Impact of Copper Nanoparticles on Inactivation and Toxicity Pathway on Model Bacteria

by

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ABSTRACT

Nanotechnology is a scientific field that has recently expanded due to its applications in pharmaceutical and personal care products, industry and agriculture. As result of this unprecedented growth, nanoparticles (NPs) have become a significant environmental contaminant, with potential to impact various forms of life in environment. Metal nanoparticles (mNPs) exhibit unique properties such as increased chemical reactivity due to high specific surface area to volume ratios.

Bacteria play a major role in many natural and engineered biogeochemical reactions in wastewater treatment plants and other environmental compartments. I have evaluated the laboratory isolates of *E. coli, Bacillus, Alcaligenes, Pseudomonas;* wastewater isolates of *E. coli* and *Bacillus;* and pathogenic isolate of *E. coli* for their response to 50 & 100 nm sized Cu nanoparticles (CuNPs). Bactericidal tests, scanning electron microscopy (SEM) analyses, and probable toxicity pathways assays were performed.

The results indicate that under continuous mixing conditions, CuNPs are effective in inactivation of the selected bacterial isolates. In general, exposure to CuNPs resulted in 4 to >6 log reduction in bacterial population within 2 hours. Based on the GR, LDH and MTT assays, bacterial cells showed different toxicity elicitation pathways after exposure to CuNPs.

Therefore, it can be concluded that the laboratory isolates are good candidates for predicting the behavior of environmental isolates exposed to CuNPs. Also, high inactivation values recorded in this study suggest that the
presence of CuNPs in different environmental compartments may have an impact on pollutants attenuation and wastewater biological treatment processes. These results point towards the need for an in depth investigation of the impact of NPs on the biological processes; and long-term effect of high load of NPs on the stability of aquatic and terrestrial ecologies.
DEDICATION

The Imam, the just Guide, Hazrat Mahdi (A.S.)

أهدي هذا العمل الى صاحب العصر و الزمان و بقية الله في أرضه الامام الحجة المنتظر المهدي (عليه السلام)
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CHAPTER 1
INTRODUCTION AND OBJECTIVES

1.1. Introduction

Nanoparticles (NPs) have recently gained the attention of the scientific community because they exhibit intrinsically unique properties that differ from bulk materials. Physical properties of bulk-sized materials, such as optical, magnetic, catalytic, thermodynamic, and electrochemical properties, remain constant; however these properties dramatically change as material size decreases and approaches the nano-scale (Baccile, 2010). A hallmark of NPs is their high specific surface area-to-volume ratio. Higher surface area results in an increase in the material’s reactivity because the number of reaction sites per unit area is increased (Theivasanthi & Alagar, 2011).

Scientists have been able to evaluate and exploit the unique properties of NPs to invent new uses and applications for such materials in various disciplines and industries, such as the pharmaceutical, cosmetics, electronics, environmental and agricultural industries (Kreuter, 1992). Similarly, NPs are widely used in the oil and gas industries (Makimura et al., 2010; Ingram et al., 2010). As a result of this widespread application, the nanotechnology consumer products inventory has grown from 212 products in 2006 to 1,317 products in 2011, nearly a 521% increase in 5 years. The U.S. National Science Foundation estimates that nanotechnology will have a $1 trillion impact on the global market and will employ over 7 million workers by 2015 (Roco, 2007).
Along with such a huge increase in the manufacturing, application, and disposal of NPs, the concentrations of NP species are expected to accumulate in and contaminate many environmental compartments (Musee, 2011). Specifically, NPs are released into surface waters, wastewaters, soils, etc., and many NP species have already been recognized as significant contaminants (Westerhoff et al., 2009). They are known to negatively impact biological activities at molecular, cellular, organismic, and ecological levels (Ren et al., 2009).

Unabated release of heavy metal-containing nanoparticles (mNPs) into the environment can potentially have harmful effects on normal ecological processes. Such NPs can alter the efficacy of beneficial microbes that are important in the normal functions of wastewater treatment processes, the natural attenuation of pollutants, and mineral cycling. Traditionally, laboratory isolates of environmentally relevant bacterial species (such as *E. coli*, *Pseudomonas* and *Bacillus*, etc.) have been used to study the impact of various types of NPs on biological and ecological systems; however, based on our knowledge no information is available on the relevance between the data for laboratory strains and environmental strains. In this study I will investigate the effects of NPs on aquatic systems by comparing data gathered on laboratory strains alongside that of relevant environmental strains of bacterial species.

Copper nanoparticles (CuNPs) are widely used in industrial applications such as semiconductors, heat transfer fluids in machine tools, metal catalysts, and even in the area of public health in biocidal preparations (Aruoja et al., 2009; Kim et al., 2011). Since CuNPs are so commonly used and released into the
environment, they are the ideal candidates for studying the potential adverse effects of NPs on aquatic systems. Understanding the impacts of NPs on organisms and surrounding ecosystems is still incomplete. Typically, ecologists study pollutants only after they have already caused significant environmental degradation and since no large NP spills have been reported, no documented ecological impact studies on NPs are currently available. Thus, the picture of the potential environmental impacts of NPs is not clear yet. One of the major challenges in generating such predictions is the complexity of NPs and their fundamental properties and behaviors.

1.2. Objectives

The main objective of this study is to investigate potential adverse effects of 50 & 100 nm CuNPs on a variety of bacterial strains in aquatic systems. Specific objectives covered by each chapter are as follows:

1.2.1. Specific Objectives

Specific objectives are as follows:

- To investigate the biocidal effects of two sizes of 50 & 100 nm CuNPs against laboratory and wastewater isolates of selected bacterial species in continuous mixing conditions
  
  i. Laboratory isolates of bacterial species: *E. coli*, *Alcaligenes*, *Pseudomonas*, *Bacillus*, *E. coli* 0157
  
  ii. Environmental isolates of bacterial species: *E. coli*, *Bacillus*

- To assess the pathways of toxicity elicitation of CuNPs in a variety of selected bacterial species utilizing the following reaction assays:
i. Lactate Dehydrogenase (LDH)

ii. Glutathione Reductase (GR)

iii. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

- To determine the concentrations of copper ions released from CuNPs in aquatic systems
  
  i. Under different environmental conditions
     1. Low versus high pH values
     2. Ambient versus elevated temperatures
     3. Light exposure versus dark
     4. Short (2 hours) versus long (200 hours) exposure times

- To determine the effects of using PBS buffer versus nano-pure water on copper ions release

- To physically determine the sites of interaction between CuNPs and bacterial cells using scanning electron microscopy (SEM)

1.2.2. Hypotheses considered in this study

- CuNPs exhibit bactericidal effects, which are size and species dependent

- Laboratory and environmental bacterial isolates similarly respond to CuNPs with inter-species variations

- Toxicity elicitation mechanism of CuNPs in bacteria is species-dependent

- High concentrations of CuNPs negatively impact normal biological activities at molecular, cellular, organismic, and ecological levels
• Toxicity of CuNPs is due to their size rather than the release of copper ions

• CuNPs are transported into bacterial plasma
CHAPTER 2
LITERATURE REVIEW

2.1. Background on Nanoparticles

2.1.1. Definition of Nanoparticles

Nanoparticles (NPs) are commonly defined as solid colloidal particles with sizes typically in the range of 10 nm to 1,000 nm in one or more dimensions. Historically, NPs used to be referred to as submicron particles; however, since the mid-1990s the term “NP” started to be used in the literature and now is fully accepted though some published articles still use the term submicron (Taylor, 2011; Kreuter, 1994).

2.1.2. History of NPs

Generally, NPs are considered a recent technological invention; however, historical evidence shows that humankind identified and was exposed to NPs a long time ago. For example, Roman glassmakers in the fourth century fabricated soda lime glass containing silver and gold NPs, however, history didn’t mention whether these NPs were naturally occurring or manmade. The famous Lycurgus Cup, which resides in the British Museum in London, is an example of how NPs were used during that period. (Moudgil, 2010). Similarly, artisans in Mesopotamia in the ninth century utilized NPs to generate a glittering effect on the surface of pots (Reiss & Hutten, 2011).

