Chronic toxicity of nano metallics on red swamp crayfish (Procambarus clarkii) in laboratory and mesocosm studies

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A Thesis
Submitted to the Graduate Faculty of the
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in partial fulfillment of the
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in

The Department of Biological and Agricultural Engineering

by
Jake Andrew Farlow
B.S., Louisiana State University, 2012
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Abstract

Nanotechnology has become integrated in commercial, industrial and medical products, and its use has grown exponentially in the past several years. Although potential applications of nanoparticles (NPs) are numerous, concerns about their water quality, environmental, and human health impacts remain unclear. Crayfish are ubiquitous to streams and wetland habitats, are used as a food source, and inhabit areas that could be impacted by water quality issues. Numerous studies have been conducted on the toxicity of various classes of agricultural pesticides and oils to crayfish (*Procambarus clarkii*) as a non-target organism. However, there is little evidence published on chronic toxicity of NP to crayfish. The first objective of this study was to estimate the 28 day toxicity and bioaccumulation of the three most produced nanoparticles, Ag, ZnO, and TiO$_2$, in a laboratory adult crayfish model. The organisms were exposed to different Ag, ZnO, and TiO$_2$ nanoparticle solutions at concentrations of 0, 100, 500, and 1000 $\mu$g/mL. AgNO$_3$ and KNO$_3$, and Zn(NO$_3$)$_2$·6H$_2$O and KNO$_3$ were used as bulk controls for Ag and ZnO treatments, respectively. Dead crayfish were removed and preserved then examined for metal accumulation and pathological changes in behavior. Metal accumulation in major organs was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Results indicate that as the concentration of NP increases the uptake of metal in tissue also increases. In this case, the chemical rankings of toxicity are as follows: AgNO$_3$ > Zn(NO$_3$)$_2$ > AgNP > ZnO > KNO$_3$ > TiO$_2$. Silver accumulated in gill tissue 3 times more in AgNO$_3$ treatments than in AgNP treatments. In abdominal tissue, silver accumulated 4 times more in AgNO$_3$ treatments than in AgNP treatments. Zinc accumulated in gill tissue 2 times more in Zn(NO$_3$)$_2$ treatments than in ZnO treatments. The second objective was to estimate the bioaccumulation of the nanoparticles: Ag, ZnO, and TiO$_2$ in a mesocosm adult crayfish model. From previous experiments, we
determined the following concentrations for each tank: AgNPs 20 mg/L, ZnO 50 mg/L, and TiO$_2$ 100 mg/L. A 0 mg/L control tank was also used. Three crayfish were removed from each tank and preserved for analysis weekly. Soil samples were taken bi-weekly. ICP-OES was used to look at the accumulation of the metals in the gill and abdominal tissues as well as the soil samples. Results indicate that bioaccumulation occurs in tissues in fluctuating trend rather than an increasing trend.

Key words: crayfish, nanoparticles, toxicity, ecology, engineering
Chapter 1
Introduction

Nanotechnologies are the science and business of manipulating matter at atomic scale (A sheet of paper is about 100,000 nm thick). Nanoparticles (NPs) are solid spherical structures ranging between 1 and 100 nm in size (1 nm = 1 x 10^-9 m) (Danhier et al. 2012). Materials produced with the aid of nanotechnologies are starting to be used in many areas of everyday life including: cosmetics, clothing fabrics, sports equipment, paints, packaging, food, etc. As applications expand, many proponents are positioning nanotechnologies as part of a greener, more sustainable future (Senjen 2009). However, it is also important to consider environmental, health and safety aspects at an early stage of nanomaterial development and use in order to more effectively identify and manage potential human and environmental health impacts from nanomaterial exposure. Moreover, nanomaterial release in the environment and aquatic ecosystems may induce malignant impacts on aquatic biota (Choi et al. 2010). The aquatic ecotoxicology of nanomaterials is a relatively new and evolving field which will be more important for future generations.

Health and safety aspects of nanomaterials have raised concerns about at least four materials: carbon nanotubes, nano silver, nano zinc, and nano titanium dioxide. There is evidence that carbon nanotubes may have an adverse effect on human health and that silver NPs and titanium dioxide NPs may be detrimental to the environment (Aitken et al. 2009). However, further research needs to be conducted because not enough is known about the human health and environment impacts of nano zinc oxide. These four are the most commonly produced nanomaterials and therefore currently have the largest potential to be harmful, as they are used in substantial quantities in consumer products (Senjen 2009).
Nano silver has become one of the most commonly used nanomaterials in consumer products, mainly as a bactericide. Undesired toxicity from nanometals is of concern both for human health, but also for other organisms and ecosystems. A study analyzing the risk to freshwater ecosystems of silver NPs incorporated into textiles and plastic predicted that in the future 15% of the total silver released into water in the European Union would come from biocidal plastics and textiles (Blaser et al. 2008). Blaser et al. (2008) predict that most of the nano silver will end up in sewage sludge, natural habitats, and at least some of it may spread to agricultural fields.

Wetlands provide food, water, and cover to a wide diversity of aquatic species in South Louisiana. With increasing global production of engineered nanomaterials, there is a chance of these nanomaterials entering wastewater streams and other aquatic environmental systems, such as wetlands. There have been studies highlighting the mobility of nano silver in the food chains of *Daphina magna* and *Nereis diversicolor* (Zhao and Wang 2010; García-Alonso et al. 2011). A significant amount of nanomaterial entering the environment is expected to reach many different organisms (Abraham et al. 2013). Even though there are potential adverse effects, a lack of understanding still exists about nanomaterials, and our knowledge on the matter must be improved especially dealing with the environment risk, transport, and fate of nanomaterials (Wijnhoven et al. 2009).

Bioaccumulation is often a good integrative indicator of the chemical exposures of organisms in polluted ecosystems (Phillips and Rainbow 1994). Bioaccumulation of metals and metalloids is of value as an exposure indicator because metals are not metabolized. But metal bioaccumulation can be complex. It is influenced by multiple routes of exposure (diet and solution) and geochemical effects on bioavailability (Luoma and Rainbow 2005). Patterns of
accumulation vary among species. This includes regulation of body concentrations of some metals by some species (Chapman et al. 1996; Rainbow 2002), and vastly different concentrations among species and environments (Rainbow 1998; Cain et al. 2004). The links between bioaccumulation and toxicity are also complex (Vijver et al. 2004; Marsden and Rainbow 2004). Toxicity is determined by the uptake of metal internally and the species specific partitioning of accumulated metal between metabolically active and detoxified forms (Rainbow 2002; Roesijadi and Robinson 1993; Wallace et al. 2003). Toxicity of the different metals and metalloids could then be characterized by how different species sequester the bioaccumulated internal concentration (Luoma and Rainbow 2005; Vijver et al. 2004). Similarly, practical techniques to test toxicity on different organism and at the ecosystem scale could be invaluable to assist in assessing and managing these materials. Further research needs to be done to determine mechanistic concepts that can capture the different geochemical and biological influences on bioaccumulation.

References


Chapter 2
Literature Review

Introduction

Techniques to assess toxicity of nanoparticles to organisms and ecosystems are needed. Crayfish, which reproduce quickly, provide a possible model for aquatic organisms. Crayfish can also be put in controlled ecosystems, called mesocosms, and look at toxicity effects in a broader environmental area. While both crayfish and crawfish are acceptable terms for members of Astacidea, crawfish is the commonly used term in the southern U.S. and crayfish is the most widely accepted term for the rest of the U.S. and the world. Both crayfish and crawfish are utilized in scientific literature, however, most scientific publications, including the American Fisheries Society, prefer crayfish for common name usage. For this reason, the term crayfish is used throughout this thesis.

Crayfish Aquaculture

Louisiana’s crayfish farming industry has grown to include more than 1,200 farms occupying more than 72,843 hectares. Louisiana accounts for 90 to 95% of the total United States production of crayfish. Total production from the 2013 season was more than 45 million kilograms and gross farm sales were $137 million (Westra 2013). Seventy to eighty percent of the crayfish produced in Louisiana is consumed in Louisiana (McClain et al. 2007).

Red swamp crayfish (*Procambarus clarkii*) are adapted to habitats with seasonal flooding and drying. The life cycle of crayfish are well suited to fluctuating periods of flooding and dewatering. In their natural habitat, rivers or swamps, sustained periods of river overflow permit crayfish to feed, grow, and mature. Commercial aquaculture of Louisiana crayfish relies on earthen ponds similar to natural habitats (McClain et al. 2007). Temporary dewatering in these habitats during summer promotes aeration of bottom sediments, reduces the abundance of
aquatic predators and allows for the establishment of vegetation that serves as cover for the
crayfish and the source of food resources when the water returns in the fall. During dry intervals,
crayfish survive by digging burrows where they can avoid predators, acquire moisture necessary
for survival and reproduce in safety (McClain et al. 2007).

Currently, cambarid crayfish aquaculture practices are based on the annual water cycles
and conditions to which crayfish have become adapted over millions of years. Flooding and
drying of crayfish ponds are similar to the natural flooding and drying cycle in Louisiana’s
Atchafalaya River Basin. The ability to control the optimal timing of these events allows crayfish
farmers to positively influence water quality and food resources (Huner and Avault 1976; Chien
and Avault 1983; Romaire and Lutz 1989; Huner 1995, 1997). Crayfish aquaculture relies on
natural reproduction within ponds. As in natural habitats, crayfish in forage-based culture ponds
depend on naturally available food web for nourishment.

Crayfish are cultivated in shallow earthen ponds 8 to 24 inches deep (McClain et al.
2007). Flat, easily drained land, with suitable levees are ideal for harvesting crayfish and
management of vegetation. The soil needs to have sufficient clay to hold water and
accommodate burrow construction. Crayfish ponds are flooded in the fall and drained in the late
spring-early summer because of the oxygen demand from decaying vegetation. In fall, winter,
and spring water exchanges are often necessary for maintenance.

Crayfish aquaculture ponds have two basic production strategies (McClain et al. 2007).
One strategy is monoculture, in which crayfish are the sole crop harvested, and production
typically occurs in the same physical location for several production cycles. The second strategy
is crop rotation, in which rice, and sometimes soybeans or other crops, are raised in rotation with
crayfish. Crayfish are either rotated with rice in the same physical location year after year, or
sometimes other crops can be rotated, with crayfish being restocked between each rotation (Huner and Avault 1976; Romaine and Lutz 1989; McClain et al. 2007).

A rice-crayfish-rice crop rotation typically uses the following system calendar: in August, the rice crop is harvested and the stubble is managed for regrowth. In October the pond is flooded, and the water quality is monitored and managed. Between November and December, crayfish harvest begins when it can be economically justified. In January and February, crayfish are harvested 2 to 4 days per week according to catch and markets. In March and April, crayfish are harvested 3 to 5 days per week until late April, then the pond is drained and readied for rice planting. In May, rice is planted and managed for rice production until August.

