deviation of *Daphnia magna* exposed to copper contaminated water following clay treatment with Na⁺montmorillonite.

FIG. 5. LC50 Values and 95% confidence limits of copper contaminated water with 0, 10, and 100 mg/L Na⁺montmorillonite.
Zn-AlLDH-Stearate clay digest analyzed on the AA for copper concentration demonstrated that clay treatment had a highly significant effect on the amount of copper present (GLM $p < 0.0001$) and each of the clays were different from one another. Copper chloride concentration was also highly significant (GLM $p < 0.0001$), but the interaction between it and clay treatment was not significant (GLM $p = 0.1561$) (Fig. 6).

![Graph showing copper concentration means ± 1 standard deviation from Zn-AlLDH-Stearate nanoclay.](image)

**FIG. 6.** Copper concentration means ± 1 standard deviation from Zn-AlLDH-Stearate nanoclay.

Solutions from three Zn-AlLDH-Stearate treatments were analyzed on the AA for copper concentrations. Clay treatments had a significant effect on how much copper was present (GLM $p = 0.0211$). There was a difference between the 10 mg/L and 100 mg/L treatment, but the 0 mg/L treatment was not different from the other two (Fig. 7). There was a highly significant difference in copper concentrations when different copper chloride concentrations were analyzed...
There was not a significant interaction between clay treatments and copper chloride concentrations (GLM $p = 0.1544$).

Daphnia body burdens of copper indicate that even with clay treatment there was still copper present in the solution. A regression comparing daphnia body burden and mean percent survival at each copper chloride concentration found a significant relationship between the two ($p = 0.013, R^2 = 0.818$). There was a downward trend in survival as body burden increased (Fig. 8).

**FIG. 7.** Copper concentration means ± 1 standard deviation of solutions after treatment with Zn-AILDH-Stearate.
Zn-AlLDH-Stearate treatment had a highly significant effect on daphnia survival and all three treatments were different from one another (GLM $p < 0.0001$). Copper chloride concentrations as well as the interaction between copper chloride concentrations and clay treatments demonstrated a highly significant effect on daphnia survival (GLM $p < 0.0001$). Survival was improved 20% with the 10 mg/L Zn-AlLDH-Stearate treatment at the 200 µg/L copper chloride concentration while the 100 mg/L Zn-AlLDH-Stearate treatment improved survival by 40% in the same concentration. At the highest copper chloride concentration there was 0% survival with the 0 mg/L clay treatment. The 10 mg/L Zn-AlLDH-Stearate treatment allowed for 5% survival in the highest copper concentration, while the 100 mg/L Zn-AlLDH-Stearate treatment had 10% survival (Fig. 9).
The 100 mg/L Zn-AlLDH-Stearate treatment more than doubled copper LC50 values of daphnia (Fig. 10). The 0 mg/L treatment had an LC0 of 92.58 µg/L copper chloride and with the 10 mg/L Zn-AlLDH-Stearate treatment the LC50 improved a little to 101.8 µg/L copper chloride. The 100 mg/L Zn-AlLDH-Stearate treatment more than doubled the LC50 at 206.43 µg/L copper chloride (Fig. 10).
Clay digest from three Zn-AlLDH-Carbonate treatments of 0 mg/L, 10 mg/L, and 100 mg/L were analyzed on the AA for copper concentration. There was a highly significant difference in copper concentration between the three treatments (GLM $p < 0.0001$). Copper chloride concentration also allowed for highly significant differences in copper concentration (GLM $p < 0.0001$). The interaction between clay treatment and copper chloride concentration was significant (GLM $p = 0.0088$) (Fig. 11).

![FIG. 10. LC50 Values and 95% confidence limits of copper contaminated water treated with 0, 10, and 100 mg/L Zn-AlLDH-Stearate.](image-url)
The AA measured copper concentrations in test solutions following three Zn-AlLDH-Carbonate treatments and results were statistically analyzed to reveal that there was a highly significant effect on copper concentration due to clay treatment and copper chloride concentration (GLM $p < 0.0001$). Clay treatments of 0 mg/L and 10 mg/L were not significant from one another. There was not a significant interaction between clay treatments and copper chloride concentrations, however (GLM $p = 0.0605$) (Fig. 12).

FIG. 11. Copper concentration means ± 1 standard deviation from Zn-AlLDH-Carbonate clay.
Copper remaining in the solution allowed for a relationship between mean percent survival and body burdens of copper in the daphnia (Fig. 13). A regression was run comparing copper body burdens in the daphnia exposed to the 100 mg/L Zn-AlLDH-Carbonate treatment to percent survival in each concentration and there was a downward trend ($p = 0.011$, $R^2 = 0.833$).

**FIG. 12.** Copper concentration means ± 1 standard deviation of solutions after treatment with Zn-AlLDH-Carbonate.

Copper remaining in the solution allowed for a relationship between mean percent survival and body burdens of copper in the daphnia (Fig. 13). A regression was run comparing copper body burdens in the daphnia exposed to the 100 mg/L Zn-AlLDH-Carbonate treatment to percent survival in each concentration and there was a downward trend ($p = 0.011$, $R^2 = 0.833$).
Treatment of 100 mg/L Zn-AlLDH-Carbonate improved daphnia survival over the 0 mg/L and 10 mg/L treatment (GLM $p < 0.0001$) (Fig. 14 and 15). The 400 µg/L copper chloride concentration following treatment of 0 mg/L Zn-AlLDH-Carbonate had 0% survival and the 100 mg/L Zn-AlLDH-Carbonate treatment improved survival by 22.5% (Fig. 14). Copper chloride concentration as well as the interaction between copper chloride concentration and clay treatment had a highly significant effect on daphnia survival (GLM $p < 0.0001$).
FIG 14. Mean survival (as percent control) ± 1 standard deviation of *Daphnia magna* exposed to copper contaminated water following clay treatment with 0, 10 and 100 mg/L Zn-AILDH-Carbonate.

FIG. 15. LC50 Values and 95% confidence limits of copper contaminated water with 0, 10, and 100 mg/L of ZN-AILDH-Carbonate.
Digested clay from three treatments of Zn-AlLDH-Nitrate (0 mg/L, 10 mg/L, and 100 mg/L) were analyzed for copper concentration using the AA and there was a highly significant difference between all treatments (GLM $p < 0.0001$). Copper chloride concentrations also demonstrated a highly significant difference in the amount of copper present in each digested clay sample (GLM $p < 0.0001$). There was a significant interaction between clay treatments and copper chloride concentrations when copper concentrations were statistically analyzed (GLM $p = 0.0034$) (Fig. 16).

![Copper Concentration Means ± 1 Standard Deviation from Zn-AlLDH-Nitrate Clay](image.png)

FIG. 16. Copper concentration means ± 1 standard deviation from Zn-AlLDH-Nitrate clay.

When test solutions were analyzed via AA for copper concentration there was much less copper in the solutions after the 100 mg/L Zn-AlLDH-Nitrate treatment compared to the 0 mg/L and 10 mg/L treatment (GLM $p < 0.0001$). Copper chloride concentrations had a highly significant effect on the amount of copper present in solutions (GLM $p < 0.0001$). There was not
a significant effect between copper chloride concentrations and clay treatment on the amount of copper present in solution ($p = 0.2771$) (Fig. 17).