2.1.3. Classification of NPs

NPs can be classified as natural or engineered (Nowack & Bucheli, 2007). Two subcategories of natural NPs are 1) nanominerals, which only exist in the
range of nano sizes, such as some clays, and Fe and Mn oxyhydroxides, and 2) mineral NPs, which can be found in both nano and micron sizes, e.g., most known minerals (Qafoku, 2010). Natural NPs may exist naturally or may form in bodies of water, or be discharged into the environment from a variety of industries specially wastewater treatment plants (Boxall, 2010). On the other hand, engineered NPs are defined as a group of materials that have certain features in common and intentionally have a physical structure that is on the order of nanometers in scale; such nanomaterials are specifically designed to allow product developers to take advantage of such a structure (Maynard, 2011). Examples of engineered NPs include but are not limited to metals and metal oxides (e.g., silver, iron, gold, and titanium oxide); metal-core quantum dots (QDs) (e.g., cadmium-telluride and gold-silica with carboxyl functional groups); and carbonaceous fullerenes (e.g., fullerenes and fullerols) (Westerhoff et al., 2008). Engineered NPs are discharged into the environment from a variety of industries and accumulate in soil and surface waters, eventually making their way to wastewater treatment plants) (Boxall, 2010)

2.1.4. Characteristics and Properties of NPs

NPs have attracted a great deal of scientific attention because of their size, which falls between the size of bulk materials and structures at the atomic or molecular scale. Thus, NPs form a link between these two size-states of materials, those at the nano-scale and their macro counterparts. Most of the chemical and physical properties of materials are size-dependent. In the case of bulk materials usually larger than 1,000 nm in size, such properties are constant, whereas they
may dramatically change as the size decreases and approaches the nano-scale (Baccile, 2010). The different and unique properties that depend on the size of NPs include optical, magnetic, catalytic, thermodynamic, and electrochemical properties.

In addition to size, the chemical composition and the shape of a NP also influence its specific properties (Sanvicens & Marco, 2008). One of the most important characteristics of NPs is the enormous increase in their surface area-to-volume ratio relative to their bulk materials and this can lead to unexpected properties. Such an increase causes NPs’ physical and chemical properties to be controlled by the effect of the surface atoms and capping agents on the NPs’ surfaces. A particle with a high surface area has a greater number of reaction sites than a particle with a low surface area, and thus, has a higher chemical reactivity (Theivasanthi & Alagar, 2011). For example, a 2 nm particle would have approximately 50% of its partially unsaturated atoms located on the surface with a specific surface area approaching 1,000 m²/g. These characteristics increase the potential of this particle to interact with chemical species it comes into contact with, either via physical adsorption (if caused by van der Waals attraction), or chemical reaction (if chemical bonds are formed) (Koper et al., 2007).

2.1.4.1. Bactericidal Properties of Copper & CuNPs

An overview of the bactericidal properties of NPs, specifically copper NPs, will be addressed since the focus of this study is the impact of copper NPs on bacterial cells. Although not much research has been conducted regarding this matter, it has been reported in the literature that NPs such as copper, silver, and
zinc have antibacterial capabilities. Such particles need to be further investigated as antibacterial agents due to their low toxicity and chemical stability in relation to other organic antibacterial agents (Esteban-Cubill et al., 2006). One of the advantages of using CuNPs to kill bacteria is that bacteria are not able to develop resistance against CuNPs as they often do against antibiotics. The small size of CuNPs allows them to directly penetrate the bacterial outer membrane; it is thought that this causes membrane rupture, consequently killing bacteria (Theivasanthi & Alagar, 2011).

The differences in cell membrane structure between gram-negative and gram-positive bacteria may determine how CuNPs interact with bacterial membranes and exert antibacterial activity. There are conflicting data on this area of research that will be discussed in Section 2.5.3.3.

Also, Theivasanthi & Alagar, 2011 reported that the method in which CuNPs are synthesized has an impact on the degree of their antibacterial activities. They showed that CuNPs synthesized by the electrolysis method are more effective in inactivating E. coli compared to those synthesized using the chemical reduction method. Therefore, CuNPs can be engineered to achieve a higher degree of bacterial inactivation.

On the other hand, copper itself is reported as one of the most toxic metals to bacteria and have been used to control bacterial diseases for years. Thurman & colleagues (1989), in their attempt to review the effects of copper and silver on bacteria and viruses, listed previous works conducted to determine bacterial disinfection due to copper ions. It was observed that copper bactericidal activity is
linked to the electrolytically formation of hydrogen peroxide. Moreover, the
degree of copper toxicity is affected by functional groups bonded to copper
molecules such as phosphates, which decrease copper toxicity, and copper
carbonates, which increase copper toxicity.

2.2. Uses and Applications of NPs

Since NPs exhibit unique properties that are different from their bulk
materials, scientists have been able to utilize them as tools in a wide range of
applications across different fields, such as the pharmaceutical, cosmetics,
manufacturing, and agricultural industries (Kreuter, 1992).

2.2.1. Pharmaceutical and Cosmetics Applications

The applications of NPs in the pharmaceutical field include improving
drugs, proteins, genes and vaccine delivery systems. The delivery is done by
dissolving, entrapping, or encapsulating the active agents within the NPs or by
adsorbing the agents onto the particles’ surfaces. Such developments in drug
delivery systems have attracted a great deal of attention in recent years and
nanotechnology is expected to solve many health-related issues (Shen et al.,
2010).

Next, several cosmetics manufacturers incorporate NPs into their products,
especially in moisturizers, hair care products, make up, sunscreen, etc. The main
NPs utilized in the cosmetics industry are liposomes, nanoemulsions, solid lipid
NPs, and metal oxide NPs. Furthermore, some reports show that NPs of silver,
copper, silicone, and silica are used as ingredients in cosmetics (Mu & Sprand,
2010).
Liposomes are small particles encapsulated within membrane bilayers of lipids and they contain, within their hydrophilic core, water-soluble agents. Liposomes are able to penetrate through skin barriers by increasing the skin’s permeability in order to deliver encapsulated agents into deeper skin layers. Liposomes are mostly used in skin care products but they are also used in some hair care products (Grebler et al., 2010).

Nanoemulsions are small-sized, usually 20–200 nm in one dimension, emulsion droplets of oil in water (o/w), or water in oil (w/o). Nanoemulsions possess unique properties, such as transparent appearance and stability against sedimentation or creaming, which make them very useful in many cosmetics and pharmaceutical applications (Solans et al., 2005).

Solid lipid nanoparticles (SLN) are spherical particles with a diameter of 10–1000 nm. SLNs possess solid lipid cores, which are able to solubilize lipophilic molecules. SLNs are used as drug delivery systems for pharmaceutical drugs and carrier systems for active ingredients in cosmetics (Wissing & Muller, 2002).

Metal oxides such as titanium dioxide (TiO$_2$) and Zinc oxide (ZnO) NPs are found in many personal care products such as toothpaste and sunscreen, as well as in food coloring and coatings for vitamin supplements. TiO$_2$ and ZnO NPs in the size ranges of 60–120 and 20–30 nm, respectively, are used in most modern sunscreen products. They are colorless particles, and this allows them, along with their small size, to more efficiently and selectively reflect or scatter ultraviolet (UV) radiation than colored particles (Filipe et al., 2009; Yu & Li, 2011). Also,
the NPs are usually coated with silicon oils, SiO$_2$ or Al$_2$O$_3$ in order to make them nonreactive with the organic components of sunscreen products and to improve their dispersion and UV absorption. In comparison, ZnO is a better sunscreen ingredient than TiO$_2$ because it is more transparent and covers a broader UV spectrum. On the other hand, TiO$_2$ could be a better ingredient if greater sun protection factor (SPF) is required due to its stability (Morabito et al., 2011).

Silver NPs, due to their high efficiency as antibacterial and antiviral agents, are used in many medical and pharmaceutical applications such as medical device coatings, wound dressings, and treatments for burns and various infections (Rai et al., 2009). Furthermore, gold and silver NPs are used in certain day and night creams to give the skin a fresher appearance (Grebler et al., 2010).

2.2.2. Manufacturing and Industrial Applications

The uses and applications of NPs in various industries have been growing at a very rapid rate in recent years and have branched out into many different and interdisciplinary fields; therefore, a review of all industrial applications is beyond the scope of this review. Alternatively, a few examples of industrial applications of NPs will be introduced in this chapter (Altavilla & Ciliberto, 2011).

Calcium hydroxide NPs have dramatically improved historical and cultural heritage conservation techniques. Historical collections of books and other ancient paper-made documents turn into brittle and fragile materials with time. Cellulose polymers of paper degrade over time through acid catalysis of cellulose hydrolysis. Calcium hydroxide NPs, due to their small size, are able to penetrate into the cellulose fibers and neutralize acidity. The excess hydroxide is
immediately converted into calcium carbonate when it reacts with the ambient CO₂ (Giorgi et al., 2002).

The use of magnetic NPs has revolutionized information storage applications. Magnetic storage devices made from iron-platinum alloyed NPs (FePtNPs) promise to have an information storage density of several terabits per square centimeter, much more than the most advanced computer hard drives. The main idea is to replace large magnetic grains in current information storage devices with one single alloy made of FePtNPs because they are magnetically hard, meaning they don’t lose magnetization direction once the external magnetic field is removed as in the case of current devices (Reiss & Hutten, 2005).