**Crayfish Life Cycle**

The red swamp crayfish (*Procambarus clarkii*) is classified as a “temperate” species that can tolerate cold winter. Crayfish in Southern Louisiana are short lived, usually living for two years or less. The hatchlings have a high survival rate (Moss et. al, 2009). Adults mate in open water in spring, after which the female burrows into the ground to spawn. Burrowing is most prevalent in Louisiana from late spring to early summer when much of the population is mature (McClain et al. 2007). Although spawning can take place in open water, burrowing provides protection while the eggs are attached underneath the mother’s abdomen. The number of eggs laid varies with female size and condition, but large crayfish can have more than five hundred eggs attached to their abdomen (McClain et al. 2007). Females with eggs are highly susceptible to predators due to the fact that they cannot move their abdomen in a normal escape response. The hatching period is dependent on temperature and usually last about three weeks (McClain et al. 2007).
As with all crustaceans, crayfish increase in size by molting or shedding their hard exoskeleton. It takes approximately eleven molts for a young crayfish to reach maturity (McClain et al. 2007). The molt cycle has five major stages but is a continuous process. During the late pre-molt stage, crayfish stop feeding and seek shelter. Molting is usually accomplished within minutes. The crayfish forms a new soft exoskeleton while a re-adsorption of the calcium from the old shell occurs. During the inter-molt phases, the exoskeleton becomes fully hardened and the crayfish feed actively to increase body tissue. The increase in crayfish size and the length of time between molts can vary greatly. The factors that affect these variables are water temperature, water quality, food quality and quantity, population density, oxygen levels and genetic influences to a lesser degree (McClain et al. 2007). Under optimal conditions, crayfish can increase up to 15% in length and 40% in weight before it has to molt again (Moss et al, 2010).

Many ecologists and aquaculturists classify crayfish as herbivores (consumers of vegetation) or detrivores (consumers of decomposed organic matter), and omnivores (consumers of both plant and animal matter) (Momot et al. 1978; Hobbs 1993; Hill and Lodge 1994; Whitledge and Rabeni 1997; Olsson et al. 2008). Crayfish also rank among the top carnivores found in lakes and streams (Momot, W. 1995). Crayfish can ingest large amounts of herbaceous and detrital materials, while searching for and ingesting animal protein. As sources of animal protein are depleted, crayfish become facultative herbivores.

**Crayfish as an Indicator Organism**

Nearly every continent, from tropical to near freezing temperatures and freshwater to saltwater, have wetland ecosystems and coastal areas inhabited by species of crayfish or similar crustaceans. In Louisiana and the Gulf Coast of the United States, wetland ecosystems are
inhabited by 39 species of crayfish (Walls 2009). Crayfish are typically epibenthic and
subbenthic in behavior, yet they might still be exposed to contaminants dissolved or suspended
in the water column and those adsorbed to surface and subsurface soils and sediments.

Southwest Louisiana has a history of contamination due to the fossil fuel refinement and
associated petrochemical industry (Pilla et al. 2009). Pollution from both organic and inorganic
(metals) occurred before environmental laws were passed and instigated in the early 1970s.
Monitoring organics and metals in crayfish, soil and water are concerning due to the intolerable
amounts of pollutants in crayfish intended for human consumption.

The production of crayfish has gained economic importance as a commercial food
product for human consumption, fish bait and laboratory organisms for biological studies (Huner
1995). Madigosky et al (1991) stated that the crayfish was recorded as a common species for
biomonitorying studies, measurement of DNA adducts, and providing more suitable biological
environmental exposure than measurement of the parent compounds. There are many reasons
why crayfish have been used as a bioindicator species. Crayfish grow rapidly and reach
harvestable size in three to four months (Banks and Brown, 2002). They have a reasonably long
life span of about 2 years. Crayfish are also in constant physical contact with the soil and water.
The crayfish have a high enough position in the food web that biomagnification and
bioaccumulation can occur by them consuming contaminated organisms that are lower on the
food web (Moss et al. 2010).

A substantial body of scientific evidence has been published on the toxicity of various
classes of agricultural pesticides to crayfish as non-target organisms (Mayer and Ellersieck 1986;
Eversole and Seller 1997; Schlenk et al. 2001; Morgan and Brunson 2002; Benli et al. 2007;
Barbee et al. 2010). Additionally several studies have addressed the toxicity of hydrocarbons in
crayfish populations. Wiikinkoski et al. (1997) evaluated the impact of warm waste water containing 500 µg/L of mineral oil on the noble crayfish (Astacus astacus) population. They concluded that A. astacus (i.e., mature males) is more tolerant of industrially contaminated systems than has been previously believed. However, Mayer and Ellersieck (1988) observed that hydrocarbon toxicity typically decreases with increasing maturity of crayfish, so immature crayfish exposed to the wastewater might have been impacted. Crayfish have also been used as a model organism for evaluating silver toxicity. Grossel et al. (2002) demonstrated that acute (96 h) exposure to silver nitrate reduced sodium/potassium-adenosine triphosphatase (Na/K-ATPase) activity, decreased sodium influx and increased sodium efflux, in a manner similar to that of teleost fish, indicating the potential of crayfish as an invertebrate model with conserved mechanisms of toxicity. However, despite potential exposure of crayfish populations to incidental and engineered nanomaterials after spills or releases into the environment, the toxicity of such chemicals is essentially unknown.

**Metal Toxicity**

Heavy metals accumulation has been studied in crayfish (Mackeviciene 2002; Sanchez-Lopez et al. 2003; Sanchez-Lopez et al. 2004; Schmitt et. al 2006; Alcorlo et al. 2006; Richert and Sneddon 2008a; Richert and Sneddon 2008b; Hamilton et al. 2008; Hagen and Sneddon 2009; Moss et al. 2010; Neelam et al. 2010; White et al. 2012). Crayfish have the ability to bioaccumulate heavy metals while simultaneously excreting them (Mackeviciene 2002; Alcorlo et al. 2006; Sneddon et al. 2007). In the water, the actual bioavailability and toxicity depends on the form and interactions with biological receptor sites on sensitive aquatic species as well as other factors such as water quality, vegetation, and sedimentation. To survive heavy metal concentrations at constant levels, three detoxification mechanisms are needed, which include
hepatic metallothioneins (MTs), vacuolar metal precipitates, and mitochondrial sequestration. MTs in invertebrates, metal buffering is considered to be the main function of the MTs, while in fish, the MTs seem to be more involved in stress-related responses due to high inducibility of their expression by different stress-related agents (van der Oost et al. 2003 and Viarengo et al. 2007). In another form of detoxification, toxic heavy metals are removed from the cytoplasm and sequestered within the vacuolar membrane in an insoluble, detoxified form. The last form of detoxification, the mitochondrion is an organelle that removes heavy metals form epithelial cytoplasm and detoxifies the metal cation by sequestering it as an insoluble precipitate (Ahearn et al. 2004).

Nanomaterial Toxicity

Nanomaterials, once associated with the high technology industries of computing, lasers and optics have recently begun to appear in variety of consumer applications ranging from textiles and food additives to sunscreen (Wissing and Müller 2002; Takhistov et al. 2006; Benn and Westerhoff 2008). Nanotechnology continues to gain the attention of advocacy groups, academic sciences, industry representatives and governmental regulatory groups, due to the current increase in production, use and disposal of the nanomaterials. All interested in nanotechnology share a similar concern that current regulations may not be sufficient in order to protect food supplies and the environment. As nanotechnology market rapidly develops, it is predicted to exceed $1 trillion annually by 2015 (Fairbrother and Fairbrother 2009).

Nanomaterial toxicity has been investigated in aquatic vertebrates through many studies (Asharani et al. 2008; Wong et al. 2010; Bilberg et al 2010; Bai et al. 2010; Xiong et al. 2011; Shaw and Handy 2011; Griffitt et al. 2012; Browning et al. 2013) and invertebrates (Blaise et al. 2008; Baun et al. 2008; Heinlaan et al. 2008; Wang et al. 2009; Wong et al. 2010; Zhao and
Nanomaterials have many characteristics that greatly differ, and scientists have not been able to reach a consensus on the effects nanomaterials have on organisms and their environments, despite the efforts made in just the last few years through an assortment of research (Luo 2007). It is also apparent from the published literature that size is not the only factor that governs nanomaterial toxicity; particle composition, surface chemistry and morphology may also be co-factors. Due to the lack of information about degradation of nanomaterials, the understanding of the possible nanoparticle bioaccumulation throughout the various trophic levels and bioaccumulation is an important aspect of nanomaterials to examine in the future. Furthermore, biodegradation of nanomaterials may result in the modification of their structure or surface characteristics, and many nanomaterials may degrade slowly or not even degrade at all (Taku 2006). To complicate the issue there is a body of scholarly work which indicates there is no clear relationship between size and toxicity which is clearly at odds with the more traditional size-dependent determination of nanomaterial toxicity (Warheit et al. 2006; Warheit et al. 2007).

While these results are somewhat confusing, some of the differences may be related to a lack of standardized methodology. In response there has been a recent call to redefine inorganic nanomaterials, not simply by size but according to size-dependent properties, in recognition that many classes of nanoparticles are indistinguishable from bulk materials for the purposes of environmental toxicology (Affan et al. 2009). Nanomaterials were defined into three classes by the National Academy in order to clarify definitions; the three classes include natural, incidental and engineered nanomaterials (Dreher 2004). Natural materials are those found in nature and are made by typical geochemical or biochemical processes; on the other hand, incidental materials
are made from the by-products of synthetic processes. Lastly, engineered materials are those synthesized by engineered processes.

Water Chemistry

The importance of water chemistry on the bioavailability of metals has been extensively investigated over many years. The main hypothesis is that the free metal ions are the bioavailable chemical species, and therefore research has focused on identifying metal speciation in water and how this is influenced by abiotic factors such as pH, and the presence of ligands in the water that may remove free ions (e.g., dissolved organic matter), anions that can complex metals or form anionic metal species (e.g., chloride and hydroxyl ions), or cations (e.g., H\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), and Na\(^+\)) that may be competing for biological uptake with trace metals (Cusimano et al. 1986; Campbell 1995; Bervoets and Blust, 2000; Wood 2001; Pyle et al. 2002; van Leeuwen and Galceran, 2004; van Leeuwen et al. 2005; Erickson et al. 2008; Shaw and Handy 2011). For elements that have different redox states, the level of oxygenation and/ or redox potential of the natural water may also be a consideration in calculating the metal. However, for most of the simple equilibrium models, the main factors are the total concentration of the metal, pH, concentrations of divalent cations (or total hardness of the water), and the electrolytes contributing to ionic strength (e.g., bulk NaCl concentration in freshwater or seawater) (Wilkinson and Buffle 2004). These simplified models are intended for practical use in predicting free metal ion concentrations, and have been applied successfully in hazard assessment for many years (e.g., corrections for water hardness or pH in metals legislation) and to predictive models of acute metal toxicity (Playle et al. 1993; Hollis et al. 1997; Paquin et al. 2002).
The environmental chemistry and ecotoxicity of engineered NPs has recently been reviewed (Handy et al., 2008a). This chemistry shares some superficial similarities with metal chemistry in those abiotic factors such as pH, the presence of divalent ions, and ionic strength can influence the colloidal behavior (aggregation) of NPs. However, the reasons for these interactions are often fundamentally different to those for dissolved metals. Current metal speciation models are equilibrium models, whereas the behavior of NPs is described in a very different way by dynamic process where the system is dependent on the amount of energy added to the NP dispersion and the physico-chemical properties of the particles (called DLVO theory) (Handy et al. 2008). These physico-chemical properties include particle size, shape (aspect ratio), and surface charge (often measured as zeta potential). The ability of a particle to colloid and aggregate with another particle will depend on these properties, as well as the kinetic energy of the particles, viscosity of the water and any drag on the particles, and the presence of other materials in natural waters such as small peptides (e.g., bacterial exudates) or macromolecules (e.g., humic acids) that may provide steric hindrance of particle–particle interactions. Clearly, this is a fundamentally different chemistry to the metal speciation models that are more familiar to the fish ecotoxicologist. The aggregation chemistry will be useful in predicting the effective surface area of the material in the context of potential interaction with the organism, but the surface chemistry (reactivity) may inform on toxicity.