Copper body burdens of daphnia exposed to the 100 mg/L Zn-AlLDH-Nitrate treatment were compared to mean percent survival in each copper chloride concentration and there was a highly significant relationship ($p = 0.003, R^2 = 0.911$) (Fig. 18).

FIG. 17. Copper concentration means ± 1 standard deviation of solutions post treatment with Zn-AlLDH-Nitrate.

Copper body burdens of daphnia exposed to the 100 mg/L Zn-AlLDH-Nitrate treatment were compared to mean percent survival in each copper chloride concentration and there was a highly significant relationship ($p = 0.003, R^2 = 0.911$) (Fig. 18).
Treatment with 100 mg/L Zn-AlLDH-Nitrate significantly improved survival when compared to the 0 mg/L Zn-AlLDH-Nitrate treatment (GLM $p < 0.0001$). At the 200 µg/L copper chloride concentration, the 0 mg/L treatment had 0% survival and the addition of 100 mg/L Zn-AlLDH-Nitrate clay brought survival up to 87.5% (Fig. 19). At the 400 µg/L copper chloride concentration there was almost complete mortality even with the 100 mg/L Zn-AlLDH-Nitrate treatment. The 100 mg/L clay increased the LC50 over the copper only by 4.20 times (Fig. 20). Copper chloride concentration had a highly significant effect on daphnia survival (GLM $p < 0.0001$), as did the interaction between clay treatments and copper chloride concentration (GLM $p < 0.0001$).
FIG. 19. Mean survival (as percent control) ± 1 standard deviation of *Daphnia magna* exposed to copper contaminated water following clay treatment with Zn-AlLDH-Nitrate.

FIG. 20. LC50 Values and 95% confidence limits of copper contaminated water with 0, 10, and 100 mg/L of Zn-AlLDH-Nitrate.
Both the 10 mg/L and 100 mg/L treatments of R-montmorillonite caused complete mortality to the *Daphnia magna* at every copper chloride concentration, so no further analysis was conducted using this clay.

When LC50 increase is examined in all of the clay treatments, there was a greater increase in LC50 values in the 100 mg/L treatments (Table 1). There was much less improvement in LC50 values in the 10 mg/L treatments. In fact, there was only a difference of 0.37 between the 10 mg/L treatments demonstrating the most and least improvement. Zn-AlLDH-Nitrate improved the LC50 the most in both the 100 mg/L treatments and 10 mg/L treatments.

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<th>clay type (100 mg/L)</th>
<th>CuCl₂ LC50 (Without treatment)</th>
<th>clay LC50 (after treatment)</th>
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<table>
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Table 1. Summary of 10 and 100 mg/L clay treatments and their corresponding copper chloride reference test LC50, LC50 with the clay addition, and amount of increase in the LC50.
Discussion

Every 100 mg/L nanoparticle clay treatment significantly reduced copper toxicity to *D. magna*. SEM images of particles indicate the most improvement in LC50 values were the smallest particle sizes. Na⁺montmorillonite and Zn-AlLDH-Nitrate demonstrated the most improvement and had the smallest aggregate sizes. This, along with analytical data from test solutions, and the clay itself reinforces my hypothesis that nanoclays reduce copper toxicity by reducing bioavailability and that smaller particles result in a greater reduction of copper bioavailability than larger particles due to differences in area per unit mass.

In every test the clay from the 100 mg/L treatments contained significantly more copper than the 10 mg/L clays and 0 mg/L filter digest. As copper chloride concentrations increased, the difference between the 100 mg/L clay addition filters and 0 mg/L addition filters became more pronounced. The more copper present in solution, the more copper the 100 mg/L clay treatment captured. (Xu et al., 2007) reported that lead adsorption onto bentonite was dependent on the concentration of bentonite and increasing amounts of clay lead to increased surface area and more binding sites for the lead. In a daphnia exposure test involving copper, clay particles, and food, the clay greatly reduced the amount of copper present via adsorption of the copper onto the clay, while the food had much less effect on copper (Jeon 2010). The clay that removed the greatest amount of copper was the Na⁺montmorillonite followed by Zn-AlLDH-Nitrate. These data correlate with the greatest improvement in daphnid survival.

The 100 mg/L clays containing the least amount of copper at the highest copper chloride concentration were the Zn-AlLDH-Stearate and Zn-AlLDH-Carbonate. The Zn-AlLDH-Stearate, and Zn-AlLDH-Carbonate all adsorbed less than half the copper of the Na⁺montmorillonite. This could be because these clays were the most hydrophobic (based on
observation). The greater hydrophobicity resulted in larger aggregate formation in suspension with less exposed surface area and binding sites. Less time dispersed as small aggregates in suspension could allow for less interaction time between the clay and the contaminant. The charge of the particle may also be a contributor to adsorption in addition to particle size. (Zhu et al., 2011) reported that when Na⁺montmorillonite has a ligand it may adsorb more copper because of an electrostatic effect due to the decreased positive charge of the mineral where ligands are sorbed. In that study, phosphate enhanced adsorption better than sulfate because the phosphate reduced surface charge more (Zhu et al., 2011). While phosphate clays were not used in this study, sulfate clays were and they were among the poorest clays at improving the daphnia LC50.

Daphnia exposed to 200 µg/L CuCl₂ solution treated with the Zn-AILDH-Carbonate clays had lower body burdens of copper compared to Na⁺montmorillonite and Zn-AILDH-Nitrate despite the fact that the Zn-AILDH-Carbonate did not bind as much copper. It is possible that the lower body burdens are due to variation in daphnid body size. One limitation of this study is that each set of daphnia were not weighed individually but rather a “mean daphnid weight” was used in body burden calculations (mean dry weight calculated from weighing 100 daphnids). With each of the clay tests, there was a significant negative relationship between mean percent survival and copper body burden. Within a clay treatment, greater copper body burdens contributed to greater mortality but this relationship was not necessarily held between clays.

The 100 mg/L Zn-AILDH-Nitrate treatment LC50 is much higher than any other clay, but the 10 mg/L treatment did not improve the LC50 as much as the 100 mg/L treatment. Similar results were seen with all clays. At the 10 mg/L concentration there may simply not be enough
clay (or binding sites) to significantly reduce copper bioavailability. The 10 mg/L treatment of clay may be able to only remove a fraction of the copper that is causing toxicity. The 10 mg/L clays did have increasing copper with increasing copper chloride concentration, but never as much copper as the 100 mg/L clays. It is interesting to note that while the 100 mg/L Na⁺montmorillonite treatment had the next highest improvement level over the 0 mg/L treatment, the 10 mg/L Na⁺montmorillonite treatment did not have a significant effect on survival. All the 10 mg/L LC50 values were extremely close to one another, but the Na⁺montmorillonite indicates that 10 mg/L treatments are not a high enough concentration to see the survival differences between the clays.