The use of metal oxide NPs has significantly boosted gas sensor technologies. In chemoresistors, commonly applied gas-sensing devices, metal oxides are typically used as gas-sensing materials, which change their electrical resistance when oxidizing or reducing gases are applied. Over the last 3 decades, intensive efforts have been made in order to improve sensitivity, selectivity, stability, and response and recovery time of such devices. Metal NPs embedded into an organic matrix provide a selective binding site for the adsorption of analyte molecules. Also, they provide the ability to control the sensor properties through molecular design, which make them very reliable gas sensors (Franke et al., 2006).

Utilizing NPs in light-emitting devices (LEDs) has opened a path toward very novel LED applications. Low cost light emitters are in high demand as they are used in so many applications ranging from advertisement (lighted displays), to
the construction and automotive industries. They are also widely used in electronics, mobile phones, e-readers, toys, etc. Most light emitters used in products currently on the market are based on organic light emitting devices (OLEDs). Nanoparticle-based LEDs are very promising because they are more robust, have a longer life span, cost much less to fabricate than OLEDs, and consume less power (Neshataeva et al., 2011).

The antimicrobial characteristics of some NPs make them suitable for use in many products like nylon, polypropylene, and other polymers because of the high demand for textile-based substrates with minimized microbial growth potential. Zinc and copper oxide NPs have been incorporated into textile fiber manufacturing due to their minimal effect on color and their clarity, surface gloss, and other properties. NPs are used in textiles such as carpet fibers, fabrics for linens used in health care and other facilities, home furnishings, etc. (Lines, 2008). Furthermore, the use of nanosized aluminum increases the energy release rate in many exothermic reactions (Altavilla & Ciliberto, 2011).

Other industrial applications of NPs include the addition of silicon dioxide NPs to tennis rackets in order to increase the strength of the material. Also, butyl-based NPs are used in coating tennis balls to reduce air permeation to make them last longer (Aston & Stephen, 2005).

2.2.3. Agricultural Applications

Agricultural and food industries have greatly benefited from nanotechnology. Nanotechnology offers many innovations for agricultural industries that enhance the quality of plant products and are capable of improving
agricultural products in a very cost-effective manner. Such innovations include rapid disease detection and molecular treatment of plant diseases, as well as enhancing the ability of plants to absorb nutrients. Likewise, advanced sensors and delivery systems have helped the agricultural industry to easily detect, inactivate, and remove many crop pathogens. Additionally, nanostructured catalysts increase the efficiency of pesticides and herbicides, effectively reducing the doses required for application (Joseph & Morrison, 2006).

2.2.3.1. Molecular Treatment of Plant Diseases

Nano-size carbon, silver, silica, and alumino-silicates are examples of NPs currently used in the field of plant disease control. Carbon nano tubes (CNTs) can be used to deliver desired molecules into plant seeds to protect them from certain diseases during growing periods (Khodakovskaya et al., 2009). Silver NPs, in addition to their excellent performance as plant-growth stimulators, are very effective in removing undesirable microorganisms in the agricultural industry. For example, a colloidal nano-silver solution, 1.5 nm diameter, is successfully able to cure large agricultural areas polluted by rose powdery mildew, a widespread and common disease of both greenhouse and outdoor grown roses that causes leaf distortion, leaf curling, early defoliation, and reduced flowering (Sharon et al., 2010). Further, Park et al. (2006) showed that a nanosized mixture of silica-silver particles exhibited high antifungal activity and controlled powdery mildews of pumpkin in both field and greenhouse tests. The results showed that pathogens disappeared from the infected leaves and the plants remained healthy.
2.2.3.2. *Nano-Based Pesticides*

Alumino-silicate nanotubes with active ingredients are very efficient when used as pesticides. One of the advantages of alumino-silicate nanotubes is that when sprayed on plant surfaces, they are easily picked up by insects’ hairs, and thus, consumed by insects. Another advantage is that these types of pesticides are more environmentally friendly because of their low toxicity to the environment (Sharon et al., 2010).

2.2.3.3. *Advanced Sensors*

Carbon nanomaterials can be used as small electro-chemical sensors that measure specific oxidation/reduction values that occur as a result of the presence of certain undesired disease-causing contaminants, pathogenic microorganisms, individual proteins, and small molecules such as residual pesticides in the plants which are harmful to both the plants and human consumers. Other types of nanosensors work by initiating enzymatic reactions to target specific chemicals and proteins in the plant (Sharon et al., 2010).

2.2.3.4. *Advanced Delivery Systems*

Encapsulation and controlled-release methods using NPs technologies have improved the use of pesticides and herbicides in agricultural industry in a similar way that nanomedicine has done for drug delivery in humans. Nanoparticle-based solutions can be used to deliver specific chemicals in a controlled and targeted manner. For example, pesticides/herbicides are formulated to contain NPs in the size range of 100–250 nm, which effectively dissolve in water and have higher target specificity. Other examples are water and/or oil-
based nanoemulsions that contain uniform suspensions of 200–400 nm pesticidal or herbicidal NPs. Such nanoemulsions can be integrated into many different media like gels, creams, and liquids in order to prevent or treat diseases or infestations, or to preserve harvested agricultural products (Joseph & Morrison, 2006).

2.2.3.5. Enhancement of Nutrient Absorption

NPs are also used to enhance yields and nutritional values of many crops and certain NPs are used as soil conditioners or fertilizers to enrich plant growth. For instance, alfalfa plants, when grown in gold NP-rich soil, absorb gold NPs through their roots which then transferred to their tissues. The gold NPs can be mechanically separated from the plant tissue following harvest (Ward & Dutta, 2003).

2.3. Concentrations of NPs in Various Environmental Compartments

With the current rise in the manufacturing, application, and disposal of NPs, they are finding their way into the environment and their concentrations in different environmental compartments is cumulatively increasing over the years (Musee, 2011). Due to limited research, little is known about the precise concentrations of ENPs in the environment. Global production estimates for nanomaterials range from 350 and 500 tons/yr for carbon nanotubes (CNTs) and nanosilver particles (AgNPs), respectively, to 5,000 tons/yr for TiO₂ NPs (Petosa, 2010). Examples of environmental compartments into which NPs are released include wastewaters, surface waters, and soils.
2.3.1. Wastewaters

The most common route for NPs to enter aquatic environments is through effluents of domestic and industrial wastewater. NPs may affect both wastewater treatment processes and agricultural soil when biosolids originating from wastewater treatment plants (WWTPs) are used as soil conditioners or fertilizers (Musee, 2011). The data reporting levels of NPs in wastewater are typically predicted values based on modeling techniques mainly due to the complexity of physiochemical and biological water parameters and limited analytical techniques available for quantifying NPs (Blaser, 2008). Kiser and coworkers (2009) reported a maximum value of titanium NPs at 2.8 mg/L in influent water of WWTPs (average 0.84 mg/L) and 8.5 mg/L in secondary solids (sludge)—the highest concentrations relative to other WWTP treatment stages. NP concentrations in the treated effluent ranged between 0.001 and 0.1 mg/L. Additionally, Kiser and coworkers reported the presence of NPs, especially titanium dioxide, in almost all WWTP treatment stages at sizes ranging from <50 nm to <70 µm. Kim and coworkers (2010) reported the presence of silver sulfide NPs (Ag₂SNPs) at sizes ranging from 5 to 20 nm in WWTP treated sludge. Further, fullerenes have been detected in WWTP effluents at concentrations of 0.2 – 1 ng/L (Howard, 2010).

2.3.2. Surface Waters

NPs routes of entry into surface waters include atmospheric deposition, soil leaching, wastewater discharges, etc. (Schaller and Fan, 2009). The major point source for contamination of surface waters is wastewater and effluents from
factories producing raw NPs, but studies of this nature are still lacking (Scown, 2010). In a model developed by Mueller and Nowack (2008), focused on estimating the concentrations of Ag and TiO₂ NPs and carbon nanotubes into the various environmental compartments in Switzerland, concentrations of Ag and TiO₂ NPs and carbon nanotubes in freshwater bodies in normal scenarios were estimated to be 0.3, 0.7, and 0.0005 µg/L, respectively. In a high emission scenario, the model predicted levels up to 16 µg/L. A direct measurement of TiO₂ NPs, from the detachment of new and aged façade paints via natural weathering, at a point of entry into a stream, was approximately 300 µg/L as reported by Kaegi et al. (2008). The principal findings from this work are in agreement with the model proposed by Mueller and Nowack (2008) that suggested weathering of paints containing NPs could be responsible for significant discharges of NPs into surface waters.