**Thesis Synopsis**

The research chapters in this Thesis were designed to examine nano metal toxicity and bioaccumulation of *P. clarkii* in the laboratory and in an outdoor mesocosm environment. The toxicity of different nano metal concentrations to crayfish in the laboratory through 28 days is examined in Chapter 3. Chapter 4 examines the toxicity of simulated nano metal “spills” to
crayfish in a semi-natural mesocosm environment for 12 weeks. Finally, Chapter 5 is a summary of the thesis and its findings as well as recommendations for future crayfish research.

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Chapter 3
Effects of Nano-scale Silver, Zinc Oxide, and Titanium Dioxide and Their Bulk Counterparts on Red Swamp Crayfish (Procambarus clarkii): Chronic Toxicity and Bioaccumulation

Objectives

The objectives of this study were to determine the bioaccumulation of silver, zinc oxide, and titanium dioxide nanoparticles in the gill and abdominal tissues of adult crayfish. Silver, zinc oxide, and titanium dioxide nanoparticles were compared to the ionic forms of these metals.

Background

There has been little scientific data published on nanometal toxicity in crayfish. In this study, crayfish are exposed to three different concentrations of nanometals and bulk metals. This study is examining toxic effects of nanoparticles to crayfish in a controlled environment.

Materials and Methods

A chronic 28-day study was done to determine the impacts of Ag, ZnO, and TiO$_2$ nanoparticles on adult crayfish Procambarus clarkii. The impacts of the bulk forms of these nanoparticles, AgNO$_3$ and ZnNO$_3$, were evaluated. KNO$_3$ was used as a control to see if the metal component or the nitrate component was more toxic. No control was used for TiO$_2$ because it is said to remain stable in solution (Qi, 2013). Each commercially purchased compound was tested independently to avoid contamination. All compounds were purchased through Sigma-Aldrich (http://www.sigmaaldrich.com/united-states.html) except for the silver nanoparticles which were purchased through NanoHorizons (http://www.nanohorizons.com/).

Suspensions of AgNP, ZnO, and TiO$_2$ were prepared with aerated single-distilled water and dispersed with a bath sonicator (JL-360, Shanghai Jieli Co., Ltd, at 100W and 40 kHz) for 20 min instead of using stabilizing agents. The shape of the NPs particles were determined using a
transmission electron microscope (TEM) (JEM-1400, JEOL, Ltd., Japan) operated at 120 kV (Figure 3.1). The particle size distribution was determined by a Nano-Zetasizer (1000 HS, Malvern Instrument Ltd., UK), which uses a dynamic light scattering (DLS) technique at 25°C and all dissolved in deionized water. During the measurement process, the effects of ionic strength, electrolyte type and electrolyte concentration on particle size were not considered since no electrolyte was added to the suspensions. Table 3.1 below shows the following characteristics of the NPs: diameter (nm), hydrodynamic diameter (d.nm), polydispersion index (PDI), and zeta potential (mv).

Table 3.1 Characteristics of nanoparticles used in this study.

<table>
<thead>
<tr>
<th>Nanoparticle Characteristics</th>
<th>Diameter (nm)\textsuperscript{a}</th>
<th>Hydrodynamic diameter (d.nm)\textsuperscript{b}</th>
<th>PDI\textsuperscript{b}</th>
<th>Zeta Potential (mV)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNP</td>
<td>55.2 ±26.5</td>
<td>378.3±30.4</td>
<td>0.24±0.05</td>
<td>-15.7±0.5</td>
</tr>
<tr>
<td>ZnO</td>
<td>93.4±20.9</td>
<td>1397.4±563.9</td>
<td>0.43±0.08</td>
<td>21.9±0.3</td>
</tr>
<tr>
<td>TiO\textsubscript{2}</td>
<td>41.7±16.4</td>
<td>401±27.4</td>
<td>0.33±0.03</td>
<td>-70.6±1.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data developed according to TEM images in our lab.
\textsuperscript{b} Particle size distribution in solutions, measured by nano-sizer.
\textsuperscript{c} Data generated by nano-sizer in lab and the pH was 7.10, 6.96, 7.02 for AgNP, ZnO, and TiO\textsubscript{2}, respectively.

A random mixture of adult male and female crayfish were obtained between March and May of 2013 from the Louisiana State University (LSU) Aquaculture Research Station, Baton Rouge, Louisiana, USA. The study was performed at the Aquaculture Engineering Laboratory in the Department of Biological and Agricultural Engineering. The test organisms were acclimated for a week to ambient laboratory conditions prior to use in the exposure tests in aerated tanks.
Figure 3.1 TEM images of suspensions of: (a) AgNPs, (b) ZnO NPs, (c) TiO$_2$ NPs. Scale bars are calibrated in nm.

Figure 3.2 Setup of laboratory experiments showing a) racks of 10 L tanks b) tank dividers with holes.
Crayfish were randomly distributed and contained in 10 L tanks that contained 4 L of test solution (Figure 3.1.a). Each tank housed 4 compartmented crayfish (Figure 3.1b). This prevented cannibalism. The average water quality parameters were: total ammonia < 4.0 ppm, pH = 7.5 ± 1.0, dissolved oxygen = 6.5 ± 0.5 ppm, and temperature = 24°C ± 2°C and were measured according to ASTM 8010 E (1992) standards. The water environment conditions of pH, dissolved oxygen (DO), and temperature were maintained within the constant range recommended by the EPA.

Each tank’s concentration was prepared using 4 L of deionized water, and the concentration of each nanoparticle can be found in Table 3.2 below.

Table 3.2 Chemical loadings for chronic metal toxicity with crayfish for 28 day exposure.

<table>
<thead>
<tr>
<th>Chemical Loadings</th>
<th>Low Concentration* (μg/mL)</th>
<th>Medium Concentration* (μg/mL)</th>
<th>High Concentration* (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>AgNP</td>
<td>100.00</td>
<td>500.00</td>
<td>1000.00</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>78.74</td>
<td>393.70</td>
<td>787.40</td>
</tr>
<tr>
<td>ZnO</td>
<td>100</td>
<td>500.00</td>
<td>1000.00</td>
</tr>
<tr>
<td>Zn(NO₃)₂ · 6H₂O</td>
<td>365.17</td>
<td>1825.83</td>
<td>3651.66</td>
</tr>
<tr>
<td>TiO₂</td>
<td>100.00</td>
<td>500.00</td>
<td>1000.00</td>
</tr>
<tr>
<td>KNO₃</td>
<td>46.88</td>
<td>1241.45</td>
<td>2482.89</td>
</tr>
</tbody>
</table>

* Each concentration was replicated three times for a total of 63 test tanks.

The metal mass component of the nanoparticle was then matched with its corresponding ionic compound mass component (Appendix Table 1 and 2). KNO₃ was used to match the nitrate component of the two bulk treatments, AgNO₃ and Zn(NO₃)₂ · 6H₂O. The lowest and the highest nitrate components were used as KNO₃ treatments. The highest concentration of KNO₃ was then divided in half to create a medium treatment for KNO₃. The concentrations can also be seen in Table 3.2 above. Each concentration in this study was replicated three times for a total of
63 test tanks. The masses were individually mixed with the DI water using manual stirring until all mass was suspended in the water. The suspension was then poured into the tanks according to concentration.

Each crayfish was weighed initially and post-mortem. This table can be found in the (Appendix Table 3). The crayfish were checked for responsiveness to a threat and to food. A piece of sinking pellet food was dropped in front of each crayfish. Responsiveness was gauged by movement of the mouth and not if the crayfish immediately ate the food. If the mouth appendages began to flutter when the food was placed in the tank, they were said to be responsive to food. Fear response was gauged by poking a disposable pipet tip at the crayfish. If the crayfish flipped its abdominal swiftly in a manner similar to escape, they were said to be responsive to fear. Each day the crayfish were fed and checked for any change in behavior or for death. Dead crayfish were collected, weighed, and then preserved using David’s Fixative (Longshaw et al. 2012). Crayfish were injected with a 10% volume/mass ratio (i.e. If the crayfish is \( \leq 10\)g, use 1mL of fixative. If the crayfish is 20g, use 2mL of fixative). They were injected with half of the measured amount into the underside of the cephalothorax (Figure 3.2.a). Then, the crayfish were injected the remaining fixative into the mandible (jaw) area (Figure 3.2b). The Control and TiO\(_2\) treatments had no mortality so a separate trials were conducted for tissue analysis. In this trial one crayfish was taken from each tank every seven days and preserved for analysis. The crayfish were then placed in food storage bags and placed in the freezer until analysis. The bags were labeled by type of chemical, tank number, crayfish number in tank, and date of preservation.
Figure 3.3 Crayfish preservation a) David’s Fixative injection into underside of the cephalothorax b) David’s Fixative injection into the mandible.
The organs that were harvested for this study were the gill and abdominal muscle. The tissue dissection protocols were as follows:

Gills:

An incision was made into the middle line of the thorax near the head. Using scissors, a cut was made through the cephalothorax from the head to the beginning of the abdomen (tail). Next, a cut was made along the cephalic groove down toward the walking legs. Using forceps, the thorax panel that covers the gill structures was firmly grasped. The panel was bent away from the crayfish and pulled to break the remaining connection.

Abdominal muscle:

An incision was made into the abdominal carapace. A cut was made down the abdominal using scissors; care was taken so as to not lacerate the abdominal muscle. The opened carapace was pried to the sides to expose the muscle. The abdominal muscle was loosened from the shell and extracted.

Each crayfish’s organ tissue underwent a digestion protocol to analyze metal content. The sample preparation protocol was modified from Moss et al. (2010). Approximately 100 mg (all masses were individually recorded) of wet tissue was removed from the entire gill structure and abdominal muscle for digestion. Gill tissue was digested using 1mL of Trace Metal Grade nitric acid (Fisher Scientific A509-P212) in a 1.5 mL microcentrifuge tube. To ensure complete digestion, each sample was allowed to sit for 24 h. Any tube exhibiting macro-level gill structure or abdominal muscle was sonicated to complete digestion.

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to calculate the amount of metal found in gill and abdominal tissue. After each tissue type from each crayfish was digested, it was added into a standard 20 mL test tube. A measured amount of
DI water was added to the tube to dilute each digestion sample. These samples were then processed by Dr. Robert Gambrell and Mr. Thomas Blanchar using ICP-OES which measured metal concentration as a function of the wavelengths emitted as the solutions were heated to plasma. The resulting values—in ppm—were analyzed with the recorded tissue mass to yield ppm/g values. These values were then converted μg/g (wet mass) to be more consistent with current research.

**Instrumentation**

All metal analyses of crayfish tissues were performed with a Varian Vista-MPX CCD simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES) with axially viewed plasma. The instrument was equipped with a mass flow controller on the nebulizer gas, cyclonic spray chamber, and 3-channel peristaltic pump. Varian's Galaxie™ ICP-Expert™ II (Agilent Technologies, Galaxie™ ICP-Expert™ II, Software, Santa Clara, California, USA) software controlled the ICP and autosampler. The wavelengths used for Ag, Zn, and Ti were 238.068 nm, 213.857 nm, and 336.122 nm, respectively.