The 10 mg/L Zn-AlLDH-Stearate filtrate contained more copper than the 0 mg/L addition at the copper chloride concentrations of 0, 25, 50, 100, and 200 µg/L. This may be due to some sort of contamination of the vessels or water samples themselves. The clay itself did not contain or release much copper as indicated by the 100 mg/L removing copper from solution. This could also be the case with some of the Zn-AlLDH-Nitrate samples, particularly in the 0, 25, 50, and 100 µg/L copper chloride concentrations where the copper levels were slightly higher with 10 mg/L Zn-AlLDH-Nitrate than 0 mg/L Zn-AlLDH-Nitrate.

One clay that did not improve survival whatsoever was the R-montmorillonite. A genotoxicity study of filtered and unfiltered clays on *Salamonella* showed R-montmorillonite® 30B introduced DNA strand breaks whether or not the sample was filtered (Sharma et al., 2010). The ammonium compound added to the clay as an organo-modifier remained in the water and caused toxicity even when the clay was filtered out (Sharma et al., 2010). The coating on the R-montmorillonite particles used in this study contained chlorine and while it may have removed
some of the metal toxicity, it induced so much additional toxicity it cannot be considered for environmental remediation.

The best predictor of how much copper toxicity could be removed from solution was the amount of clay used (10 mg/L vs. 100 mg/L). Even though clay type did not significantly affect survival, it is obvious that some clays removed more copper than others, with the best being Zn-AlLDH-Nitrate. Zn-AlLDH-Nitrate can be produced easily and with uniform structure, unlike naturally occurring Na⁺ montmorillonite, making it a real candidate for removing copper toxicity. It seems the Zn-AlLDH-Nitrate has potential for being used in drinking water filters and possibly for remediation of effluent. Zn-AlLDH-Nitrate should be tested on other metals to see the effectiveness of toxicity removal for these as well.

Zn-AlLDH-Nitrate demonstrated a real possibility as being used for a remediation tool, but this application may not be practical on a large scale, due to the large amount of clay required and the need to filter it out after it reacts with the water. The filtering method does show great promise for drinking water or other relatively small amounts of water. Perhaps in third world countries where metal-contaminated drinking water is a problem this could be one solution (Charlet et al., 2011).

Another possible use for this type of product could be for TIE characterizations. Use of nanoparticle Zn-AlLDH-Nitrate could become part of the confirmation process of metals or a particular metal. The EPA Phase 1 TIE manual states EDTA chelation as a way to establish metals are a problem, along with C₁₈ solid phase extraction and filtering with pH adjustments. Once the Phase 1 TIE implicates metals as a toxicant, it can be difficult to establish what metal or metals are responsible. This is especially true if the suspected metal is causing chronic rather than acute toxicity, because the effect level may be lower than the detection limit of the
analytical devise being used. Nanoparticle clay, particularly the Zn-AILDH-Nitrate offers an innovative solution to this problem because the metal toxicants that are bound to the clay can be filtered and concentrated in a fashion similar to what was done for this experiment making detection much easier.
CHAPTER 3
EFFECTS OF COEXPOSURE TO COPPER CHLORIDE AND LDH NANOCCLAYS ON

*Daphnia magna*

Literature Review

Forty percent of hazardous waste in the United States is a combination of organic and metal compounds (Sandrin and Maier, 2003). Metals can increase the oxidative stress to organisms and inhibit enzymes (Sandrin and Maier, 2003). Metals in the environment may also react with organic matter, clay minerals, and even organic pollutants, which all affect their bioavailability (Sandrin and Maier, 2003).

Copper is a common pollutant in water from urban and agricultural runoff. As an essential element, copper assists with metabolic activities and acts as a catalyst for cellular respiration. Enzymes containing copper include cytochrome c oxidase and lysl oxidase (initiates cross-linking of collagen through oxidation of other enzymes). Besides oxygen-metabolism, these enzymes allow for electron transport via copper redox reactions (Francis and Harrison, 1988). The same properties that allow for redox reactions can allow ROS (reactive oxygen species) to form. ROS has a damaging, oxidative effect on cells, which is promoted when excess copper leads to excess reactions (Minghetti et al., 2009). Another mechanism of copper toxicity is through the displacement of zinc in proteins (Minghetti et al., 2009). Copper replaces the zinc receptor disallowing zinc enzymes (involved in many metabolic activities) to bind (Francis and Harrison, 1988). Cu-ATPases are proteins, found in all eukaryotes, that have evolved to deal with copper transport and help prevent detrimental reactions (Minghetti et al., 2009). Their functions are to transfer copper from the carrier to the receptor in the cupro-enzymes and also to regulate copper homeostasis (Minghetti et al., 2009). Too much copper exposure can cause these
homeostatic systems to fail. In fish, copper toxicity occurs when copper/gill interactions allow for an electrolyte imbalance in the sodium channels to occur (Gheorghiu et al., 2010). This disrupts the internal ion balance and leads to death (Gheorghiu et al., 2010).

The EPA’s *Quality Criteria for Water 1986 – Copper* manual states that invertebrates are more sensitive than fish to copper toxicity. *D. Magna* and *C. dubia* were in the top six for most sensitive species when species mean acute values were calculated (EPA 2007). Daphnids had a genus mean acute value of 4.05 ug/L of dissolved copper. Acute toxicity decreases with increased hardness, alkalinity, and total organic carbon. *Americamysis bahia*, commonly studied in laboratories, has a chronic LOEC of 77 ug/L. EPA limits are based on water hardness. For example, at a hardness of 100, the four-day average copper level should not exceed 12 ug/L. Increased hardness is able to diminish copper and other metals bioavailability because cations, such as calcium, sodium, and magnesium, compete with the metal for uptake by the organism (Clifford and McGeer, 2008). In a zinc toxicity study with *Daphnia magna*, calcium was found to have the greatest toxicity reducing effect, followed by magnesium, and lastly sodium (Clifford and McGeer, 2008).

Natural organic matter (NOM) is a mixture formed from decomposed plants, animals, and microorganisms (Gheorghiu et al., 2010). NOM can reduce a metals bioavailability by binding to it and forming metal-NOM complexes (Gheorghiu et al., 2010). Copper tends to have strong covalent bonds to organic matter ligands found in NOM (Usman et al., 2005). Organic matter, such as humic substances, has negative charges that allow for the positively charged copper ions to coagulate on them (Usman et al., 2005). It is important when studying copper toxicity to take into account additional factors such as hardness, pH, and DOC (dissolved
organic carbon), that could affect the test outcome because of the effect on bioavailability (Ryan et al., 2009).

A nanoparticle is a particle having at least one dimension under 100 nm (Klaine et al., 2008). Nanoparticles in air or water are commonly referred to as colloids (Klaine et al., 2008). Colloids can be defined as small particles (generally < 1um) that are not affected by gravity (Gustafsson and Gschwend, 1997). They are important in aqueous environments because of their ability to affect transport and bioavailability of nutrients or pollutants (Ju-Nam and Lead, 2008). While there is an abundance of naturally occurring colloids, the potential effects of manufactured nanoparticles on the environment are relatively unknown (Ju-Nam and Lead, 2008).