2.3.3. Soils

Most of the NPs released into soils originate from non-point sources—these are probably the most important but they are the most difficult to control. On the other hand, NPs from point sources such as nanomaterial emissions from production and manufacturing facilities or transport processes, landfills, and runoff from manufacturing facilities or roads are basically easier to monitor (Nowack & Bucheli, 2007). There is no reported data of NP concentrations or values per gram of soil in the literature.
2.4. Environmental Implications of NPs

Even though research on usage, manufacturing, characterization, and applications of NPs is well established, understanding the impacts of NPs on organisms and surrounding ecosystems is still in its infancy. Typically, ecologists study pollutants only after they have already caused significant environmental degradation and since no large NP spills have been reported, no documented ecological impact studies on NPs are currently available. Thus, the picture of the potential environmental impacts of NPs is not clear yet. The lack of available information in this area and the urgent need for such information has led to new government-funded research opportunities to assess the environmental impacts of NPs. The goal is to develop a proactive, mechanistic understanding of both the long-and short-term environmental impacts of NPs on organisms and ecosystem processes, especially when exposed to lower concentrations. Also, conducting intensive empirical research and risk assessments for NPs is required.

Consequently, ecologists face significant challenges to predict the impact of NPs on the environment. One of the major challenges in generating such predictions is the complexity of NPs and their fundamental properties and behaviors, even though a great deal of research in this area has been achieved over the last two decades. This challenge is mainly linked to the existence of NPs in multiple forms and sizes. As explained earlier, the chemical, mechanical, electronic, and magnetic properties of NPs are dependent upon their size and can dramatically change as their size changes—and this largely determines their environmental
influence. It is essential to mention that the relationship between a NP’s properties and its size is what makes it commercially valuable.

2.5. Toxicity of NPs

2.5.1. Physicochemical Properties Affecting Toxicity of NPs

It is well documented in the literature that physical and chemical properties of NPs play a big role in determining any adverse effects and toxicity of the NPs. These properties include their surface reactivity, shape and aggregate structure, modified surface characteristics, solubility, and crystal structure (Lai & Sayre, 2009).

2.5.1.1. Surface Reactivity

The most important factor determining NP toxicity is surface reactivity. Increased surface reactivity of NPs is related to their increased surface area, due to their nanosize, along with other physicochemical properties (Warheit, 2010). However, some other factors may overcome surface reactivity as in the case where rats exposed to TiO₂ NPs did not experience more cytotoxic effects to their lungs compared with rats exposed to crystalline silica, which are larger particles with a higher surface reactivity (Warheit et al., 2006). In general, however, non-dissolved metal and metal oxide NPs are thought to cause toxicity due to their high surface reactivity (Morrow, 1992).

2.5.1.2. Shape and Aggregate Structure

Shape is another important factor in determining toxicity mechanisms of NPs. The various shapes of NPs, including NP aggregates, affect their deposition and absorption in the body and its response. For example, different aggregate
structures of single-walled carbon nanotubes (SWCNTs) have been associated with different regional responses of mice lungs (Shvedova, 2005). Furthermore, CNTs, nanofibers, nanowires, and nanorods are long and thin with a high aspect ratio, and therefore, may behave and exhibit some of their pulmonary effects similar to asbestos fibers, through the ‘frustrated phagocytosis’ pathway (the cellular process by which solid particles are engulfed by the cell membrane). Alternatively, some of the long and thin CNTs/nanofibers have the tendency to aggregate into intertwined, coiled, and particle-like materials, which may behave like black carbon particles and be toxic via the ‘lung overload’ mechanism and/or due to their surface reactivity. Therefore, depending on their aggregation state and physicochemical properties, CNTs/nanofibers can produce toxicity through multiple mechanisms (Johnston et al., 2010).

2.5.1.3. Surface Characteristics Modification

Modification of NPs’ surface characteristics can significantly affect their properties and therefore their toxicity mechanisms. For instance, hydroxylated fullerene, an example of a modified NP, showed less toxicity than unmodified fullerene and induced a different mechanism of cell death (Isakovic et al., 2006) than its unmodified counterpart. In contrast, increasing the sidewalls of (SWCNT) decreases its toxicity (Sayes et al., 2006).

2.5.1.4. Solubility

Most NPs are insoluble, or weakly soluble; however, in some reported cases NPs were able to dissolve in culture medium or biological fluids and taken up by cells. Such differences in dissolution rates can significantly affect the
toxicity of NPs (Lai, 2011). A good example of how NP solubility affects toxicity to cells can be demonstrated by ZnO NPs. Toxicity and cellular uptake paths for dissolved ZnO NPs were shown to be different from those for non-dissolved ZnO NPs because of their different abilities to release Zn$^{2+}$. Xia (2008) has reported that ZnO NPs’ toxicity is due to the release of Zn$^{2+}$ ions along with cellular production of reactive oxygen species (ROS) and oxidative stress. Additionally, gene expression profiling has revealed the differences between dissolved and non-dissolved ZnO NPs (Poynton et al., 2011). Also, the high biopersistance (hard to remove) of fibers, a well-known carcinogen, is associated with its solubility and clearance kinetics (Bernstein et al., 2001). Similarly, the cytotoxicity of copper oxide and copper NPs also depends on their intracellular solubility (Studer et al., 2010). Other studies have shown that some dissolved metal and metal oxide NPs may also be genotoxic (Totsuka et al., 2009).

2.5.1.5. Crystal Structure

A study in rats exposed to NPs of the same composition but different crystal structures showed different toxicity, cell proliferation, and inflammation responses in the lung. The NPs used in this study were P25 ultrafine TiO$_2$ and ultrafine rutile TiO$_2$ of similar sizes and surface areas. Lung toxicity, inflammation, and adverse tissue effects developed due to exposure to P25 ultrafine TiO$_2$ NPs, whereas, in contrast, only transient inflammation developed in the lungs following exposure to ultrafine rutile TiO$_2$ NPs. The greater adverse effects of the P25 ultrafine TiO$_2$ NPs were explained by the larger production of
ROS in lung cells exposed to this particular crystal structure (Warheit et al., 2007).

2.5.2. Copper Chemistry

Copper is a soft, orange-brown metal (chemical element) whose exploitation by mankind goes back to ancient history. Anthropological and historical evidences show that Egyptians and Babylonians practiced copper chemistry as early as 4,000 B.C (Hatfield, 1997). For example, some Egyptians artifacts, which survived up to the present time, showed that ancient Egyptians used copper to make bands, nails, and other tools which were used in ship building and also water at that historical period was conveyed using copper pipes (Lucas, 1948).

The chemical symbol of copper is Cu, which is an abbreviation of the Latin word: cyprium or cuprum. Cuprum means the metal of Cyprus, the location where ancient Romans used to mine copper (Allison, 1954).

Copper exists in both forms: pure or parts of minerals. The pure copper is found is poly-crystal form. Examples of copper containing minerals include copper sulfides, copper carbonates, copper oxides, etc (Rickwood, 1981). Copper element is numbered 29 in the periodic table with an atomic weight of 63.546. The most common forms of copper compounds are salts associated with oxidation states +1 and +2, which are called cuprous and cupric, respectively.

Table (1) lists some important properties of copper. Copper is known for its slow reactivity with atmospheric oxygen rather than reactivity with water.
Copper reactivity with oxygen results in the formation of brown-black copper oxide layer (Holleman & Wiberg, 2001)

Table 1: Properties of copper

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Orange-brown</td>
</tr>
<tr>
<td>Melting Point</td>
<td>1083.4 +/- 0.2°C</td>
</tr>
<tr>
<td>Boiling Point</td>
<td>2567°C, with a valence of 1 or 2</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>8.96 (20°C)</td>
</tr>
<tr>
<td>Electrical conductor</td>
<td>Good</td>
</tr>
<tr>
<td>Heat Conductor</td>
<td>Good</td>
</tr>
</tbody>
</table>

2.5.3. *Complex ions of copper*

Metal ions when bonded with a number of other molecules, ligands, form complex ions. Metal ions usually act as Lewis acid and the other molecules act as Lewis base. Basic examples of ligands include water, ammonia, and chloride ions. Complexity of complex ions depends on type and number of bonds formed between metal ions and ligands. An example of copper complex ions is the formation of a light blue precipitate when copper sulfate is dissociated into Cu²⁺ and SO₄²⁻ ions in water and NH₃ is added to the solution. Addition of ammonia to water forms ammonium and hydroxide ions. Hydroxide ions combine with Cu²⁺ ions to form Cu(OH)₂ precipitate. Cu²⁺ ions also bond with NH₃ to form Cu(NH₃)₄²⁺ (complex ions) (Oliphant, 2003). The following chemical equations explain the previous reactions to form copper complex ions:

\[
\text{CuSO}_4(s) \rightleftharpoons \text{Cu}^{2+}(aq) + \text{SO}_4^{2-}(aq)
\]

\[
\text{NH}_3(aq) + \text{H}_2\text{O}(l) \rightleftharpoons \text{NH}_4^+(aq) + \text{OH}^-(aq)
\]

\[
\text{Cu}^{2+}(aq) + 2 \text{OH}^-(aq) \rightleftharpoons \text{Cu(OH)}_2(s)
\]

\[
\text{Cu}^{2+}(aq) + 4 \text{NH}_3(aq) \rightleftharpoons \text{Cu(NH}_3)_4^{2+}(aq)
\]
2.5.4. Toxicity Mechanisms of NPs