The ICP-OES had a detection limit of 2 ppb. EPA recommends (as an initial step in the exposure assessment process) use of a residue value of ½ level of detection (LOD), as appropriate, for samples with no detectable residues if it is known or believed that these samples have been treated with a chemical agent. The use of ½ LOD (i.e., 1 ppb) for non-detectable residues in samples is widely used in the risk assessment community and is advocated by EPA (EPA, 1998a) when the appropriate conditions are met.

Brightfield microscopy was used to visually investigate the uptake of the metal exposures in the crayfish’s gill tissues. The microscope was used to take the gill filament pictures. Each crayfish had one gill (podobranch) chosen to be indicative of the entire gill structure. Multiple
gill filaments from each podobranch were laid on glass slides. HyClone™ Dulbecco's Phosphate Buffered Saline (Thermo Scientific™ SH30264.01) was used to maintain the proper osmolarity of the gill filament cells under the cover slip. 2x objectives were used to magnify individual tips of gill filaments. All gill micrographs were captured with a Nikon Eclipse TS100 microscope camera (Figure 3.4). MetaMorph® Advanced version 7.7.5.0 (Molecular Devices, Inc., MetaMorph® Advanced, Software, Downington, Pennsylvania, USA) software was used to capture digital images.

Figure 3.4 Brightfield microscopy digital images of crayfish gill tissue using same contrast. The following images are shown above: a) control day 7 b) control day 14 c) control day 21 d) AgNP 100 µg/mL day 7 e) AgNP 100 µg/mL day 14 f) AgNP 100 µg/mL day 28 g) AgNP 1000 µg/mL day 4 h) AgNP 1000 µg/mL day 7 i) AgNP 1000 µg/mL day 13 j) AgNO₃ 78.74 µg/mL 3 hours k) AgNO₃ 393.7 µg/mL 3 hours l) AgNO₃ 787.4 µg/mL 6 hours.
Data Analysis

SAS version 9.3 (SAS Institute Inc., SAS 9.3, Software, Cary, North Carolina, USA) was used to analyze the data. All tables and graphs were constructed using Microsoft Excel 2010 (Microsoft, Microsoft Excel 2010, Software, Redmond, Washington, USA).

Change in mass averages were analyzed using one-way ANOVA comparing variances of each treatment change in mass averages to the control change in mass averages. F-statistic gives the P-value for the test of equal variances. If the probability (Pr > F) is less than 0.05 ($\alpha =0.05$), there is evidence that the variances for the two groups are different. Therefore, the Satterthwaite (Unpooled) variance estimator is used to suggest areas of significance. If the probability (Pr > F) is greater than 0.05 ($\alpha =0.05$), the Pooled (Equal) variance estimator is used to suggest areas of significance.

Gill and abdominal tissue metal concentration averages were also analyzed using one-way ANOVA comparing variances of each treatment metal concentration averages to the control metal concentration averages. F-statistic gives the P-value for the test of equal variances. If the probability (Pr > F) is less than 0.05 ($\alpha =0.05$), there is evidence that the variances for the two groups are different. Therefore, the Satterthwaite (Unpooled) variance estimator is used to suggest areas of significance. If the probability (Pr > F) is greater than 0.05 ($\alpha =0.05$), the pooled (Equal) variance estimator is used to suggest areas of significance.

Results

The survival of crayfish in the treatments, AgNP and AgNO$_3$ are showed in Figure 3.4. Survival graphs were used to evaluate the toxicity of each chemical used. Ionic AgNO$_3$ was far more toxic than its nanoparticle form of AgNP. There was only one crayfish (8.33%) that survived the 28 day study in silver treatments, the lowest concentration of 100 µg/mL AgNP.
Table 3.3 Results of chronic metal toxicity with Crayfish for 28-d exposure.

<table>
<thead>
<tr>
<th></th>
<th>Concentration exposed to crayfish</th>
<th>Survival (%)</th>
<th>Change in Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>Control (Ag)</td>
<td>100</td>
<td>1.66±1.77</td>
</tr>
<tr>
<td></td>
<td>Control (Zn)</td>
<td>100</td>
<td>1.66±1.77</td>
</tr>
<tr>
<td></td>
<td>Control (Ti)</td>
<td>100</td>
<td>1.66±1.77</td>
</tr>
<tr>
<td><strong>AgNP</strong></td>
<td>100 µg/mL</td>
<td>8.33</td>
<td>2.78±1.05</td>
</tr>
<tr>
<td></td>
<td>500 µg/mL</td>
<td>0</td>
<td>2.60±0.85</td>
</tr>
<tr>
<td></td>
<td>1000 µg/mL</td>
<td>0</td>
<td>2.31±0.96</td>
</tr>
<tr>
<td><strong>AgNO₃</strong></td>
<td>78.74 µg/mL</td>
<td>0</td>
<td>2.02±0.95</td>
</tr>
<tr>
<td></td>
<td>393.7 µg/mL</td>
<td>0</td>
<td>2.18±2.24</td>
</tr>
<tr>
<td></td>
<td>787.4 µg/mL</td>
<td>0</td>
<td>2.66±0.93</td>
</tr>
<tr>
<td><strong>ZnO</strong></td>
<td>100 µg/mL</td>
<td>16.67</td>
<td>1.95±1.01</td>
</tr>
<tr>
<td></td>
<td>500 µg/mL</td>
<td>16.67</td>
<td>1.66±1.41</td>
</tr>
<tr>
<td></td>
<td>1000 µg/mL</td>
<td>8.33</td>
<td>1.32±1.95</td>
</tr>
<tr>
<td><strong>Zn(NO₃)₂</strong></td>
<td>365.17 µg/mL</td>
<td>0</td>
<td>2.48±0.91</td>
</tr>
<tr>
<td></td>
<td>1825.83 µg/mL</td>
<td>0</td>
<td>1.86±2.64</td>
</tr>
<tr>
<td></td>
<td>3651.66 µg/mL</td>
<td>0</td>
<td>1.70±3.34</td>
</tr>
<tr>
<td><strong>TiO₂</strong></td>
<td>100 µg/mL</td>
<td>100</td>
<td>1.16±0.58</td>
</tr>
<tr>
<td></td>
<td>500 µg/mL</td>
<td>100</td>
<td>1.58±0.60</td>
</tr>
<tr>
<td></td>
<td>1000 µg/mL</td>
<td>100</td>
<td>0.67±1.88</td>
</tr>
<tr>
<td><strong>KNO₃</strong></td>
<td>46.88 µg/mL</td>
<td>66.67</td>
<td>2.11±0.79</td>
</tr>
<tr>
<td></td>
<td>1241.45 µg/mL</td>
<td>50</td>
<td>2.15±0.99</td>
</tr>
<tr>
<td></td>
<td>2482.89 µg/mL</td>
<td>0</td>
<td>2.11±0.79</td>
</tr>
</tbody>
</table>

Figure 3.5 shows the survival of crayfish in the treatments, ZnO and Zn(NO₃)₂. Again the ionic Zn(NO₃)₂ was more toxic that its nanoparticle form of ZnO. No crayfish survived the any of Zn(NO₃)₂ treatments. Two crayfish (16.67%) survived in each of the 100 µg/mL and 500 µg/mL treatments of ZnO, while only one crayfish (8.33%) survived the highest concentration of 1000 µg/mL ZnO. Figure 3.6 and Figure 3.7 show the survival of crayfish in the treatments, TiO₂ and KNO₃. Every treatment of TiO₂ had a 100% survival rate. TiO₂ had little to no toxicity. No crayfish survived the highest concentration (1000 µg/mL) of KNO₃, but 66.67% and 50% of crayfish survived 100 µg/mL and 500 µg/mL, respectively. KNO₃ was not as toxic as the other
ionic compounds of AgNO$_3$ and Zn(NO$_3$)$_2$. Table 3.3 shows each treatments’ respective survivability.

Figure 3.5 Crayfish survival when exposed to a) silver nanoparticles and b) silver in bulk form. AgNO$_3$ was highly toxic and plotted in hours. Silver nanoparticles have a lower toxicity than AgNO$_3$.

The changes of mass were averaged for each treatment with a standard deviation (Appendix Table 3). Table 3.3 also shows each treatments change in mass average. No treatments’ change in mass average was statistically significant ($p < 0.05$) from the control.
Figure 3.6 Crayfish survival when exposed to a) zinc nanoparticles and b) zinc in bulk form. The ZnO nanoparticles have a lower toxicity than Zn(NO₃)₂.

The results of the ICP-OES analysis of gill tissue concentrations can be found in Table 3.4, 3.6, 3.8. The tables show 3, 7, 14, 21, 28 day averages of gill tissue concentrations of each treatment metal.

Both AgNP and AgNO₃ silver concentration averages were all statistically significant from the control. Silver accumulated 3 times more in AgNO₃ treatments than in AgNP treatments. The significance of the gill zinc concentration averages for ZnO treatments increased as the study progressed. All of the Zn(NO₃)₂ treatments gill concentration averages were also
statistically significant (p < 0.001) from the control. Zinc accumulated 2 times more in Zn(NO\textsubscript{3})\textsubscript{2} treatments than in ZnO treatments.

![Graph 1](image1)

**Figure 3.7** Crayfish survival when exposed to titanium nanoparticles. Titanium is not highly toxic.

![Graph 2](image2)

**Figure 3.8** Crayfish survival when exposed to potassium nitrate. The nitrate component has moderate toxicity.
Table 3.4 Results of ICP-OES for average silver content in crayfish gill tissue for a 28-d exposure\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Concentration exposed to crayfish</th>
<th>Gill Tissue Average Silver Content ((\mu g/g))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 day</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>0 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>AgNP</td>
<td></td>
</tr>
<tr>
<td>100 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>500 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>1000 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td></td>
</tr>
<tr>
<td>78.74 (\mu g/ml)</td>
<td></td>
</tr>
<tr>
<td>393.7 (\mu g/ml)</td>
<td></td>
</tr>
<tr>
<td>787.4 (\mu g/ml)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Statistical difference compared with the control treatments (* \(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), t test). \(x\) = no crayfish died or no crayfish were left.

Table 3.5 Results of ICP-OES for average silver content in abdominal tissue for a 28-d exposure\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Concentration exposed to crayfish</th>
<th>Abdominal Tissue Average Silver Content ((\mu g/g))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 day</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>0 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>AgNP</td>
<td></td>
</tr>
<tr>
<td>100 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>500 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>1000 (\mu g/ml)</td>
<td>x</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td></td>
</tr>
<tr>
<td>78.74 (\mu g/ml)</td>
<td></td>
</tr>
<tr>
<td>393.7 (\mu g/ml)</td>
<td></td>
</tr>
<tr>
<td>787.4 (\mu g/ml)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Statistical difference compared with the control treatments (* \(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), t test). \(x\) = no crayfish died or no crayfish were left.
Table 3.6 Results of ICP-OES for average zinc content in crayfish gill tissue for a 28-d exposure.