In aquatic organisms the potential for biological impacts may be determined based on the particle’s size, structure, chemical composition, shape and aggregation, as well as aquatic factors such as pH, hardness, and organic matter (Nel et al., 2006, Handy et al., 2008). Sea water may allow for more aggregation than freshwater due to the additional ionic charges from the salt that lead to more particle collisions, and thus, more aggregation (Handy et al., 2008). pH can have a similar effect depending on the surface charge of the particles in question (Handy et al., 2008 and Alloy et al., 2011). Nanoparticles in the presence of chemical contamination can have unexpected results. In a 2010 study involving fullerenes and two pesticides fullerenes decreased acute toxicity of the pesticide tribufos, but increased acute toxicity of bifenthrin (Brausch et al., 2010). It is not understood why the toxicity patterns differed (Brausch et al 2010). This is relevant because despite knowing a nanoparticle’s characteristics and potential impact on aquatic organisms, the interactions that take place with other compounds in the aqueous environment may cause unknown organism or population effects.
Nanoparticles are already being utilized for the decontamination and removal of chemicals including lead and bisphenol A (Grazyna et al., 2009). Nanomaterials are useful in water purification for reducing chemical toxicants, bacteria, and viruses (Grazyna et al., 2009). Silver is known to have antibacterial properties and Fe₃O₄, with a mesoporous silica coating, has the capacity for waste remediation (Grazyna et al., 2009). Another possibility for bioremediation comes through the proposed use of nanoparticle clay to remove metals from water (Usman et al., 2005).

Layer double hydroxides (LDH) are anionic clays that have a structure consisting of positively charged layers separated by anions and water molecules (Mousty 2010). They have chemical and physical properties similar to cationic clays (Mousty 2010). They may be hydrophobic or hydrophilic depending on their constituents or the constituents of the environment in which they are found (Besse-Hogan et al., 2009), and can be formed in a wide range of compositions with different anions sandwiched between the metal cation layers (Hibino 2011). LDH have a demonstrated ability to absorb a wide range of contaminants including chromium, selenium, and arsenic, through ion-exchange and absorption mechanisms (Xu et al., 2007). LDH clays can even adsorb negatively-charged bacteria cells between their positively charged layers leading to increased biodegradation of non-sorbed materials like atrazine (Besse-Hoggan et al., 2009).

LDH nanoparticle clays, with their increased surface area, offer even more potential for remediation. The surface of clay can be changed with organic treatments to render the clay more hydrophobic and more compatible with polymers (Sharma et al., 2010). Changing coatings or ligands in the clay can alter its charge and ability to sorb materials (Zhu 2011). For example, when phosphate ligands were added to Fe-deformed Na+montmorillonite, copper adsorption was
enhanced more than when sulfate ligands were added (Zhu 2011). Coating and the anions used in the clay can also raise or lower solubility of the clay. For example, Zn-Al LDH clays have lower solubility than Mg-Al LDH clays, so they are more stable and more durable in aqueous solution (Hibino 2011). It is not known how the solubility will affect toxicity remediation in solution, or if the different coatings or anions used in the LDH clay will contribute to toxicity themselves.

However, clays themselves may be detrimental to aquatic organisms if they are ingested. When daphnia were exposed to carbon nanotubes the main uptake mechanism for the organisms was through feeding (Roberts et al., 2007). Daphnia are non-selective feeders within a certain particle size range (Kirk and Gilbert, 1990). The addition of clay and increased turbidity on zooplankton populations have been shown to have detrimental effects (Cuker, 1993). Clay addition can decrease light attenuation, therefore decreasing photosynthesizing algae (food for zooplankton). Adding clay can also alter zooplankton feeding ability (Cuker, 1993). Ingestion of clay particles causes a reduction in the amount of phytoplankton that can be ingested by D. magna (Kirk and Gilbert, 1990). Robinson et al. tested three clays, montmorillonite, kaolinite, and a natural clay from South Carolina, with pulsed exposures to D. magna to determine if there was a relationship between these exposures and organism response (Robinson et al., 2010). The authors demonstrated survival and growth of Daphnia magna were adversely affected by clays with montmorillonite (the smallest clay tested) having the greatest effect on survival (Robinson et al., 2010). Coatings added to clay compound potential toxicity. A 2010 comet assay with ammonium treated montmorillonite had dose-response genotoxic effects while the untreated montmorillonite had no genotoxic effects (Sharma et al., 2010). Even after the clay was filtered out the ammonium compound remained in solution and caused
genotoxic effects on the same level of magnitude as the clay/ammonium compound mix (Sharma et al., 2010).

Clay that has adsorbed metals may pose a greater risk to aquatic life. A microcosm study by Meader et al. demonstrated that algae contaminated with copper resulted in increased transport of copper into the daphnids (Meador et al., 1993). Copper-contaminated clays may also have the same detrimental effect. Small particles have a greater surface area to weight ratio than larger particles, potentially increasing the exposure (Klaine et al., 2008). Nano-particle clay may be able to adsorb even more metal because of its small size and increased surface area. While these clays may adsorb water column Cu and make it less bioavailable, those copper ions may be re-solubolized in the acidic environment of the gut during coexposure.

The goal of this research was to determine the effect of coexposure to clay nanoparticles and copper chloride toxicity on *Daphnia magna*. To accomplish this goal, the following hypothesis was tested. Smaller clay particles will increase copper uptake by *D. magna*, because of increased rates of ingestion and greater copper adsorption per unit mass of clay.

Methods

Dilution water and control water used for the test was reverse osmosis water that was also filtered through deionization (DI) resin and reconstituted to a pH of 7.8, hardness of 140, and alkalinity of 50 (hard synthetic freshwater, HSFW) (USGS.com). High-density polyethylene containers were used to store all test media. Copper chloride was obtained from Fisher Scientific. Copper dilutions of 0, 25, 50, 100, 200, and 400 µg/L were used for each test. These dilutions will be referred to as copper-contaminated water throughout this paper. Copper chloride stock was .05 grams of copper chloride into .5 L DI water. Dilutions were produced by spiking 1 L of reconstituted lab water with an appropriate amount of copper chloride stock.
Copper chloride stock was made fresh weekly and stored in a high-density polyethylene 1 L container at 4 °C when not in use. To prepare the 25 µg/L dilution, .25 mls of stock was added to a 1 L volumetric flask and the volume was brought up to to 1 L with HSFW. The 100 µg/L dilution was 1 ml of stock into 1 L of HSFW, etc.

Clays were selected based on hydrophobicity, which affects particle behavior and size in aqueous solution. The clays used were (in increasing order of hydrophobicity)

Na’montmorillonite, R-montmorillonite, Zn-AILDH-Nitrate, Zn-AILDH-Carbonate, and Zn-AILDH-Stearate. A Metler AE 240 scale was used to weigh 20.0 and 200.0 mg of each clay. These were added to 2 L of copper-contaminated water so each copper dilution had either 10.0 mg/L or 100.0 mg/L of clay. The solutions were initially hand-shaken for 30 seconds and then allowed to react for two hours prior to test initiation. Adsorption of Cr (VI) can reach equilibrium adsorption with C. bentonite after 2 hours and mo (VI) can reach equilibrium with bentonite after 1 hour (Atia 2008). 10 mg/L and 100 mg/L per liter clay blanks were prepared by adding clay in those amounts to 1L of HSFW. All blanks underwent the same treatment as the copper-contaminated water. The purpose of the blanks was to observe if the clay themselves induced toxicity.