Lai (2011), in a review of the toxicity of NPs, listed 5 potential cytotoxicity mechanisms of NPs: (1) interaction with plasma membrane may cause instability associated with ion transport, signal transduction, and cell death; (2) interaction with mitochondria may alter metabolism or interfere with antioxidant defenses and ROS production. For example, Li et al., 2003 have demonstrated that NPs preferentially mobilize to mitochondria and induce oxidative stress and mitochondrial damage; (3) binding to DNA may damage DNA and arrest cell cycle division and protein synthesis; (4) interaction with cytoskeleton may halt vesicular trafficking and cause mechanical instability and cell death; and (5) interaction with proteins, lipids, and other biomolecules may lead to different types of ‘corona’ and biological effects through protein unfolding, fibrillation, thiol cross-linking, and loss of enzyme activity.

2.5.4.1. Toxicity Mechanisms in Terrestrial Ecologies

NPs are taken up and adsorbed to plant surfaces through a plant’s nano- and/or micrometer-scale openings. Usually above ground parts of the plant take up NPs at a rate greater than underground parts because of the deposition of airborne NPs onto its shoot surfaces. NPs find their way into epicuticular cavities, waxy protective structures found on plant surfaces, and other types of plant appendages (Dietz & Herth, 2011).

For example, Birbaum and coworkers (2010) exposed young maize (Zea mays) plants to 400 mg aerosol CeO₂ NPs (37 nm in size) for 20 min to investigate NP deposition on shoot surfaces. NPs were found to be tightly
associated with the plants’ shoot surfaces and couldn’t be removed even by surface washing.

Uptake into the plant body depends on NPs’ size and surface properties and the permeation properties of plants’ cuticles and waxy layers covering outer surfaces of leaves, fruits, and epidermal cells (Schreiber, 2011). Also, larger NPs can be taken up through cuticle-free areas such as hydathodes (secretion mechanism), the stigma of flowers, and stomates (gas exchange pores). Pores of opened stomates can be as big as 10 µm in diameter in some plants (Humble & Hsiao, 1970). NPs have been reported to penetrate into the intercellular gas space of leaves by passage through the stomates and are then deposited on the cell wall of the substomatal cavity or neighboring cells after deposition of airborne CeO₂ NPs (Birbaum et al., 2010).

As mentioned earlier, NPs are more readily taken up by above ground parts of plants because organs like roots develop waxy protection layers in the inner surface of the cell wall called suberin. The role of suberin is to prevent apoplastic (diffusion space outside cell membranes) bypass flow of solutes and water from the soil to the central cylinder (Steudle & Peterson, 1998). However, it has been reported that newly formed lateral roots in the more basal root zone allow apoplastic bypass; thus, NPs can enter the central cylinder along with water and nutrients (Faiyue et al., 2010).

Zinc oxide NPs (ZnONPs) 20 nm in size were used in hydroponics (growing plants using mineral nutrients) of ryegrass and higher concentrations of
ZnONPs were found in the roots than in the shoots. Severe damage and collapsed cells were observed in the roots (Lin & Xing, 2008).

Further, luminescent NaYbEr₄ NPs [ytterbium (Yb), erbium (Er)] 45 nm in size were used in growing Phalaenopsis spp. and Arabidopsis thaliana seedlings. These NPs emit luminescence between 650 and 710 nm after photon upconversion, if excited at 974 nm. Initially, the NPs were concentrated in the roots, but 6 days later, they reached the central cylinder and spread throughout the plant. Luminescence was observed in leaves and flower stalks, indicating that the NPs were able to translocate over long distances but only after a long period of time (Hischemoller et al., 2009).

Other potential pathways for NPs to enter plants besides lateral root junctions are mechanical injuries in the roots and wounding from below ground herbivores (organisms that are anatomically and physiologically adapted to eat plant-based food). These are known entry routes for bacteria and therefore, may also be efficient routes for NP uptake (James & Olivares, 1998).

2.5.4.2. Toxicity Mechanisms in Aquatic Life

The mechanisms of NPs’ toxicity to aquatic life are poorly investigated; however, an understanding of the toxicity mechanisms of metal ions in fish may be used to extrapolate pathways of NP uptake by fish. Possible mechanisms through which NPs are toxic to fish may involve endocytosis pathways, diffusional pathways, mucus pathways, direct toxicity to gills and intestines, etc. The mechanisms of metal uptake across biological membranes in fish involve carrier-mediated transport on metal ion transporters; however, NPs are too big to
use ion transporters, or paracellular diffusion pathways, and therefore, the most likely potential uptake route is by endocytosis pathways, which is a process by which cells absorb NPs by engulfing them. However, more investigation and modeling work is needed in this field because endocytosis is a known uptake pathway for trace metals only. Furthermore, for very small NPs (<20 nm) that have a hydrophilic surface coating, it remains theoretically possible for these materials to diffuse through the cell membrane. Thus, diffusional route of entry that is both a function of particle size and the hydrophobicity of the particle surface can be considered another uptake pathway for relatively smaller NPs (Shaw & Handy, 2011). Also, NPs can utilize the protective role of mucus as an appropriate uptake route for NPs, which has been already observed in the case of carbon nanotubes precipitation in the gill mucus of trout (Smith et al., 2007). Similar to metal ions, the uptake sites such as gills and intestine could also be a target organ for toxic effects. Some NPs are respiratory toxicants in fish and produce gill pathologies which are similar to those for metal ions. Similar to surface acting metals, it remains possible that some NPs can exert toxic effects without appreciable uptake of the metal into the internal organs like in the case of short waterborne exposures to TiO2 (Federici et al., 2007). Moreover, Handy and coworkers (2008), in an attempt to review the possible mechanisms of absorption, distribution, metabolism, and excretion (ADME) for NPs in fish, identified numerous knowledge gaps on the ADME of NPs. The main reason behind these gaps is the lack of routine methods for the direct measurement of NPs in fish tissues in order to establish body distributions and target organs. Although there
are current applied methods involving electron microscopy of dissected tissues, they are considered very labor-intensive ways of determining the presence or absence of nanometals.

2.5.4.3. Toxicity Mechanisms in Bacteria

Many reports in the literature suggest that NPs cause toxicity to bacteria by disrupting their cellular membrane (Musee et al., 2011). Membrane disruption leads to a bacterial cell’s reduced ability to control the movement of substances in and out of the cell; as a result cellular metabolic disturbances occur, which may then lead to death. Membrane disruption can be caused by either strong electrostatic interaction between highly positively charged NPs, due to their high surface area, and a negatively charged cell membrane, or by oxidative stress on the membrane by reactive oxygen species (ROS), which are generated when NPs interact with the bacterial membrane (Shrivastava et al., 2007).

Several studies have shown that gram-negative bacteria are more resistant to NPs than their gram-positive counterparts; however, other studies have shown just the opposite (Musee et al., 2011). The first group of studies concluded that gram-negative bacteria are highly resistant to NPs due to the composition of their membrane, which consists of multilayers of tightly packed lipopolysaccharide, phospholipids, and protein molecules. The membrane of gram-positive bacteria consists only of several layers of peptidoglycan, which is thought to make it less effective as a protective barrier (Fan et al., 2002; Yoon et al., 2007; Premanathan et al., 2010; & Brayner et al., 2006). On the other hand, other reports argued that gram-negative membranes are highly negatively charged and therefore they
attract positively charged NPs more than their gram-positive counterparts—thus more electrostatic interactions are expected which lead to cellular rupture. Also, the greater thickness of gram-positive bacteria cell membranes may make them more protective than those of gram-negative bacteria, the latter of which possess protective lipids and polysaccharides that are not strongly linked together and that lack rigidity (Jiang et al., 2009; Zhang et al., 2007; & Nair et al., 2009). Furthermore, another study showed that ZnO NPs have similar effects on both gram-negative and gram-positive bacteria (Huang et al., 2008). Musee and coworkers (2011) argued in their review on the antibacterial effects of NPs that bacterial resistance to NPs is not only linked to membrane composition, but it also may involve the physicochemical state and type of NPs, inter-species differences, and test conditions. Therefore, detailed investigations of NPs’ properties, different bacterial species characteristics, as well as testing conditions need to be studied.