<table>
<thead>
<tr>
<th>Concentration exposed to crawfish</th>
<th>3 day</th>
<th>7 day</th>
<th>14 day</th>
<th>21 day</th>
<th>28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0 µg/ml</td>
<td>x</td>
<td>42.96±4.92</td>
<td>39.69±6.05</td>
<td>37.04±5.50</td>
</tr>
<tr>
<td><strong>ZnO</strong></td>
<td>100 µg/ml</td>
<td>x</td>
<td>170.87±51.41</td>
<td>153.08±41.52</td>
<td>144.03±25.11**</td>
</tr>
<tr>
<td></td>
<td>500 µg/ml</td>
<td>x</td>
<td>612.15±12.27</td>
<td>927.71±202.92*</td>
<td>862.60±138.90***</td>
</tr>
<tr>
<td></td>
<td>1000 µg/ml</td>
<td>x</td>
<td>1800.56±602.85</td>
<td>1507.03±344.80**</td>
<td>1984.05±530.09**</td>
</tr>
<tr>
<td><strong>Zn(NO₃)₂</strong></td>
<td>365.17 µg/ml</td>
<td>x</td>
<td>1763.09±207.32***</td>
<td>2456.00±345.08***</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>1825.83 µg/ml</td>
<td>x</td>
<td>2893.47±314.35***</td>
<td>3461.88±338.74***</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>3651.66 µg/ml</td>
<td>x</td>
<td>2964.40±185.71***</td>
<td>3155.98±250.24***</td>
<td>x</td>
</tr>
</tbody>
</table>

*a Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test). x = no crayfish died or no crayfish were left.

Table 3.7 Results of ICP-OES for average zinc content in abdominal tissue for a 28-d exposure.

<table>
<thead>
<tr>
<th>Concentration exposed to crawfish</th>
<th>3 day</th>
<th>7 day</th>
<th>14 day</th>
<th>21 day</th>
<th>28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0 µg/ml</td>
<td>x</td>
<td>34.94±3.58</td>
<td>31.83±2.38</td>
<td>29.61±2.00</td>
</tr>
<tr>
<td><strong>ZnO</strong></td>
<td>100 µg/ml</td>
<td>x</td>
<td>36.18±5.05</td>
<td>37.29±3.11</td>
<td>37.65±2.38*</td>
</tr>
<tr>
<td></td>
<td>500 µg/ml</td>
<td>x</td>
<td>53.72±0.44*</td>
<td>70.43±6.86**</td>
<td>62.95±6.61**</td>
</tr>
<tr>
<td></td>
<td>1000 µg/ml</td>
<td>x</td>
<td>108.62±32.60</td>
<td>89.47±16.80*</td>
<td>92.85±14.97**</td>
</tr>
<tr>
<td><strong>Zn(NO₃)₂</strong></td>
<td>365.17 µg/ml</td>
<td>x</td>
<td>46.67±7.24</td>
<td>49.61±8.26*</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>1825.83 µg/ml</td>
<td>x</td>
<td>82.57±12.78*</td>
<td>70.88±10.42**</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>3651.66 µg/ml</td>
<td>x</td>
<td>125.97±47.40**</td>
<td>119.71±43.86***</td>
<td>x</td>
</tr>
</tbody>
</table>

*a Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test). x = no crayfish died or no crayfish were left.
Table 3.8 Results of ICP-OES for average titanium content in crayfish gill tissue for a 28-d exposure\(^a\).

<table>
<thead>
<tr>
<th>Concentration exposed to crayfish</th>
<th>3 day</th>
<th>7 day</th>
<th>14 day</th>
<th>21 day</th>
<th>28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.28±0.04</td>
<td>0.24±0.03</td>
<td>0.27±0.03</td>
<td>0.30±0.05</td>
</tr>
<tr>
<td>TiO(_2)</td>
<td></td>
<td>0.27±0.05</td>
<td>0.78±0.16</td>
<td>0.43±0.12</td>
<td>0.82±0.42</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>x</td>
<td>0.60±0.08</td>
<td>0.58±0.17</td>
<td>0.45±0.13</td>
<td>1.13±0.52</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>x</td>
<td>2.82±0.94</td>
<td>2.39±0.88*</td>
<td>2.10±0.71*</td>
<td>1.91±0.54*</td>
</tr>
<tr>
<td>1000 µg/ml</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test). x = no crayfish died or no crayfish were left.

Table 3.9 Results of ICP-OES for average titanium content in abdominal tissue for a 28-d exposure\(^a\).

<table>
<thead>
<tr>
<th>Concentration exposed to crayfish</th>
<th>3 day</th>
<th>7 day</th>
<th>14 day</th>
<th>21 day</th>
<th>28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.18±0.00</td>
<td>0.34±0.14</td>
<td>0.28±0.10</td>
<td>0.26±0.08</td>
</tr>
<tr>
<td>TiO(_2)</td>
<td></td>
<td>0.16±0.01</td>
<td>0.16±0.01</td>
<td>0.17±0.01</td>
<td>0.22±0.05</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>x</td>
<td>0.89±0.54</td>
<td>0.72±0.31</td>
<td>0.61±0.24</td>
<td>1.03±0.50</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>x</td>
<td>0.18±0.00</td>
<td>0.34±0.14</td>
<td>0.28±0.10</td>
<td>0.26±0.08</td>
</tr>
<tr>
<td>1000 µg/ml</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test). x = no crayfish died or no crayfish were left.

The TiO\(_2\) treatment only had statistically significant titanium concentration averages at the highest concentration (1000 µg/mL) of TiO\(_2\) and for 14, 21, and 28 day gill tissue averages. KNO\(_3\) tissue metal concentration averages were not determined because KNO\(_3\) was used as a control to match the nitrate components with AgNO\(_3\) and Zn(NO\(_3\))\(_2\).
The results of the ICP-OES analysis of abdominal tissue concentrations can be found in Table 3.5, 3.7, 3.9. The tables show 3, 7, 14, 21, 28 day averages of abdominal tissue concentrations of each treatment metal. Abdominal tissue metal concentration averages for all the metals were lower in metal concentration than the gill tissue metal concentration averages.

The AgNP treatments average abdominal silver concentrations were all statistically significant from the control. Similar to the gill tissue, AgNO₃ abdominal silver concentration averages were statistically significant (p < 0.001) from the control. Silver accumulated 4 times more in AgNO₃ treatments than in AgNP treatments. All of the abdominal zinc concentration averages of the 500 μg/mL ZnO were statistically significant from the control. The other two treatments of ZnO (100 μg/mL and 1,000 μg/mL) had increasing statistically significant abdominal zinc concentration averages as the study progressed. All of the Zn(NO₃)₂ treatments gill concentration averages were also statistically significant from the control, except for the 7 day 365.17 μg/mL treatment of Zn(NO₃)₂ average abdominal zinc concentration. None of the TiO₂ treatments were statistically significant from the control. Again, KNO₃ tissue metal concentration averages were not determined.

Crayfish that were treated with TiO₂ exhibited aggressive behavior throughout the study. When prodded, the crayfish would move into a defensive position and snap their pincers. In TiO₂, crayfish abdominal muscle was in the flexed position for the entire study and could be due to the buildup of titanium on the exoskeleton of the crayfish. On the other hand, all other crayfish in other treatments were similar in behavior to the control.

**Discussion**

The NP showed stability and uniform dispersion throughout the test period. No agglomeration and precipitation were observed except titanium. NPs stability is a major concern
in nanotechnology and nanotoxicology. An unstable NP will precipitate to a metal clump which affects the test interpretations. However, this study was designed to simulate a chemical spill using industrial sourced NPs. Hence it is ideal to use water as a carrier solvent to test nanoparticle toxicity. Use of water-insoluble NPs could be less effective from phase separation. Starch and BSA are biocompatible agents that possess the advantages of being non-toxic and good water soluble stabilizing agents for NPs.

The results suggest the toxicity of NPs to aquatic species is concentration-dependent. Laboratory studies show that the nanoparticle and bulk forms of silver and zinc are toxic to crayfish (*Procambrus clarkii*). Titanium NPs showed little to no toxicity. Potassium in bulk form had a similar or lower toxicity than bulk silver and zinc which shows that the nitrate is moderately toxic. The chemical rankings of toxicity are as follows: AgNO₃ > Zn(NO₃)₂ > AgNP > ZnO > KNO₃ > TiO₂. The impact of NPs and bulk treatments on metal tissue concentration were highest in gill tissue followed by abdominal tissue.

AgNO₃ was the most toxic of all the chemicals used and was far more toxic than its’ NP form. The crayfish in AgNO₃ survived at the max 7 days of the 28 day experiment. The tissue digestion shows the AgNO₃ crayfish having a higher silver content (µg/g) in both gill and abdominal tissue than that of the AgNP crayfish. Both AgNP and AgNO₃ treatments stayed in uniform dispersion throughout the test period and was gray in color and opaque. As the test went on, the crayfish developed a blue-gray sheen on the exoskeleton.

Freshwater fish are possibly affected the most by silver, and the effects are due to the disturbance of osmoregulation in the gill (Lima et al. 1982; Nebeker et al. 1983; Bianchini et al. 2002; Bilberg et al. 2009). The mechanism of AgNPs is undefined, but it is known that NPs cause respiratory toxicity in rodents (Handy and Shaw 2007; Sung et al. 2008) Similar to rodent
NP respiratory toxicity, it is very likely that the gills of fish are also vulnerable to NPs. The toxicity of silver is dependent mostly on matter that it can form covalent, colloidal, or complex bond; this characteristic is due to silver’s cationic nature and how it strongly associates with various ligands, while it is located in natural waters (Ratte 1999). It is still ambiguous if AgNPs are a directly correlated to the enhanced toxicity, even though the toxicity of AgNPs may be justified by the release of Ag ions. For example, Navarro et al. (2008) presented evidence that toxicity is mainly the result of Ag ions, and this research also showed that AgNPs contribute to toxicity by a source of dissolved Ag ions. In contrast, Fabrega et al. (2011) showed a specific NP effect that could not be explained by dissolved Ag ions.

There is little information on surface chemistry and aggregation state of nanomaterials; therefore, not much is known about the sources of enhanced or inhibited toxic effect in comparison to Ag ions. One hypothesis is that the toxicity will be affected by NP aggregation by decreasing the rate of dissolution, uptake by organism, and stability of the NP against aggregation. This hypothesis is supported by recent research done by Reinsch et al. (2012), and it showed that growth is more inhibited by aggregated AgNPs compared to dispersed AgNPs. This occurrence is possibly due to a less complete sulfidation of the AgNPs in the aggregates.

Zn(NO$_3$)$_2$ was the second most toxic of all the chemicals used and was far more toxic than its’ NP form. The tissue digestion shows the Zn(NO$_3$)$_2$ crayfish having a higher zinc content ($\mu$g/g) in both gill and abdominal tissue than that of the ZnO crayfish. ZnO was found to be less toxic than AgNPs. Both ZnO and Zn(NO$_3$)$_2$ treatments stayed in uniform dispersion throughout the test period and was white-blue in color and slightly transparent. As the test went on, the crayfish developed a white-blue sheen on the exoskeleton.
The hardness of dilution water, the dissolved oxygen concentration, and temperature are some of the environmental factors that modify the toxicity of zinc compounds to aquatic species (Skidmore 1964). To have consistency among results, all of environmental factors in laboratory experiments were controlled to obtain consistency among results. When there are acute toxic concentrations of zinc, the gill tissue of a fish is destroyed most likely killing the fish, but when there are chronic toxic concentration, a stress is induced which causes death. Evidence has pointed to dissolved Zn ions being the main attributor to the toxicity of ZnO (Franklin et al. 2007, Aruoja et al. 2009, Brunner et al. 2006; Wong et al. 2010); yet, some evidence of nanoparticle specific toxicity has also caused researchers to proceed with caution against such generalization of the toxicity mechanisms of nanomaterials (Wang et al. 2008; Heinlaan et al. 2008).