A *Daphnia magna* acute toxicity test was run on the copper-contaminated solutions with and without the addition of clay. *Daphnia magna* were cultured in-house and monthly reference-toxicity testing took place with sodium chloride as the reference toxicant. A control of HSFW was run with every test. Tests were conducted in a 20° C room with a 16 hours light, 8 hour dark light cycle. Test chambers consisted of 100mL plastic cups containing 40mL of test solution. Each concentration had 5 replicates with 8 organisms each, giving a total of 40 per treatment. *D. magna* were loaded into the tests using a glass pipette and the pipette was changed out between
concentrations to prevent cross-contamination. *D. magna* used in tests were all <24 hours old. Each test chamber was fed a 1mL suspension of the green algae, *Selenastrum capricornutum*, along with YTC daily. Tests ended after 96 hours and survival was recorded daily.

Copper was measured using a SpectrAA 600, Atomic Absorption Spectrometer with Zeeman correction following EPA method number 7010 “Graphite Furnace Atomic Absorption Spectrophotometry”. For copper the recommended wavelength is 327.4, slit width is 0.5 nm, temp char = 2300. Graphite tubes were replaced after 300 firings and the inert gas used was either argon or helium, depending on what was available.

A calibration curve of 6 ppb, 15, ppb, 25 ppb, and 45 µg/L was used with a copper sulfate solution as the standard. The copper sulfate stock was made by adding 30 mg copper which was 117.87 mg of copper sulfate to 1 L of water. Each day new standard was made by adding 10 µL of stock to 9.99 ml of make-up water. Make-up water was .1% nitric acid diluted with milli-Q water and was used to dilute samples that were run on the AA. Each sample was run twice and all values were recorded in a lab notebook.

Four replicates of five *D. magna* were exposed to each copper/clay concentration for 24 hours for the purpose of measuring their copper content via atomic absorption spectroscopy. The same temperature, light cycle, test cup volume, volume of solution, and feeding regime was followed as used for the bioassays. All daphnia were <24 hours old at test initiation. Following exposure they were poured over a fine-micron nylon mesh, rinsed with DI water, and tweezed into a centrifuge vial to be digested for AA analysis.

The *D. magna* were digested using the aid of a conventional microwave. Microwave digestion allows for quick decomposition of the sample with little loss of volatile elements (Oliveira, 2003). It can also use a small amount of reagent (Oliveira, 2003) which was desirable
due to the small sample that required digestion. 100 µL of 70% nitric acid were added to each vial containing *D. magna*. Vials were capped and secured in a microwave proof container, then heated at 50% power for 15 second intervals until all *D. magna* were fully digested. Digestion was determined by visual inspection and was complete when the mixture was a clear yellow fluid. The *D. magna* digest was run on the AA as a 1:10 ration of digest to makeup water. This was necessary because the daphnia contained such high levels of copper. 50 µL of digest were pipetted into an AA vial with an epindorf pipette and then 500 µL of makeup water were added to it.

Approximately 6 mls of every copper/clay aqueous sample was stored in a freezer until use in a polyethylene, capped test tube. 990 µL of 0 µg/L, 25 µg/L, and 50 µg/L concentrations were added to each AA cuvet along with 10 µl of 70% acid. Concentrations of 100 µg/L, 200 µg/L, 400 µg/L had to be more dilute to be within range for the AA. The 100 µg/L was diluted 50% with 1% HNO₃, so 500 µL of 100 µg/L solution was added to 500 µL of make-up water in the AA cuvet. The 200 µg/L was diluted by adding 250 µL of 200 µg/L solution to 750 µL of make-up water and the 400 µg/L was diluted by adding 125 µL of sample to 875 µL of make-up water. All samples had a pH of <2.0 before being placed on the AA.

Clay solutions in concentrations of 10 mg/L and 100 mg/L were prepared using hard synthetic freshwater. Each clay solution was hand shaken for 30 seconds and allowed to react for at least 2 hours. A carbon-lacey grid was dipped into each solution with forceps and allowed to dry for 15 minutes.

Particle aggregates were measured with the aid of a FEI Quanta 200 Scanning Electron Microscope (SEM) and data package. The SEM was run in ESEM mode with a 0.08 torr and
spot size of 5. At least 20 images of different aggregates from each clay solution were captured and measured using image j software.

NOECs and LOECs were determined using Toxstat Version 3.4 (University of Wyoming). EPA’s Probit or Trimmed-Spearman Karber Method (when appropriate) were used to determine LC50s and confidence limits. Regression analysis was run using Excel 2007 to measure the relationship between mean percent survival and copper body burden in daphnia. SAS Learning Addition 4.1 was used for all other statistical analysis including ANOVA, Tukey Multiple Range Test, and Student-Newman-Keuls test. A p value of <0.05 indicates a significant response with an alpha level of .05 and 95% confidence limits.

Results

Copper concentration was measured using the AA in test solutions following three Na+montmorillonite treatments. The 100 mg/L Na+montmorillonite clay treatment reduced copper concentration compared to the 0 mg/L treatment (GLM $p = 0.0014$). There was no difference between the 0 mg/L treatment and 10 mg/L treatment (Fig. 26).
Daphnids exposed to the 100 mg/L treatment were analyzed for body burdens of copper. Daphnia from the 200 µg/L concentration could not be analyzed due to loss of sample.

Regression analysis indicated there was not a significant relationship between mean percent survival and body burden (GLM $p = 0.0654$) (Fig. 27) however it appears the response is outside the linear range of the toxicity curve.

FIG. 26. Copper concentration means ± 1 standard deviation of solutions after treatment with Na$^+$ montmorillonite.
The 100 mg/L Na\textsuperscript{+} montmorillonite treatment improved daphnia survival significantly over the 0 and 10 mg/L treatments (GLM $p < 0.0001$), particularly at the 50 µg/L, 100 µg/L, and 200 µg/L copper chloride concentrations. At the 100 µg/L copper chloride concentration the 0 mg/L clay treatment only had 27.5% survival, but the addition of 100 mg/L Na\textsuperscript{+} montmorillonite increased this to 100% survival. In the 200 µg/L copper chloride concentration the 0 mg/L Na\textsuperscript{+} montmorillonite treatment had 2.5% survival and the 100 mg/L Na\textsuperscript{+} montmorillonite treatment allowed for 67.5% survival (Fig. 28). Copper chloride concentration as well as the interaction between copper chloride concentration and clay treatment also had a highly significant effect on daphnia survival (GLM $p < 0.0001$). LC50 values were improved from 71.19 µg/L copper chloride in the 0 mg/L Na\textsuperscript{+} montmorillonite treatment to 224.95 µg/L copper chloride in the 100 mg/L Na\textsuperscript{+} montmorillonite treatment (Fig. 29).

**FIG. 27.** Copper concentrations in daphnia exposed to a 100 mg/L Na\textsuperscript{+} montmorillonite treatment compared to mean percent survival of each concentration. ANOVA $p = .0654$ $R^2 = 0.729$
FIG. 28. Mean survival (as percent control) ± 1 standard deviation of *Daphnia magna* exposed to copper-contaminated water and a clay treatment of Na⁺ montmorillonite.