Generation of ROS occur after NPs penetrate inside a cell membrane which causes peroxidation of many cellular constituents (Kim et al., 2007). Theoretically, bactericidal effects of NPs should be similar in both gram-negative and gram-positive bacteria once they are inside the cell; however, the evidence contradicts this hypothesis. Oxidative stress inside the cell could be due to either the release of metal ions species from NPs or direct interaction between NPs and cellular constituents. Also, ROS can cause DNA strand breakage and protein inactivation, which leads to cellular metabolic disruption followed by cell death (Musee et al., 2011). For example, AgNPs inside the cell have a greater affinity
for sulfur- and phosphorus-containing sites like DNA at which they initiate their oxidative attack (Pal et al., 2007).

Many other factors influence the mechanisms of NP toxicity in bacteria, such as the size of the NPs and the bacterial culture population. More detailed investigations are needed in order to determine the effect of different sized NPs individually, as the degree of antibacterial activity among the same NP species of different sizes is not necessarily similar (Musee et al., 2011). Likewise, highly dense bacterial cultures showed less susceptibility to antibacterial NPs than less dense cultures because the rate of growth is dramatically reduced if low density cultures are exposed to NPs whereas in higher density cultures the growth rate is slowly reduced and therefore, more bacterial cells are being reproduced (Nair et al., 2009).

2.5.5. Methods of Determining the Toxicity of NPs

In NPs toxicity studies, how and when NPs are mixed up with bacterial cells are critical factors in the experimental planning. In most of the previous studies regarding the impact of NPs on bacterial species, the toxicity measurement methods were not standardized, and this is thought to be the main reason behind inconsistent toxicity data found in the literature. For example, in one study evaluating the potential of silver NPs as an antibacterial agent against *E. coli* and another study evaluating the toxicological impact of ZnO NPs on *E. coli* cells, bacterial cells were cultured on agar plates supplemented with NPs without mixing; and it wasn’t mentioned whether NPs were added initially to the agar medium or directly to the plates (Brayner et al., 2006; Sondi & Salopek-Sondi,
2004). Such a method is questionable because the NPs were not distributed throughout the solution; therefore, complete interaction between the bacteria and NPs could not occur. In a different study evaluating the bactericidal effects of silver NPs, bacterial cells were grown to a log phase; then, silver NPs were added to the solution and left for 30 min (Morones et al., 2005). The reported data likely does not represent the actual antibacterial activity of silver NPs because the fact that NPs tend to settle down very fast was not considered in addition to the short contact/exposure time.

In a recent study on the toxicity of ZnONPs toward gram-positive bacteria, the antibacterial activity of ZnO NPs was estimated by a micro-titer plate–based method (sterile 96-well micro-titer plate) (Lipovsky et al., 2011). Again, fast sedimentation of NPs doesn’t allow uniform contact/exposure time for NPs throughout the bacterial population. Allahverdiyev and colleagues (2011), in their attempt to examine the antimicrobial effects of TiO$_2$ and Ag$_2$O NPs against drug-resistant bacteria, treated bacterial cultures at the beginning of the logarithmic phase with a ZnO/TiO$_2$ NP solution and then samples were removed for assay. Such a method, even though there is some mixing involved, doesn’t allow equal exposure time and thorough mixing. In a study of the impact of gold NPs on *Salmonella*, a solution of GNPs was mixed with sodium phosphate buffer and 5 different concentrations of bacterial cells; however, the overall contact time and the method of mixing were not mentioned in the article (Wang et al., 2011). On the other hand, Ashutosh et al. (2011) did report the contact time for the treatment of *E. coli* cells with various concentrations of ZnO and TiO$_2$ NPs; however, the
method of treatment and mixing was not mentioned. Therefore, because of the lack of consistency in the experimental plan in the context of contact between NPs and bacteria, studies performed under homogeneous mixing conditions that ensure uniformed contact conditions throughout the reaction vessel are necessary.
CHAPTER 3

IMPACT OF COPPER NANOPARTICLES ON A VARIETY OF BACTERIA: ASSESSMENT OF INACTIVATION AND TOXICITY PATHWAYS

Abstract

The widespread use of nanoparticles (NPs) in industrial and consumer products has resulted in the emergence of NP species as significant environmental contaminants, and wastewater is one of the primary conduits of such contamination. Metals in NP forms have high surface areas, which confer different physical, chemical properties and biological capabilities to these materials than are found in their elemental forms. Similarly, the environmental fate, transport, and toxicity of NPs are significantly different than their elemental counterparts.

Bacteria play a major role in many natural and engineered biogeochemical reactions in wastewater treatment plants and other environmental compartments. I have evaluated the laboratory isolates of Bacillus, Alcaligenes, Pseudomonas, and wastewater isolate of Bacillus for their response to 50 & 100 nm sized Cu nanoparticles (CuNPs).

In general, exposure to CuNPs resulted in 4 to >6 log reduction in bacterial population within 2 hour. After 2hr exposure to 50CuNPs, the laboratory isolates of Alcaligenes and Pseudomonas, showed 5.75 and 6.64 log reduction, respectively; whereas under similar conditions treatment with 100CuNPs resulted in 5.97 and 6.58 log reduction, respectively. Comparison of laboratory and wastewater isolates of Bacillus for their response to 50CuNPs indicated 4.84 and
4.16 log reduction, respectively; and under similar conditions treatment with 100CuNPs resulted in 4.35 and 3.61 log reduction, respectively. Based on the GR, LDH and MTT assays, bacterial cells showed different toxicity elicitation pathways after exposure to CuNPs. Therefore, it can be concluded that the laboratory isolates are good candidates for predicting the behavior of environmental isolates exposed to CuNPs. Also, high inactivation values recorded in this study suggest that the presence of CuNPs in different environmental compartments may have an impact on pollutants attenuation and wastewater biological treatment processes.
3.1. Introduction

NPs have different physical and chemical properties and different impact on biological species than their elemental sources. They are characterized for relatively high surface area, which confer the higher reactive properties, hardness and electrical conductivity (Utamapanya et al., 1991; Ren et al., 2009). Due to these novel characteristics NPs are used in a broad range of industries such as electronic, pharmaceutical, cosmetic, oil, gas, environmental, agriculture and materials science applications (Nowack & Bucheli, 2007; Makimura et al., 2010; Ingram et al., 2010). As a result of this wide application, the inventory of nanotechnology based consumer products has grown from 212 products in 2006 to 1,317 products in 2011, which is nearly 521% increase in 5 years. The U.S. National Science Foundation estimates that nanotechnology will have a $1 trillion impact on the global market and will employ over 7 million workers by 2015 (Roco, 2007).

Widespread use of NPs in agricultural, industrial and consumer products has resulted in the release of huge quantities of NPs in environment and surface water. In recent years, NPs species have been recognized as significant contaminants in wastewater and sludge (EPA 2003; Westerhoff et al., 2009). They are known to impact biological activities at molecular, cellular, organism, and ecological levels (Ren et al., 2009).

Unabated release of heavy metal-containing nanoparticles (mNP) into the wastewater can have harmful effect on the normal bio-ecological processes. Such NP can alter the efficacy of beneficial microbes critical in the normal functions of
wastewater treatment processes also play significant role in natural attenuation of pollutants and mineral cycling. Traditionally, laboratory isolates of common pathogenic and indicator bacteria (such as *E. coli*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, etc.) have been used to study the impact of various types of NPs on the biological and ecological systems; however, as far as I know at present no information is available on the relevance of laboratory strains for studying the impact of NPs in wastewater and sludge.

CuNPs are widely used in industrial applications such as semiconductors, heat transfer fluids in machine tools, metal catalysts, and even in the area of public health as biocidal preparations (Aruoja et al. 2009; Kim et al., 2011). CuNPs being one of the primary engineered NPs in industrial applications, makes them good candidate for studying the potential adverse effects of NP on bio-geo-ecology of surface and waste waters. Copper can impact a biological cell in three major ways: a) Acting on cell wall or cell membrane, b) Interacting with cellular enzymes that counter the effect of toxic radicals, c) Interfering with the cellular powerhouse. Brown and colleagues (1995) analyzed the DNA sequence of copper resistant determinant (*pco*) in *E. coli* and reported that when *E. coli* cells are exposed to high concentration of Cu$^{2+}$, cellular uptake of copper is initially insignificant due to copper export. They also identified four mutations in the copper-resistance determinant (*pco*) of *E. coli* which eliminated copper resistance and these four mutations contained six open reading frames, designated *pco-* ABCDRS. Finally, characterization of chromosomal mutants defective in cellular copper management was conducted and two of these mutants became
inactive and the other two mutants pco complemented the defective genes.