Differential gene expression profiling in *D. magna* has shown that dissolved Zn$^{2+}$ and ZnO from particle-dependent effects both have distinct modes of action. Furthermore, environment exposure of ZnO may be identified by three biomarker genes including a multicystatin, ferritin, and C1q, and these genes may be used to distinguish between NPs and ionic exposure (Poynton et al. 2010). Although many controversies exist on the origin of this particle dissolution-related toxicity, many factors are considered to trigger toxic effects. These factors include Zn-dependent reactive oxygen species formation following particle dissolution (Xia et al. 2008; Feris et al. 2010); disruption of cellular zinc homeostasis which leads to lysosomal and mitochondria damage and ultimately cell death; and inhibition of enzyme activity (Xia et al. 2008).

In the study, the TiO$_2$ solutions were originally white and opaque. The crayfish developed a white sheen on the exoskeleton TiO$_2$ NPs had no measurable effects on crayfish at low doses
(100–500 μg/mL), whereas there were significant effects at higher levels (1000 μg/mL). These findings are consistent with the results from in vitro toxicity tests on a rat liver-derived cell line (Hussain et al. 2005). Xiong et al. (2011) suggest that ·OH generated by TiO₂ NPs may result in oxidative stress and damage but the toxicity of ·OH is not lethal. Therefore, it can be concluded that, at high concentrations, TiO₂ NPs had a dose–response toxicity.

Conclusion

In this research I characterized the toxicity and behavior of NPs (AgNP, ZnO, and TiO₂) and bulk metals (AgNO₃, Zn(NO₃)₂ and KNO₃) to Procambarus clarkii in an aqueous exposure medium. From the results of this study, it can be concluded that: (1) The chemical rankings of toxicity are as follows: AgNO₃ > Zn(NO₃)₂ > AgNP > ZnO > KNO₃ > TiO₂; (2) the chronic toxicity of AgNPs and bulk AgNO₃ to crayfish were significantly higher compared to the control; (3) the chronic toxicity of ZnO and bulk Zn(NO₃)₂ to crayfish were significantly higher compared to the control; (4) TiO₂ NPs were able to cause toxicity effects without causing mortality. This study has taken a first step in the direction of investigating the ecotoxicity of metal oxide NPs to Procambrus clarkii and highlights the need for integrated toxicological assessment of metal oxide NPs in aquatic systems. Thus, further research into the properties of NPs with different chemical compositions and the NP behaviors of environmental water chemistry as related to the toxicity mechanisms of NPs, are required to evaluate the aquatic eco-toxicity of metal-oxide NPs and to determine definitively whether their toxicity is caused by nano-effects.

Future work, in addition to the lethal toxicity test, may include molecular biomarker response tests which may be essential to determine the sublethal toxicities of the test chemicals to crayfish and provide insights into toxic mechanisms of NPs. By monitoring several
biomarkers, including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA) and protein carbonyl contents, the disturbances of the oxidative defense system caused by exposure to NPs and their bulk particle suspensions can be observed in the gill, gut, exoskeleton, and hepatopancreas (liver) tissue in crayfish.

References


Chapter 4
Bioaccumulation of Silver, Zinc Oxide, and Titanium Dioxide Nanoparticles in Red Swamp Crayfish (Procambarus clarkii) In Mesocosm Studies

Objectives

The objectives of this study were to determine the bioaccumulation of silver, zinc oxide and titanium dioxide nanoparticles in the gill and abdominal tissues of adult crayfish. This study was preformed over 12 weeks where crayfish were exposed to the outdoor environment but contained within large fiberglass tanks.

Background

There has been little scientific data published on nanometals toxicity on crayfish. In this study, crayfish are exposed to concentrations of silver, zinc, and titanium nanoparticles. This study is simulating a “chemical spill” in which the crayfish would be exposed to in natural habitats. The mesocosm allows for the containment of crayfish in semi-natural environment. This study simulates a “real world” situation representing a possible chemical spill.

Materials and Methods

A chronic 12-week mesocosm study was done to determine the impacts of Ag, ZnO, and TiO₂ nanoparticles on adult crayfish Procambarus clarkii. Each commercially purchased compound was tested independently of each other to avoid contamination. All compounds were purchased through Sigma-Aldrich (http://www.sigmaaldrich.com/united-states.htmL) except for the silver nanoparticles which were purchased through NanoHorizons (http://www.nanohorizons.com/). Suspensions of AgNP, ZnO, and TiO₂ were prepared with aerated single-distilled water and dispersed with a bath sonicator (JL-360, Shanghai Jieli Co., Ltd, at 100W and 40 kHz) for 20 min instead of using stabilizing agents. The shape of the NPs
50 particles were determined using a transmission electron microscope (TEM) (JEM-1400, JEOL, Ltd., Japan) operated at 120 kV (Figure 4.1).

![TEM images](image)

Figure 4.1 TEM images of suspensions of: (a) AgNPs, (b) ZnO NPs, (c) TiO$_2$ NPs. Scale bars are calibrated in nm.

The particle size distribution was determined by a Nano-Zetasizer (1000 HS, Malvern Instrument Ltd., UK), which uses a dynamic light scattering (DLS) technique at 25°C and all dissolved in deionized water. During the measurement process, the effects of ionic strength, electrolyte type and electrolyte concentration on particle size were not considered since no electrolyte was added to the suspensions. Table 4.1 below shows the following characteristics of the NPs: diameter (nm), hydrodynamic diameter (d.nm), polydispersion index (PDI), and zeta potential (mv).

Table 4.1 Characteristics of nanoparticles used in this study.

<table>
<thead>
<tr>
<th>Nanoparticle Characteristics</th>
<th>Diameter (nm)$^a$</th>
<th>Hydrodynamic diameter (d.nm)$^b$</th>
<th>PDI$^b$</th>
<th>Zeta Potential (mV)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNP</td>
<td>55.2±26.5</td>
<td>378.3±30.4</td>
<td>0.24±0.05</td>
<td>-15.7±0.5</td>
</tr>
<tr>
<td>ZnO</td>
<td>93.4±20.9</td>
<td>1397.4±563.9</td>
<td>0.43±0.08</td>
<td>21.9±0.3</td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>41.7±16.4</td>
<td>401±27.4</td>
<td>0.33±0.03</td>
<td>-70.6±1.4</td>
</tr>
</tbody>
</table>

$^a$Data developed according to TEM images in our lab.

$^b$Particle size distribution in solutions, measured by nano-sizer.

$^c$Data generated by nano-sizer in lab and the pH was 7.10, 6.96, 7.02 for AgNP, ZnO, and TiO$_2$, respectively.
Mesocosm studies were conducted at the LSU AgCenter Aquaculture Research Station (30.369715, -91.178373). Five fiberglass tanks (3.6 meters diameter x 1.3 meters high) were used in the experiments. The tanks were located outside and exposed to the weather (Figure 4.2). The tanks were filled with 15±5 centimeters of Mississippi River Alluvial soil, and samples were taken for content analysis before the experiments. The tanks were then drained and dried out for a week. To simulate a 15 centimeter water depth of a crayfish pond, 1,665 L of well water were added to each tank. A Kent / AMCO C700 positive displacement water meter was used to measure the 1,665 L of water for each tank.

To simulate a common crayfish pond, Jupiter rice was grown as a source of food for the crayfish. As per LSU AgCenter publication “Rice Varieties & Management Tips 2012”, the rice was water seeded 140 kg/ha. Based on the area of the tanks, each tank was seeded with 0.15 kg per tank on March 15. The tanks also had *Typha latifolia* (cattails) growing in them as well. Mesocosms that required aeration as a component of the experimental protocol were supplied with air from one ceramic diffuser (Model # AS15L, Aquatic Eco-Systems, Apopka, FL, USA) per mesocosm to maintain DO levels above 3 mg/L (Matherne et al, 2013).

One hundred crayfish were added to four tanks on April 30, 2013. A random mixture of adult male and female crayfish were provided by the LSU AgCenter’s Aquaculture Research Station. The crayfish were allowed to acclimate for a week before the nanoparticles were added. A control tank was also used. To simulate crayfish moving into a region of pollution silver nanoparticles were added one week before the crayfish were introduced. Overall there were five tanks total: Control, AgNP before crayfish added, AgNP after crayfish added, ZnO, and TiO₂. The concentrations of these nanoparticles were determined from the previous laboratory experiments.
Figure 4.2 Fiberglass mesocosm tanks with rice, cattails, and pyramid traps in place.

From those experiments, the following concentrations were determined for each tank: AgNP 20 mg/L, ZnO 50 mg/L, and TiO$_2$ 100 mg/L (Table 4.2). These concentrations were determined from previous experiments and scaled down from disaster scenarios. These concentrations allowed for a chronic 12-week study.

Crayfish were sampled weekly. Two pyramid crayfish traps were placed in each tank to capture crayfish (Figure 4.2). Three crayfish samples were taken from each tank and placed in a food storage bags then covered with ice. Each individually bagged crayfish were then placed in a freezer to euthanize and prevent spoilage until the preparation for analysis.
Table 4.2 Chemical loadings of mesocosm tanks for 12 week study. LSU Aquaculture Research Station tank description is also indicated.

<table>
<thead>
<tr>
<th>Chemical Loadings</th>
<th>Tank</th>
<th>Exposure (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (D3)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AgNP After (F9)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>AgNP Before (F11)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>ZnO (E4)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>TiO₂ (C4)</td>
<td>100</td>
</tr>
</tbody>
</table>

The organs that were harvested for this study were the gill and abdominal muscle. The tissue dissection protocols were as follows:

Gills:

An incision was made into the middle line of the thorax near the head. Using scissors, a cut was made through the cephalothorax from the head to the beginning of the abdomen (tail tissue). Next, a cut was made along the cephalic groove down toward the walking legs. Using forceps, the thorax panel that covers the gill structures was firmly grasped. The panel was bent away from the crayfish and pulled to break the remaining connection.

Abdominal muscle:

An incision was made into the abdominal carapace. A cut was made down the abdominal using scissors; care was taken so as to not lacerate the abdominal muscle. The opened carapace was pried to the sides to expose the muscle. The abdominal muscle was loosened from the shell and extracted.

Each crayfish’s organ tissue underwent a digestion protocol to analyze metal content. The sample preparation protocol was modified from Moss et al. (2010). Approximately 100 mg (all masses were individually recorded) of tissue was removed from the entire gill structure and abdominal muscle for digestion. Gill tissue was digested using 1 mL of trace Metal Grade nitric
acid (Fisher Scientific A509-P212) in a 1.5 mL microcentrifuge tube. To ensure complete
digestion, each sample was allowed to sit for 24 h. Any tube exhibiting macro-level gill
structure or abdominal muscle was sonicated to complete digestion.

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to
calculate the amount of metal found in gill and abdominal tissue. After each tissue type from
each crayfish was digested, it was added into a standard 20 mL test tube. A measured amount of
DI water was added to the tube to dilute each digestion sample. These samples were then
processed by Dr. Robert Gambrell and Mr. Thomas Blanchard using ICP-OES which measured
metal concentration as a function of the wavelengths emitted as the solutions were heated to
plasma. The resulting values—given in ppm—were analyzed with the recorded tissue mass to
yield ppm/g values. These values were then converted μg/g to be more consistent with current
research.