FIG. 29. LC50 Values and 95% confidence limits of copper-contaminated water with 0, 10, and 100 mg/L Na⁺ montmorillonite.
Solutions from three Zn-AlLDH-Stearate treatments were analyzed on the AA for copper concentrations (Fig. 30). Clay treatments had a significant effect on how much copper was present (GLM $p = 0.0257$). The 100 mg/L treatment solution contained less copper than the other two treatments, which were not different from one another. There was not a significant interaction between clay treatments and copper chloride concentrations (GLM $p = 0.0577$).

Although the 100 mg/L Zn-AlLDH-Stearate treatment reduced the amount of copper in solution there was still enough to be measured in daphnia body burdens. A regression comparing daphnia body burden in the 100 mg/L Zn-AlLDH-Stearate treatment with mean percent survival of each concentration demonstrated a statistically significant relationship between the two (Fig. 31). When survival decreased, body burdens of copper increased.
Both the Zn-AlLDH-Stearate 10 mg/L treatment and 100 mg/L treatment both improved survival over the 0 mg/L treatment (GLM \( p < 0.0001 \)). In the 100 µg/L copper chloride concentration the 0 mg/L clay treatment had 27.5% survival and the 10 mg/L and 100 mg/L clay treatments had 75% and 85% survival, respectively. Approximately the same percentage increase in survival is seen in the 200 µg/L copper chloride concentration (Fig. 32). The LC50 for the 10 mg/L and 100 mg/L Zn-AlLDH-Stearate treatments were very similar (Fig 33). Both the 10 mg/L and 100 mg/L clay treatments more than doubled the LC50 from the copper only. The copper chloride concentration along with the interaction between copper chloride concentration and clay treatment were both highly significant (GLM \( p < 0.0001 \)). With increasing copper chloride concentrations survival decreased, but the interaction between clay treatments and copper chloride concentration allowed for significantly more survival in some copper chloride concentrations, such as the 100 and 200 µg/L copper chloride concentrations.
FIG. 32. Mean survival (as percent control) ± 1 standard deviation of *Daphnia magna* exposed to copper contaminated water and a clay treatment of Zn-AILDH-Stearate.

FIG. 33. LC50 Values and 95% confidence limits of copper contaminated water with 0, 10, and 100 mg/L Zn-AILDH-Stearate.
Copper-contaminated solutions with three Zn-AlLDH-Carbonate treatments were analyzed on the AA for copper concentration (Fig. 34). Clay treatment had a highly significant effect on copper concentration, as did copper chloride concentration (GLM $p < 0.0001$). All three treatments had significantly different copper concentrations from one another. The interaction between clay treatment and copper chloride concentration was significant (GLM $p < 0.0028$).

Copper remaining in solution after treatment with Zn-AlLDH-Carbonate had an adverse effect on daphnia survival. A regression was run comparing mean percent survival in each copper chloride concentration with body burdens of copper and there was a significant relationship showing a downward trend in survival with increased body burdens of copper (Fig. 35).
Survival data was analyzed to ascertain if clay treatments, copper chloride concentration, and the interaction between the two had an effect. Clay treatment did not have an effect on daphnia survival (GLM $p = 0.0815$), but copper chloride concentration had a highly significant effect on daphnia survival (GLM $p < 0.0001$). As copper chloride concentration increased daphnia survival decreased. The interaction between clay treatment and copper chloride concentration was not significant (GLM $p = 0.1219$). At the 50 µg/L copper chloride concentration, the 0 mg/L Zn-AILDH-Carbonate treatment had 70% survival, while the 10 mg/L Zn-AILDH-Carbonate treatment had lower survival at 62.5%. The 100 mg/L Zn-AILDH-Carbonate treatment had 87.5% survival in the same copper-contaminated concentration (Fig. 36). The LC50 of the 0 mg/L Zn-AILDH-Carbonate treatment was 67.04 µg/L copper chloride solution and the 10 mg/L Zn-AILDH-Carbonate treatment was 63.56 µg/L copper chloride solution. The

FIG. 35. Copper concentrations in daphnia exposed to a 100 mg/L Zn-AILDH-Carbonate treatment compared to mean percent survival of each concentration. ANOVA $p = .029$. $R^2 = 0.736$
100 mg/L Zn-AlLDH-Carbonate treatment had an LC50 of 92.31 µg/L copper chloride solution (Fig. 37).

FIG. 36. Mean survival (as percent control) ± 1 standard deviation of *Daphnia magna* exposed to copper contaminated water and a clay treatment of Zn-AlLDH-Carbonate.

FIG. 37. LC50 Values with 95% confidence limits of copper contaminated water with 0, 10, and 100 mg/L of Zn-AlLDH-Carbonate.
**Zn-AlLDH-Nitrate.** When test solutions treated with Zn-AlLDH-Nitrate were analyzed for copper concentration there was not a significant effect on copper concentration from clay treatment (GLM $p = 0.6185$). The interaction between clay treatment and copper chloride concentration was not significant (GLM $p = 0.5779$) (Fig. 38).

![Graph showing copper concentration means ± 1 standard deviation of solutions after treatment with Zn-AlLDH-Nitrate.](image)

**FIG. 38.** Copper concentration means ± 1 standard deviation of solutions after treatment with Zn-AlLDH-Nitrate.

Daphnia from the 100 mg/L Zn-AlLDH-Nitrate treatment were compared with mean percent survival in each copper chloride concentration and there was not significant relationship (Fig. 39).
Survival data was statistically analyzed and clay treatment had a highly significant effect on daphnia survival (GLM $p < 0.0001$) with the 10 mg/L Zn-AlLDH-Nitrate treatment improving survival over the 0 and 100 mg/L Zn-AlLDH-Nitrate treatments, which were grouped together. In the 50 µg/L copper chloride concentration, the 0 mg/L Zn-AlLDH-Nitrate treatment had 70% survival, the 10 mg/L Zn-AlLDH-Nitrate treatment had 90% survival, and the 100 mg/L Zn-AlLDH-Nitrate treatment had 52.5% survival (Fig. 40). The interaction between copper chloride concentration and clay treatment was also highly significant (GLM $p < 0.0001$) at affecting survival. The 100 mg/L Zn-AlLDH-Nitrate treatment LC50 was lower than the 10 mg/L Zn-AlLDH-Nitrate treatment, and wasn’t much higher than the 0 mg/L Zn-AlLDH-Nitrate treatment. The LC50 for the 0 mg/L Zn-AlLDH-Nitrate treatment was 61.52 µg/L copper.

![FIG. 39. Copper concentrations in daphnia exposed to 100 mg/L Zn-AlLDH-Nitrate compared to mean percent survival of each concentration. ANOVA $p = 0.122$. $R^2 = 0.489$](image)
chloride, the 10 mg/L treatment was 150.28 µg/L copper chloride, and the 100 mg/L treatment was 72.17 µg/L copper chloride (Fig 41).