The objective of this study was to investigate the adverse effects of CuNPs on the laboratory isolates of *Bacillus*, *Alcaligenes*, and *Pseudomonas* and field isolates of *Bacillus*. Then, the toxicity effects on laboratory strain of *Bacillus* were visualized after exposure, using scanning electron microscopy (SEM). The concentrations of Cu$^{2+}$ were determined under different conditions, such as pH, temperature, light exposure, etc. To the best of our knowledge, this is the first study reporting the biocidal effects of two different sizes of CuNPs against the laboratory and wastewater isolates of selected bacterial species, as well as determining the pathways of CuNP toxicity elicitation.

3.2. Materials and Methods

3.2.1. Bacterial Strains

Environmentally relevant strains of gram-positive and gram-negative bacteria were tested for their response to CuNP. Laboratory strains; *Bacillus* (ATCC 23059), *Alcaligenes* (ATCC 8750), and *Pseudomonas* (ATCC 10145) were obtained from the American Type Culture Collection. As a representative of environmental bacteria a strain of *Bacillus* isolated from a wastewater lagoon (Maricopa, AZ) was included in the study for comparison purposes. The environmental isolate was presumptively identified by colony morphology on selective media and verified by polymerase chain reaction (PCR).

3.2.2. Culture Preparation

Bacterial cultures were grown using nutrient broth (NB). A volume of 1.0 ml of each strain was taken from over-night cultured stocks and suspended in 9.0
ml of the corresponding broth media. The inoculated broth tubes were incubated in a shaker-incubator (New Brunswick Scientific C24, Edison, NJ) (150 RPM @ 37°C) to achieve a log phase bacterial culture.

Bacterial cells were grown to an optical density of 0.8 to 1.0 at 600 nm, measured using a spectrophotometer (Hach DR/4000U, Loveland, CO). Bacterial cells were harvested by centrifugation for 10 min at 1000xg and 22°C; supernatants were discarded and pellets were re-suspended in phosphate buffer (0.5M PBS).

3.2.3. Exposure to CuNPs

Experiments were performed using an axially rotating mixer (40 rpm) to ensure NPs are kept evenly dispersed in the reaction mixture, throughout the experiment. Use of the axially rotating mixer is critical in experiments using CuNPs as they tend to settle down in peripheral zone in stir mixer.

The CuNPs of two sizes: 50 nanometer (50CuNPs) and 100 nanometers (100CuNPs) were obtained from (Sigma-Aldrich, Saint Louis, MO). Each type of NPs (50CuNPs and 100CuNPs) was added to the individual reaction tube at a final concentration of 6mM (0.0036mg per 10mL).

Total number of 50CuNPs in each tube = (total mass) x (density)⁻¹ x (volume)⁻¹
= (0.0036 mg) x (1x10²¹ nm³) x (8.94)⁻¹ x (1,000 mg)⁻¹ x (6.5x10⁴ nm³)⁻¹
= 6.2x10⁹ 50CuNPs

Total number of 100CuNPs in each tube = (total mass) x (density)⁻¹ x (volume)⁻¹
= (0.0036 mg) x (1x10²¹ nm³) x (8.94)⁻¹ x (1,000 mg)⁻¹ x (5.2x10⁵ nm³)⁻¹
= 7.7x10⁸ 100CuNPs
Individual reaction tubes containing 50 or 100 nm CuNPs were spiked with washed bacterial cells at a concentration of cells $1 \times 10^9$ cells per ml. The control reaction tube contained similar concentrations of bacteria with no CuNPs added. Periodic samples (20, 40, 60, 90 & 120 min) were collected from each reaction tube and duplicates of each sample were analyzed for viable bacterial cells using membrane filtration on agar plates and incubated at 37°C for 24 hours.

3.2.4. Membrane Filtration

Membrane filtration was used to analyze the samples collected from different treatments. The data provided the numbers of viable bacteria at each sampling time. Samples were filtered through 47 mm cellulose acetate membrane with 0.47 µm pore size. Membranes were placed on appropriate selective agar plates and incubated at 37°C for 24-48 hours. After incubation, colonies were counted and data was recorded as viable counts.

3.2.5. CuNPs Impact on Bacteria in Wastewater Samples

To examine the impact of the CuNPs on bacteria in the environment, wastewater samples were collected from Scottsdale Wastewater Treatment Plant (Scottsdale, AZ). Samples from different treatment stages were collected (primary, secondary, and tertiary treated wastewater). Each wastewater sample was analyzed for viable bacterial cells using the membrane filtration technique. The membrane was transferred on nutrient agar plates and incubated at 37°C for 24 hours. After collecting the initial control sample, 50 & 100 nm CuNPs at a final concentration of 6mM (0.0036mg per10mL of wastewater) were added to individual reaction tubes containing each type of wastewater sample with
continuous rotational mixing. Samples were collected at 20 & 120 min. and analyzed for viable bacterial cells. The mixing of the reaction tube was stopped after 120 min. and then samples were collected at 24, 48, & 72 hours and again analyzed for viable bacterial cells. The mixing was resumed after 72 hours and samples were collected at 96 hours for enumerating viable bacterial cells.

3.2.6. CuNP Toxicity Pathways

Copper is known to impact biological cells in three different ways: by interacting with ligands on the surface of bacterial cells; by impairing cellular defense mechanisms, and by interfering with the cellular energy pathway. To identify the toxicity pathway, washed cells of selected bacterial species were exposed to both types of CuNPs and after the specified exposure time, cells were analyzed for the parameters of toxicity pathways. The control experiments included analyses of unexposed cells for all toxicity parameters.

3.2.6.1. LDH Assay

The impact of CuNPs on the integrity of bacterial cell membrane was studied using the Cellular In Vitro Toxicology Assay Kit, Lactic Dehydrogenase, (Sigma-Aldrich, Saint Louis, MO). The kit was used according to the manufacturer’s instructions with slight optimization for detecting the total LDH in bacteria cells and samples suspension.

3.2.6.2. GR Assay

The cellular level of glutathione reductase, an enzyme that fights against radicals, was determined using the Glutathione Assay Kit, (Cayman Chemical Company, Ann Arbor, MI). The kit was used according to the manufacturer’s
instructions with slight optimization. The absorbance readings for GR activity were normalized using the following given equation in the GR assay protocol:

The actual extinction coefficient for NADPH at 340 nm adjusted for the path length of the solution in the cuvette = 0.00622 μM⁻¹ cm⁻¹ x 1 cm = 0.00622

3.2.6.3. MTT Assay

The integrity of energy cycle in the target bacterial cell was determined using the MTT Cell Proliferation Assay Kit that was obtained from American Type Culture Collection (ATCC) (Manassas, VA). The kit was used according to the manufacturer’s instructions with slight optimization.

3.2.7. Scanning Electron Microscope

The physical impact of 50 & 100 nm CuNPs toxicity on the laboratory strain of Bacillus was visualized using a Scanning electron microscope (SEM) (Focused Ion Beam – Nova 200 – NanoLab - FEI) (Hillsboro, OR). Laboratory isolates of Bacillus were washed with phosphate saline buffer (PBS). Separate aliquots of washed bacterial cells were exposed to 50 & 100 nm CuNPs. After the specified exposure time, bacterial cells were removed from reaction solution and placed on aluminum stud and coated with gold by sputter coater (Denton Vacuum) (Moorestown, NJ). The prepared samples were observed under the scanning electron microscope. As a control, non-treated Bacillus cells were also visualized using the same protocol and experimental conditions. To determine whether CuNPs transported within the cell, exposed cells were washed 15 times by allowing CuNPs to settle down for 2 min and supernatant was transferred to a new tube in each wash. The washed cells were then observed under SEM with
XLD scan capabilities. The CuNP outside bacterial cells are visible; however the CuNPs inside the bacterial cell are localized using Cu specific peaks in XLD scans.

3.2.8. Release of \( \text{Cu}^{2+} \) from CuNPs

The kinetics of the release of \( \text{Cu}^{2+} \) released from 50 & 100 nm CuNPs in aqueous solution (0.5M PBS buffer) was studied using Cole-Parmer Laboratory Cupric Ion Selective Electrode (Cu\(^{2+}\)-ISE) (Vernon Hills, Illinois). The Cu\(^{2+}\) release experiments were performed under different conditions such as light exposure, temperature, contact time, and medium acidity. The Cupric Ion Selective Electrode was used according to the manufacturer’s instructions.

3.2.9. Complex Ions Formation

The possibility of Cu\(^{2+}\) complex formation in 0.5M PBS solution versus nano-pure water was determined by comparing copper electrode calibration curves prepared using both solutions. Standard calibration curves were prepared according to manufacturer’s instructions.