Soil samples were taken biweekly. The soil was collected using a grab sampler. Each
sample was placed in a food storage bag then brought back to the lab freezer until analysis. Soil
samples were oven dried at 70°C for 24 h (Fisher Scientific, Isotemp Oven Model 750F) (Zhao et
al. 2012). One gram of each soil sample was digested with 5 mL of trace metals grade nitric acid
in an 80 mL glass test tube. The samples were then placed under a vent hood and refluxed for 24
hours. Then 50 mL of DI water was added to each sample and allowed to sit for at least 24 hours
for solids to settle. The samples were then pipetted into 20 mL tubes for analysis. The resulting
values—given in ppm—were analyzed with the recorded mass to yield ppm/g values. These
values were then converted μg/g (wet mass) to be more consistent with current research.
Instrumentation

All metal analyses of crayfish tissues were conducted with a Varian Vista-MPX CCD simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES) with axially viewed plasma. The instrument was equipped with a mass flow controller on the nebulizer gas, cyclonic spray chamber, and 3-channel peristaltic pump. Varian's Galaxie™ ICP-Expert™ II software controls the ICP and auto sampler. The wavelengths used for Ag, Zn, and Ti were 238.068 nm, 213.857 nm, and 336.122 nm, respectively.

The ICP-OES had a detection limit of 2 ppb. EPA recommends (as an initial step in the exposure assessment process) use of a residue value of ½ level of detection (LOD), as appropriate, for samples with no detectable residues if it is known or believed that these samples have been treated with an agent. The use of ½ LOD (1 ppb) for nondetectable residues in samples is widely used in the risk assessment community and is advocated by EPA (EPA 1998a) when the appropriate conditions are met.

Data Analysis

SAS version 9.3 (SAS Institute Inc., SAS 9.3, Software, Cary, North Carolina, USA) was used to analyze the data. All tables and graphs were constructed using Microsoft Excel 2010 (Microsoft, Microsoft Excel 2010, Software, Redmond, Washington, USA).

Gill and abdominal tissue metal concentration averages were also analyzed using one-way ANOVA comparing variances of each treatment metal concentration averages to the control metal concentration averages. F-statistic gives the P-value for the test of equal variances. If the probability (Pr > F) is less than 0.05 (α =0.05), there is evidence that the variances for the two groups are different. Therefore, the Satterthwaite (Unpooled) variance estimator is used to
suggest areas of significance. If the probability (Pr > F) is greater than 0.05 (α = 0.05), the pooled (Equal) variance estimator is used to suggest areas of significance.

Results

Soil analysis results show that soil metal concentrations fluctuated over the 12-week study (Table 4.3). Traces of silver were only found in the silver test tanks, whereas traces of zinc and titanium were found in all treatment tanks. The soil samples showed a fluctuation of metal concentrations rather than bioaccumulation.

The results of the ICP-OES analysis of gill tissue concentrations can be found in figures 4.3a, 4.4a, and 4.5a. Both AgNP After and AgNP Before gill silver concentration averages were statistically significant from the control for 4 and 7 weeks, respectively. AgNP Before treatment had a higher statistical significance (p < 0.01) for silver concentration averages than AgNP After treatment (p < 0.05) silver concentration averages. ZnO treatment only had statistical significance (p < 0.05) from the control 3 weeks out of the first four weeks of the study. The TiO₂ treatment did not have any statistical significance from the control for the entire study.

The results of the ICP-OES analysis of abdominal tissue concentrations can be found in figures 4.3b, 4.4b, and 4.5b. Both AgNP After and AgNP Before abdominal silver concentration averages were statistically significant from the control for 5 and 6 weeks, respectively. AgNP Before treatment had a higher statistical significance (p < 0.001) for silver concentration averages than AgNP After treatment (p < 0.01) silver concentration averages.
Table 4.3 Results of biweekly soil analysis from crayfish mesocosm studies.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Prior to Dosing</th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 5</th>
<th>Week 7</th>
<th>Week 11</th>
<th>Week 11 Study Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ag</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>29.59</td>
<td>18.77</td>
<td>23.19</td>
<td>29.45</td>
<td>18.55</td>
<td>20.27</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>80.96</td>
<td>68.21</td>
<td>93.52</td>
<td>91.41</td>
<td>75.78</td>
<td>82.36</td>
</tr>
<tr>
<td>AgNP After</td>
<td>Ag</td>
<td>*</td>
<td>*</td>
<td>0.19</td>
<td>5.38</td>
<td>0.44</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>25.28</td>
<td>22.75</td>
<td>24.35</td>
<td>25.16</td>
<td>26.79</td>
<td>22.48</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>80.29</td>
<td>89.34</td>
<td>80.85</td>
<td>78.99</td>
<td>79.71</td>
<td>69.57</td>
</tr>
<tr>
<td>AgNP Before</td>
<td>Ag</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>2.96</td>
<td>*</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>20.02</td>
<td>27.62</td>
<td>17.53</td>
<td>25.13</td>
<td>19.18</td>
<td>32.22</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>62.53</td>
<td>84.44</td>
<td>72.16</td>
<td>88.66</td>
<td>75.33</td>
<td>102.16</td>
</tr>
<tr>
<td>ZnO</td>
<td>Ag</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>18.60</td>
<td>28.83</td>
<td>39.49</td>
<td>33.87</td>
<td>27.83</td>
<td>28.46</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>42.18</td>
<td>71.92</td>
<td>80.83</td>
<td>60.83</td>
<td>77.12</td>
<td>77.44</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Ag</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>28.51</td>
<td>22.77</td>
<td>28.45</td>
<td>20.85</td>
<td>28.68</td>
<td>39.77</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>79.87</td>
<td>83.50</td>
<td>76.16</td>
<td>73.85</td>
<td>71.98</td>
<td>71.25</td>
</tr>
</tbody>
</table>

* Below detection limit.

The ZnO treatment did not have any statistical significance from the control for the entire study. The TiO₂ treatment only had statistical significance (p < 0.05) from the control 1 week out of the study and had similar averages to that of the control.
Figure 4.3 Silver content (μg/g) in crayfish a) gill and b) abdominal tissue in 12-week mesocosm study. Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test).
Figure 4.4 Zinc content (μg/g) in crayfish a) gill and b) abdominal tissue in 12-week mesocosm study. Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test).
Figure 4.5 Titanium content (μg/g) in crayfish a) gill and b) abdominal tissue in 12-week mesocosm study. Statistical difference compared with the control treatments (* p < 0.05, **p < 0.01, ***p < 0.001, t test).
Discussion

NPs enter into the environment in various pathways. NPs may be accidentally released or emitted during manufacture; however, the manner in which they typically enter the environment is determined by their end use. Some sunscreens contain NPs, which provides an illustration of a pathway NPs use to enter the environment. During washing or swimming, sunscreen may come off, causing the NPS to enter the sewage system or bodies of water. Then, the unused sunscreen will either be incinerated or ultimately end up in a landfill. Little is known currently about the end of life fate of NPs. Direct disposal of nanomaterial may occur as landfill and incineration or during wastewater treatment (Fairbrother and Fairbrother 2009). Upwards of 95% of NPs used in cosmetics, paints, and coatings may enter wastewater as runoff during application or during the product’s deterioration through time (Mueller and Nowack 2008). When the NPs enter the environment, they act very differently compared to their intended use. For example, they may aggregate, attach themselves to other soil contaminants, or be absorbed into solid particles, bioaccumulated or biomagnified. Even though there have been a number of reports of the ecotoxicological effects on aquatic and soil organisms, there still remains much to be learned about the NPs true effect. It is also apparent from the published literature that size is not the only factor that governs NP toxicity; particle composition, surface chemistry and morphology may also be co-factors. Due to the lack of information about degradation of NPs, the understanding of the possible NP bioaccumulation throughout the various trophic levels and bioaccumulation is an important aspect of NPs to examine in the future.

Therefore, the replication of chemical spill concentrations and their distribution between actual field conditions in Louisiana is critical in the evaluation of the crayfish toxicity to the simulated mesocosm studies. For a worst case scenario, a single semi-truck and a single train
tank car have a maximum allowable legal gross weight in the United States of 80,000 kg and 129,295 kg, respectively (23 U.S.C. § 127, 49 CFR § 179.13). Such a scenario is given that crayfish production ponds (4–8 ha) will contain 12,000 to 60,000 m³ (12E6 to 60E6 L) of water (Huner and Barr 1980). If either semi-truck or train car were to overturn near these crayfish ponds it would cause catastrophic damage to the ecosystem with concentrations ranging from 1,000 mg/L to 6,000 mg/L for a semi-truck overturn and 2,000 mg/L to 10,000 mg/L for a single train car overturn. From previous research, these concentrations would ultimately be too toxic for any crayfish to survive. Due to time and funding, the NP concentrations used in the mesocosm studies were scaled down significantly to observe bioaccumulation rather than mortality.

Our mesocosm studies results show that the nanoparticle forms of silver and zinc do bioaccumulate in crayfish. Titanium NPs showed little to no bioaccumulation over the course of the study. The chemical rankings of bioaccumulation of metal in the crayfish gill tissue are as follows: ZnO > AgNP Before > AgNP After > TiO₂; and the rankings of bioaccumulation of metal in the crayfish abdominal tissue are as follows: AgNP After > AgNP Before > ZnO > TiO₂. The impact of AgNP and ZnO NPs treatments on tissue metal concentrations were similar in gill and abdominal tissues. TiO₂ NPs treatments had higher tissue metal content in the gill tissue followed by the abdominal tissue.

Inorganic and organic ligands will react with Ag due to the lack of thermodynamic stability in most environmental conditions. (Xiu et al. 2011; Liu et al. 2010). Silver is prone to be very reactive with sulfide, chloride, and organic matter (Levard et al. 2011a; Levard et al. 2011b; Liu and Hurt 2010). AgNPs are quite small in size, which, in turn, causes the kinetics of corrosion to be much faster in comparison to bulk silver. This characteristic reduces the lifetime of the metallic state of Ag in nature. There are many known corrosion agents present in nature;
therefore environmental transformations of AgNPs will definitely affect surface properties of these NPs, while also affecting their transport, reactivity, and toxicity in soils and aqueous systems (Liu et al. 2010).

Information is very limited on the ecotoxicological effects of ZnO across all taxa, and when compared to TiO2, a NP that shares many of the same properties, the research on ZnO is quite scarce. (Kahru and Dubourguier 2010). There have been far less toxicity studies on aquatic invertebrates compared to bacteria, and the studies that do exist center around freshwater crustaceans such as Daphnia magna (Blinova et al. 2010; Heinlaan et al. 2008; Wiench et al. 2009; Zhu et al. 2009) and Thamnocephalus platyurus (Blinova et al. 2010; Heinlaan et al. 2008). These two species showed that toxicity of ZnO NPs was due to solubilized Zn ions as suggested by recombinant Zn-sensor bacteria. Blinova et al. (2010) also compared ZnO NPs toxicity in natural waters to artificial freshwater, and this study demonstrated that organic matter present in the natural river water did not significantly affect ZnO NPs toxicity. These results were in line with the data obtained when the Zn sensor bacteria only showed small disparities in bioavailable Zn ion concentrations in both artificial and natural waters. No studies of chronic effects, such as growth and reproduction, on D. magna have been reported. On the other hand, a chronic study on the marine amphipod Corophium volutator showed that waterborne ZnO NPs (35±10 nm) at 1 mg/L had significant effects on survival, growth, and reproduction, and the toxicity in this study was not solely due to solubilized Zn$^{2+}$ (Fabrega et al. 2011).