FIG. 40. Mean survival (as percent control) ± 1 standard deviation of Daphnia magna exposed to copper contaminated water and a clay treatment of Zn-AlLDH-Nitrate.
Both the 10 mg/L and 100 mg/L treatments of R-montmorillonite caused complete mortality to the *daphnia magna* at every copper chloride concentration, so no further analysis was conducted using this clay.

When LC50 increase is examined in all of the clay treatments, there was a greater increase in LC50 values in the 100 mg/L treatments (Table 2). 100 mg/L Zn-AlLDH-Nitrate treatments had the least improvement in the LC50 of all the 100 mg/L treatments, but the 10 mg/L treatment demonstrated the most improvement in LC50 of all the 10 mg/L treatments. There was less improvement in LC50 values overall in the 10 mg/L treatments. Na’montmorillonite improved LC50 the most in the 100 mg/L treatments, but had one of the lowest LC50 values in the 10 mg/L treatment.

![FIG. 41. LC50 Values and 95% confidence limits of copper contaminated water with 0, 10, and 100 mg/L of Zn-AlLDH-Nitrate.](image)
<table>
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<th>clay type (100mg/L)</th>
<th>CuCl₂ LC50 (Without treatment)</th>
<th>clay LC50 (after treatment)</th>
<th>increase in LC50 (times)</th>
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<table>
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<th>clay LC50 (with treatment)</th>
<th>increase in LC50 (times)</th>
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<td>63.56</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 2: Summary of 10 and 100 mg/L clay treatments and their corresponding copper chloride reference test LC50, LC50 with the clay addition, and amount of increase in the LC50.

Discussion

Each clay tested, excluding Zn-AILDH-Carbonate and R-montmorillonite, demonstrated a significant reduction in copper toxicity to *Daphnia magna*. Of the four clays that significantly reduced daphnia toxicity only Na⁺montmorillonite and Zn-AILDH-Carbonate had greater toxicity reduction in the 100 mg/L treatment than the 10 mg/L treatment. Na⁺montmorillonite was the smallest aggregate size in solution the Na⁺montmorillonite toxicity result disputes the hypothesis.

Ignoring the Na⁺montmorillonite, the order of hydrophobicity from least to most, was the same order of toxicity removal from least to most. The order of hydrophobicity in the four remaining from least to most is Zn-AILDH-Nitrate, Zn-AILDH-Carbonate, and Zn-AILDH-Stearate and their corresponding amount of increase in LC50 values is 1.17, 1.19, and 2.19 times, respectively. The hypothesis that smaller clay particles will increase copper uptake by *D.*
magna because of increased rates of ingestion was true in this study with the exception of Na⁺montmorillonite based on toxicity data.

For the majority of clays, data comparing daphnia body burdens of copper to mean percent survival in each copper chloride concentration also support the hypothesis. Zn-AlLDH-Nitrate did not have a significant relationship between daphnia body burdens of copper and mean percent survival, but Zn-AlLDH-Carbonate, and Zn-AlLDH-Stearate.

The 100 mg/L Zn-AlLDH-Nitrate treatment demonstrated the least improvement in survival over the 0 mg/L treatment of any clay tested, although the 10 mg/L Zn-AlLDH-Nitrate treatment demonstrated the most improvement in survival of all the 10 mg/L clay treatments. This could help confirm the hypothesis because Zn-AlLDH-Nitrate also had very small aggregates in solution and perhaps they were being ingested and releasing copper, although copper body burden measurements do not support this. It has been demonstrated that single and multi-walled nanotubes cause daphnia toxicity by clogging up their digestive system (Roberts et al., 2007, Edington et al., 2010). This could have been the case with the Zn-AlLDH-Nitrate as it appeared to be more soluble and remain in solution longer than the other clays tested. Robinson et al found colloidal clay could be removed from the daphnia gut in as little as 30 minutes, but with constant exposure as in this case, it could interfere with food assimilation (Robinson et al., 2010). However, if interference with food assimilation were the case, an effect should also be seen in the blank and lowest copper-chloride concentration as clay concentrations were constant. It is possible that 100 mg/L Zn-AlLDH-Nitrate was just enough clay to be at a threshold concentration for a toxic effect and the additional copper levels allowed for additivity. Pulsed exposures of Na⁺montmorillonite have caused 7-day LC50 values as low as 5.17 mg/L, so it is not unreasonable to expect toxicity in the 100 mg/L treatment. In the Robinson et al study the
most detrimental effect to daphnia was caused by the clay with the smallest particle size and these were particles in the micrometer range (Robinson et al., 2010).

Another possibility for the discrepancy between the Zn-AlLDH-Nitrate 10 and 100 mg/L treatments is that Zn-AlLDH-Nitrate may be releasing a nitrogen-containing compound that is only toxic to daphnia at a level reached during the 100 mg/L treatment. Again, this concentration may be at a threshold level that allows for additive copper toxicity in the higher copper-chloride concentrations. EPA’s Aquatic Life Freshwater Quality Criteria book states that daphnia can show effects due to ammonia in the range of 0.304 to 1.2 mg/L. Ammonia values and other nitrogen-containing compound concentrations were not measured so it is not known if these could have led to the observed toxicity. Future studies may want to examine why this clay helps reduce copper toxicity at lower concentrations, but induces toxicity at a higher concentration. Low doses of Zn-AlLDH-Nitrate offer possibility for copper contamination remediation, but may be dangerous at high concentrations in the environment. Understanding the mechanisms of toxicity could help to reduce that risk.

Na⁺montmorillonite aggregates appeared to settle to the bottom of the test chambers relatively quickly (within a few hours), minimizing daphnia exposure time to the copper-laden clay and allowing it to be the exception to the hypothesis. We did not see the toxic effects that Robinson et al. found in their studies of pulsed clay, but these were static-renewal tests and there was minimal resuspension of clay in the test chambers as took place in the Robinson et al study (Robinson et al., 2010). If test chambers had been constantly agitated it may have led to increased reaction between clay and copper and possibly greater reduction of copper toxicity. Alternatively, agitation of the test chambers may also have led to increased toxicity due to greater potential for ingestion by the daphnids. If clay is to be used as a remediation tool in an
open water environment, it is likely that clay would be introduced through pulsed exposures and perhaps this scenario should be tested.

Both the 10 mg/L and 100 mg/L Zn-AlLDH-Stearate treatments were almost as effective at reducing copper toxicity on *Daphnia magna* as the 10 mg/L Zn-AlLDH-Nitrate treatment. The 10 mg/L Zn-AlLDH-Stearate treatment increased the daphnia LC50 2.26 times over the 0 mg/L treatment and the 10 mg/L Zn-AlLDH-Nitrate treatment increased the LC50 2.44 times. The 100 mg/L Zn-AlLDH-Stearate treatment improved the daphnia LC50 value 2.19 times over the 0 mg/L treatment, while the 100 mg/L Zn-AlLDH-Nitrate treatment only improved the LC50 value by 1.17 times (not a significant improvement). This data indicates that there may be a broader range of effective doses for Zn-AlLDH-Stearate than Zn-AlLDH-Nitrate making it a better option for real-world application purposes. Future studies should examine lethal and sublethal toxicity limits of Zn-AlLDH-Stearate.

One clay that did not improve survival whatsoever was the R-montmorillonite. A genotoxicity study of clays on *Salamonella* showed Cloisite® 30B introduced DNA strand breaks (Sharma et al., 2010). The ammonium compound added to the clay as an organo-modifier remained in the water and caused toxicity even when the clay was filtered out (Sharma et al., 2010). The coating on the R-montmorillonite particles used in this study contained chlorine and while it may have removed some of the metal toxicity it induced so much additional toxicity it cannot be considered for environmental remediation.

Zn-AlLDH-Stearate and Zn-AlLDH-Carbonate treatments had significant effects on copper concentration in solution and that was also confirmed by significant regression relationships comparing daphnia body burdens of copper to mean percent survival in each copper chloride concentration. The data from this study demonstrating reduced copper concentration in
solutions containing some clays, along with reduced copper toxicity in the presence of some clays may have implications for the biotic ligand model. In an experiment involving 24 factor level combinations including pH, dissolved organic carbon concentration, water hardness, and dissolved organic matter all factors had significant effects on copper toxicity (Ryan et al., 2009). There was an especially strong interactive effect between dissolved organic carbon and pH (Ryan et al., 2009), so similar interactions could be discovered with TSS (AA measure of clay in solution) and other biotic ligand components, leading to a better fitting model for toxicity prediction.

If engineered nanoparticle clays are to be used for metal decontamination in aquatic environments more hydrophobic clays seem to hold the most promise for remediation. While large concentrations of Na⁺ montmorillonite (100 mg/L) improved daphnia survival the most of any treatment the 10 mg/L treatment was almost the worst. Zn-AILDH-Stearate was second place at toxicity removal no matter the concentration (10 or 100 mg/L). This clay can be produced easily with a uniform structure, unlike naturally occurring Na⁺ montmorillonite, making it a candidate for removal of copper toxicity in aquatic environments. The clay’s hydrophobicity limits their contact with pelagic organisms, but it is unknown what the effects befall other organisms near the surface or shore and if those effects would be more or less toxic than the copper toxicity itself. The hydrophobicity of these clays that limited their contact to daphnia in this experiment could concentrate metal toxicity wherever they would accumulate in a real world application perhaps affecting sensitive organisms, populations, or even species.
CHAPTER 4
OVERALL CONCLUSIONS

This experiment examined copper chloride toxicity in *Daphnia magna* under two different exposure scenarios to nanoparticle clay. Both scenarios used three different clay treatments (0, 10, and 100 mg/L) with 6 clays (in order of hydrophobicity) Na´montmorillonite, R-montmorillonite, Zn-AlLDH-Nitrate, Zn-AlLDH-Carbonate, and ZN-AlLDH-Stearate. The goal of this research was to ascertain if nanoparticle clays could reduce copper toxicity to *Daphnia magna* by reducing copper bioavailability and if so, to also determine which clays reduced toxicity the most. In the coexposure scenario the goal was to determine the effects of coexposure to clay nanoparticles and copper chloride toxicity on *Daphnia magna*. To accomplish the first goal the hypothesis that nanoclays reduce metals toxicity by reducing bioavailability was tested and that smaller particles will result in greater reduction of copper bioavailability than larger particles due to differences in surface area per unit mass. To determine the coexposure effects the hypothesis that smaller clay particles will increase copper uptake by *D. magna*, because of increased rates of ingestion and greater copper adsorption per unit mass of clay was applied.

Nanoparticle clays do reduce copper toxicity, but the situation in which it is being used should determine which application is appropriate. If the sample can be filtered following clay treatment it is ideal to have higher amounts of clay and less exposure time. This scenario allowed for the highest LC50 values overall. If this treatment is to be used for remediation of a water body or in a situation where the clay cannot be filtered out it is more ideal to use a lower dose of clay.
When filtered and coexposure 100 mg/L clay treatments were compared, the majority of the filtered treatments improved LC50 values over the coexposure treatments. The only clay that violated this trend was the Zn-Al-S. This clay had the least improvement in LC50 value when filtered from solution, but followed only behind Na\textsuperscript{+} montmorillonite at improving LC50 values in the coexposure scenario. In the coexposure tests the longer adsorption time may have allowed for a longer copper adsorption by the Zn-Al-S, leading to higher LC50 than in the filtered experiment. Visual inspection indicated that most of the aggregates were floating on top of the solution, rather than in the water column, making them less available for daphnia to ingest.

Unlike the 100 mg/L treatments, the 10 mg/L coexposure treatments improved LC50 values over the filtered treatments, with the exception of Zn-AlLDH-Carbonate. It was one of the more hydrophilic clays with smaller aggregates allowing for more ingestion and copper toxicity to the daphnia. The relationship between body burden of copper in the 100 mg/L treatment of Zn-AlLDH-Carbonate and mean percent survival confirm this.

Zn-AlLDH-Nitrate had drastically different results when filtered treatments and coexposure treatments of 100 mg/L were examined. The filtered treatment demonstrated the most LC50 improvement of any treatment tested at 4.2 times the 0 mg/L treatment, but the coexposure treatment was the worst performing of any of the 100 mg/L treatments with an improvement in LC50 value of 1.17 times. Zn-AlLDH-Nitrate had one of the smallest aggregate sizes and when clay digest was analyzed also adsorbed more copper than most of the other clays. It is unknown why the filtered 100 mg/L treatment reduced toxicity the most and the coexposure 100 mg/L treatment was not significantly different from the 0 mg/L treatment, however a release of nitrogen is a possible explanation.
The R-montmorillonite clay used in this project induced more toxicity than the copper alone in all exposure scenarios. All treatments of R-montmorillonite caused complete mortality in all of the test solutions. It was discovered after toxicity testing that the clay was treated with chlorine. When considering a nanoclay for remediation coatings and characteristics of the clay itself should be considered prior to use.

In both clay treatments (10 mg/L and 100 mg/L) and exposure scenarios there was improvement in LC50 values over the 0 mg/L treatment for nearly all clays. This suggests that while clay nanoparticles themselves may have some detrimental effects on organisms, the benefits when they are utilized for remediation of copper pollution outweigh the negative impacts. These results were similar to another study by Jeon et al where copper toxicity to *Daphnia magna* were greatly reduced with the presence of food and clay due to decreased bioavailability and enhanced resistance due to nutrition (Jeon 2010).

It has been shown that clay can affect algae volume in the water column, therefore affecting zooplankton that feed on it (Cuker et al., 1993). Clay reduces zooplankton due to limited light for algae growth, but also limits visual acuity of predators like crustaceans (Cuker et al., 1993). In that study, 33.1 mg/L per day of Na⁺ montmorillonite were added to fish enclosures. If 33.1 mg/L per day has an adverse effect on phytoplankton it would be interesting to see what effects would occur at 10 mg/L per day or what the effects would be if copper-contaminated water was used. That study did not use nanoparticle clay which may have differing effects from clay in the micrometer range. Kirk et al noted that course 50-100 mg/L of coarse clay caused large reductions in cladoceran populations, but fine clay sometimes even increased their population (Kirk et al., 1990). Future studies should focus on effects of copper and nanoparticle clay for remediation at other trophic levels.
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