Only a few studies have been published to text the toxicity of TiO2 NPs using freshwater invertebrates as test organisms. While some studies reported acute effects of TiO2 NPs on D. magna (Adams et al. 2006; Hund-Rinke and Simon 2006; Lovern and Klaper 2006; Lovern et al. 2007; Wiench et al. 2009), Griffitt et al. (2008) found no measurable toxic effects on Daphnia
pulex. Similar results for TiO$_2$ and ZnO NPs showing strong aggregation were obtained by Adams et al. (2006). While the problem of aggregation and sedimentation of nano-scale particles causing a decrease in substance concentrations has been evaluated (Crane et al. 2008; Handy et al. 2008; and Klaine et al. 2008), no feasible method has been developed to insure constant concentrations over the complete test period. The test dispersions in the mesocosm study were only dispersed one time, and this fact has to be kept in consideration when compared to the results of other studies. For example Velzeboer et al. (2008) stated that their lack of toxic effects by nano-scale particles (including TiO2) in part as the result of settling of particle aggregations. In a recent study, Filella et al. (2008) demonstrate that the presence of *Daphnia* can alter the aggregate size of natural mineral colloids.

Their size, compactness, deposition, and transport in the water column and porous media is controlled by the NPs coagulation (Zhou et al. 2012). Over the last several years, a great amount of work has been devoted to investigating the role of solution chemistry, for example, the pH, ionic strength, monovalent/divalent/trivalent ion ratio, NP concentration, presence of organic matter in affecting NP coagulation (Chen et al. 2006; Salah et al. 2008; Domingos et al. 2009; French et al. 2009; Zhou and Keller 2010; Buettner et al. 2010; Li et al. 2010; Chowdhury et al. 2010; Keller et al. 2010; Liu et al. 2011; Bian et al. 2011; Huynh and Chen 2011). However, one crucial component in controlling NP coagulation that has been overlooked is the interaction between naturally occurring clay particles and the engineered NPs (Hotze et al. 2010). Clay is one of the most common byproducts of chemical weathering and the main constituent of soils, and due to clay’s abundance and ubiquity, the interaction it has is likely to affect, if not regulate, the mobility of these NPs once they are released in the environment.
Our findings suggest that the use of clay as sediment substrate potentially has more of an effect on the NPs than the Mississippi Alluvial soil. Further conversely, the surficial sediment zone is a dynamic, chemical, and biological system that is sensitive to redox potential (Chapman et al. 1998). Increasing redox potential just slightly, may result in the oxidation of metal complexes, augmenting the release of metals from sediments and clay to pore waters, which increases the bioavailability of metals (Zhuang et al. 1994; Peterson et al. 1996). This characteristic due to redox potential is particularly important because surficial sediments contribute drastically to the exposure of benthic organisms to contaminants. The redox potential in sediments may be increased by several processes: 1) temporal and spatial changes in rates of particle deposition and microbial reduction of sulfate, which result in the seasonal and spatial variation of acid-volatile sulfide (AVS) (Besser et al. 1996; Hansen et al. 1996; Liber et al. 1996), 2) bioturbation and bioirrigation by the burrowing, feeding, tubing, excretion, respiration, and locomotion activities of benthic animals (crayfish in this case) (Tessier et al. 1996; Matisoff 1995; Gagnon et al. 1996; Peterson et al. 1996), and 3) sediment resuspension caused by storms, flooding, tidal exchanges or currents, dredging, and trawling (Chapman et al. 1998). Even though knowledge regarding the stability of clay minerals/oxide mixtures exists in the soil science literature (Ferreiro et al. 2011; Goldberg and Glaubig 1987; Tombacz et al. 2001), the applicability of such knowledge in clay minerals/engineered NP systems needs to be further tested.

Contaminated soils and waters pose a major environmental and human health problem, which may be partially solved by the emerging phytoremediation technology. This cost-effective plant-based approach to remediation takes advantage of the remarkable ability of plants to concentrate elements and compounds from the environment and to metabolize various molecules
in their tissues (Salt et al. 1998). This technology makes use of the naturally occurring processes by which plants and their microbial rhizosphere flora degrade and sequester organic and inorganic pollutants (Pilon-Smits 2005). Toxic heavy metals and organic pollutants are the major targets for phytoremediation. Inorganics cannot be degraded, but they can be phytoremediated via stabilization or sequestration in harvestable plant tissues. Cattail (Typha latifolia) root tissue samples would provide a great start to future research in a mesocosm environment.

In comparing laboratory and mesocosms studies, results indicate that the silver and zinc NPs are toxic, but are less toxic than their corresponding bulk form. Bioaccumulation does occur in both studies, but in mesocosms studies NPs can bind to plants or sediment which is why we do not see an increasing bioaccumulation trend. Overall, both studies give us insight about nanoparticle toxicity.

Conclusion

In this research we characterized the bioaccumulation of NPs (AgNP, ZnO, and TiO$_2$) to Procambrus clarkii in a semi-natural mesocosm. From the results of this study, it can be concluded that: (1) The chemical rankings of bioaccumulation of metal in the crayfish gill tissue are as follows: ZnO > AgNP Before > AgNP After > TiO$_2$ and the rankings of bioaccumulation of metal in the crayfish abdominal tissue are as follows: ZnO > AgNP After > AgNP Before > TiO$_2$; (2) bioaccumulation in crayfish tissue occurs in a fluctuating trend rather than in an increasing trend; (3) soil samples were not conclusive with a bioaccumulation trend. The present study has taken a first step in the direction of investigating the ecotoxicity and bioaccumulation of NPs to Procambrus clarkii and highlights the need for integrated toxicological assessment of NPs in aquatic systems. Thus, further research into the properties of NPs with different chemical compositions and the NP behaviors of environmental water chemistry and sedimentation as
related to the toxicity mechanisms of NPs, are required to evaluate the aquatic ecotoxicity of NPs and to determine definitively whether their toxicity is caused by nano-effects.

Future work, in addition to the lethal toxicity test, may be scaling down the study so that clay/sediment mixtures can be observed and sampled. Crayfish are known burrowers which can cause the sediment to release bound metals making them more bioavailable in the environment. By monitoring this activity with different sediment samples, we may determine which sediment sample is best for remediation if any. Future research could be crucial information for remediation of a potential chemical spill.

References


Chapter 5
Conclusions

Overall, both studies showed that NPs are toxic and bioaccumulate in *P. clarkii*. This thesis investigated various toxic effects on *P. clarkii* with several commonly manufactured NPs (AgNPs, ZnO NPs, TiO$_2$ NPs). Due to the lack of information about degradation of NPs, the understanding of the possible NP bioaccumulation throughout the various trophic levels and bioaccumulation is an important aspect of NPs to examine.

Chapter 3 examined the chronic toxicity of different nano metal and bulk metal concentrations to crayfish in a controlled laboratory setting through 28 days. The results suggest that nano metals are less toxic than bulk metals and the toxicity of NPs to aquatic species is concentration-dependent. The impact of NPs and bulk treatments on metal tissue concentration were highest in gill tissue followed by abdominal tissue.

Chapter 4 examined simulating a “chemical spill” which the crayfish might be exposed to in the wild. The replication of chemical spill concentrations and their distribution between actual field conditions in Louisiana is critical in the evaluation of the crayfish toxicity to the simulated mesocosm studies. The results suggest that bioaccumulation in crayfish tissue occurs in a fluctuating trend rather than in an increasing trend. Soil samples were not conclusive with a bioaccumulation trend and should be investigated further.

Both studies have taken a first step in the direction of investigating the ecotoxicity of metal oxide NPs to *P. clarkii* and highlights the need for integrated toxicological assessment of metal oxide NPs in aquatic systems. Thus, further research into the properties of NPs with different chemical compositions and the NP behaviors of environmental water chemistry as related to the toxicity mechanisms of NPs, are required to evaluate the aquatic eco-toxicity of metal-oxide NPs and to determine definitively whether their toxicity is caused by nano-effects.
This novel research showed that NP bioaccumulation does occur in crayfish tissues and tissue silver and zinc content in nanoparticle treatments were significantly higher than the control.
### Appendix

Appendix Table 1 Silver and titanium loading calculations.

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<td>µg/mL</td>
<td>µg/mL</td>
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Appendix Table 2 Zinc and potassium loading calculations.

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Appendix Table 3 Crayfish change in mass calculations.

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Appendix Table 3 (continued) Crayfish change in mass calculations.

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Appendix Figure 1 Brightfield microscopy digital images of crayfish gill tissue using same contrast. The following images are shown above: a) control day 7 b) control day 14 c) control day 21 d) control day 21 e) AgNP 100 μg/mL day 7 f) AgNP 100 μg/mL day 14 g) AgNP 100 μg/mL day 28 h) AgNP 500 μg/mL day 4 i) AgNP 500 μg/mL day 7 j) AgNP 500 μg/mL day 13 k) AgNP 1000 μg/mL day 4 l) AgNP 1000 μg/mL day 7 m) AgNP 1000 μg/mL day 13 n) AgNO₃ 78.74 μg/mL 3 hours o) AgNO₃ 78.74 μg/mL 9 hours p) AgNO₃ 393.7 μg/mL 3 hours q) AgNO₃ 787.4 μg/mL 3 hours r) AgNO₃ 787.4 μg/mL 6 hours s) ZnO 100 μg/mL day 7 t) ZnO 100 μg/mL day 14.
Appendix Figure 1 (continued) Brightfield microscopy digital images of crayfish gill tissue using same contrast. The following images are shown above: a) ZnO 100 μg/mL day 21 b) ZnO 100 μg/mL day 28 c) ZnO 500 μg/mL day 7 d) ZnO 500 μg/mL day 14 e) ZnO 500 μg/mL day 21 f) ZnO 500 μg/mL day 28 g) ZnO 1000 μg/mL day 7 h) ZnO 1000 μg/mL day 14 i) ZnO 1000 μg/mL day 28 j) Zn(NO₃)₂ 365.17 μg/mL day 4 k) Zn(NO₃)₂ 365.17 μg/mL day 8 l) Zn(NO₃)₂ 365.17 μg/mL day 13 m) Zn(NO₃)₂ 1825.83 μg/mL day 5 n) Zn(NO₃)₂ 1825.83 μg/mL day 13 o) Zn(NO₃)₂ 1825.83 μg/mL day 25 p) Zn(NO₃)₂ 3651.06 μg/mL day 4 q) Zn(NO₃)₂ 3651.06 μg/mL day 14 r) TiO₂ 100 μg/mL day 7 s) TiO₂ 100 μg/mL day 14 t) TiO₂ 100 μg/mL day 21.
Appendix Figure 1 (continued) Brightfield microscopy digital images of crayfish gill tissue using same contrast. The following images are shown above: a) TiO$_2$ 100 $\mu$g/mL day 28 b) TiO$_2$ 500 $\mu$g/mL day 7 c) TiO$_2$ 500 $\mu$g/mL day 14 d) TiO$_2$ 500 $\mu$g/mL day 21 e) TiO$_2$ 500 $\mu$g/mL day 28 f) TiO$_2$ 1000 $\mu$g/mL day 7 g) TiO$_2$ 1000 $\mu$g/mL day 14 h) TiO$_2$ 1000 $\mu$g/mL day 21 i) TiO$_2$ 1000 $\mu$g/mL day 28.
Appendix Figure 2 TEM images of diameter measurement of nanoparticles. The following images are shown above: a,b,c,d) AgNPs, e,f,g) TiO2 NPs, h,i,j,k) ZnO NPs.
Vita

Jake Andrew Farlow is a homegrown tiger born in Baton Rouge, Louisiana. He graduated *magna cum laude* from Lafayette High School, Lafayette, Louisiana, in May 2008. Then he attended Louisiana State University with a TOPS scholarship and completed his Bachelor of Science in Biological Engineering in December 2012. He then entered into an accelerated Master’s program at Louisiana State University and completed his Master of Science in Biological and Agricultural Engineering in May 2014. He currently seeks the happiness in life.