3.3. Results and Discussion

3.3.1. Inactivation of Bacterial Cells by CuNPs

The bactericidal impact of 50 & 100 nm CuNPs on laboratory and wastewater isolates of different bacterial isolates was investigated and results are presented in Figures 1, 2, 3 and 4. Although 50 & 100 nm CuNPs had similar cumulative bactericidal impact after 120 min contact time, the short term inactivation kinetic for both types of NPs was different for \textit{Bacillus, Alcaligenes}, and \textit{Pseudomonas}. The highest level of bactericidal effect for 50 & 100 nm
CuNPs against laboratory isolates of *Alcaligenes*, and *Pseudomonas* was noted at 90 min. Against laboratory and field isolates of *Bacillus*, the highest level of bactericidal effect of 50 & 100 nm CuNPs was recorded at 60 and 40 min, respectively (Table 2). When bacterial cells were exposed to 100CuNPs, a sudden decrease in bactericidal activity was recorded at 60–90 min contact time. Following this time window, bactericidal activity resumed and lasted until the end of assay. No such shift in bactericidal activity was recorded for 50CuNPs (Fig. 1, 2, 3, and 4).

**Fig. 1.** Bactericidal Effects of 50 & 100 nm CuNPs on the Laboratory Isolate of *Bacillus*.
Note: Line graphs represent accumulative inactivation and bar graphs represent inactivation between time intervals.
Fig. 2. Bactericidal Effects of 50 & 100 nm CuNPs on the Wastewater Isolate of *Bacillus*.
Note: Line graphs represent accumulative inactivation and bar graphs represent inactivation between time intervals.

Fig. 3. Bactericidal Effects of 50 & 100 nm CuNPs on the Laboratory Isolate of *Alcaligenes*.
Note: Line graphs represent accumulative inactivation and bar graphs represent inactivation between time intervals.
Fig. 4. Bactericidal Effects of 50 & 100 nm CuNPs on the Laboratory Isolate of Pseudomonas.
Note: Line graphs represent accumulative inactivation and bar graphs represent inactivation between time intervals.

Table 2. Comparison of inactivation results of wastewater and laboratory isolates of selected bacterial species

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Inactivation Rate</th>
<th>Total Reduction Log_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus (Laboratory Isolate)</td>
<td>60 40</td>
<td>4.16 3.61</td>
</tr>
<tr>
<td>Bacillus (Wastewater Isolate)</td>
<td>60 40</td>
<td>4.84 4.35</td>
</tr>
<tr>
<td>Alcaligenes (Laboratory Isolate)</td>
<td>90 90</td>
<td>5.75 5.97</td>
</tr>
<tr>
<td>Pseudomonas (Laboratory Isolate)</td>
<td>90 90</td>
<td>6.64 6.58</td>
</tr>
</tbody>
</table>
3.3.2. Wastewater Samples Inactivation Results

The bactericidal impact of 50 & 100 nm CuNPs in primary, secondary and tertiary treated wastewater samples (collected on April 10 & 25, 2012) was investigated and results are presented in Figures 5 & 6. After 2 hour mixing with 50 or 100 nm CuNPs, the concentration of bacteria in the primary treated wastewater sample was reduced from 6.3 to 2.7 & 3.1 logs, respectively. Similarly, concentration of bacteria in secondary treated wastewater samples was reduced from 3.9 to 2.1 & 2.3 logs, respectively. A greater impact on bacterial inactivation in the samples was observed for 50 compared to 100CuNPs. After mixing was stopped, concentration of bacterial cells was increased in primary and secondary treated wastewater samples. This is thought to be due to the high level of organic matter in these samples which resulted in bacterial regrowth. When the mixing was resumed after 72 hours, concentration of bacteria was reduced again, which points out the importance of the mixing conditions for observing the impact of CuNPs. On the other hand, concentration of bacteria in the tertiary treated wastewater sample collected on April 10, 2012 remained unchanged throughout the experiment. This is thought to be due to the persistence of bacteria found in the sample which survived primary, secondary, & tertiary treatment processes. However, the tertiary treated wastewater sample collected on April 25, 2012 was free of culturable bacteria. The difference between the bacterial concentrations in the tertiary treated wastewater samples collected in different days might be linked to the efficiency of treatment processes at the wastewater treatment plant.
Furthermore, the bactericidal impact of 50CuNPs on *E. coli* in the sterilized primary and secondary treated wastewater samples was investigated and results are presented in Figure 7. After 2 hour mixing time, *E. coli* concentration in the sterilized primary treated wastewater sample was reduced from 9.3 to 4.8 logs which were in a similar trend to the first set of non-sterilized wastewater samples. Similarly, concentration of *E. coli* cells in the sterilized secondary treated wastewater samples was reduced from 9.3 to 5.9 logs. After the mixing was stopped, concentration of *E. coli* cells was increased in the sterilized primary and secondary treated wastewater samples. This is thought to be due to the high level of organic matter in these samples which resulted in bacterial regrowth. When the mixing was resumed after 72 hours, concentration of *E. coli* was reduced again which points out the importance of the mixing conditions for observing the impact of CuNPs.

Fig. 5. Bactericidal Effects of 50CuNPs on the primary, secondary, & tertiary treated wastewater samples.
Fig. 6. Bactericidal Effects of 100CuNPs on the primary, secondary, & tertiary treated wastewater samples.

Fig. 7. Bactericidal Effects of 50CuNPs on *E. coli* cells spiked in the sterilized primary & secondary treated wastewater samples.
3.3.3. **Toxicity Pathways**

This section presents the results of LDH, GR and MTT assays performed on different bacterial cells after exposure to CuNPs. These activities represent three stages of energy cycle (Kreb’s cycle) in a normal bacterial cell (Fig. 8)

Fig. 8. Tricarboxylic acid cycle (TCA cycle/Kreb’s Cycle): modified from Wikipedia.

### 3.3.3.1. LDH Assay

The results of LDH assay for all bacterial strains after exposure to CuNPs for 120 min are presented in Figures 9, 10, 11, and 12. In general, the total LDH levels in all bacterial cells decreased over time except for *Pseudomonas* cells exposed to 50CuNPs where they remained unchanged (Table 3). The total LDH activities measured are inversely proportional to the remaining viable cells in the sample as inactivation increases, LDH activity decreases. LDH activities in the laboratory isolate of *Bacillus* were less in magnitude than the rest of the strains and such difference in magnitude could be due to the difference in the stress
response pathway used by bacterial strains to handle excessive copper. For example, copper stress results in the expression of copper-binding proteins (CuBPs) on the surface of some bacterial species such as *Pseudomonas* and *Vibrio*; however, no such CuBPs are expressed on the surface of *Bacillus* (Rensing and Gross, 2003). Therefore, further molecular work is required to determine exact nature of such responses by different bacterial strains.

Table 3: Summary of LDH activities for the bacterial strains exposed to 50 & 100 nm CuNPs for 120 min.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LDH (50 nm)</th>
<th>LDH (100 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus</em> (Lab. Isolate)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><em>Bacillus</em> (Field Isolate)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><em>Alcaligenes</em> (Lab. Isolate)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><em>Pseudomonas</em> (Lab. Isolate)</td>
<td>↔</td>
<td>↓</td>
</tr>
</tbody>
</table>

Fig. 9. Enzymatic activities in the cells of the laboratory isolate of *Bacillus* after exposure to CuNPs for 120 min
Fig. 10. Enzymatic activities in the cells of the wastewater isolate of *Bacillus* after exposure to CuNPs for 120 min

Fig. 11. Enzymatic activities in the cells of the laboratory strains of *Alcaligenes* after exposure to CuNPs for 120 min
3.3.3.2. GR Assay

The results of glutathione reductase (GR) assay for all bacterial cells after exposure to CuNPs for 120 min are presented in Figures 9, 10, 11, and 12. Exposure to 100 nm CuNPs resulted in the GR activities in Bacillus (field and laboratory isolates) and Alcaligenes cells; however, for 50 nm CuNPs, activities were noted only in field isolate of Bacillus. In general, the GR activities declined over time until stopped at the end of exposure time. No GR activity was noted in the laboratory isolate of Pseudomonas cells exposed to 50 & 100 nm CuNPs and Bacillus and Alcaligenes cells exposed to 50 nm CuNPs. The variations in GR induction levels among different species could be ascribed to factors other than CuNPs. It is likely that GR activities noted in cells were only partially induced by CuNP exposure. For example part of the glutathione may be derived from the medium, which in this case contains yeast extracts that are ordinarily rich in glutathione. Even though samples were washed, it is possible some glutathione
from medium was still attached to the cells and interfered with the GR activity readings. Also, levels of GR in bacteria are species-specific where a wide range of bacteria lacks glutathione and others induce it in very small concentrations (Fahey et al., 1978).

3.3.3.3. MTT Assay

The results of the MTT assay for all bacterial strains, after exposure to CuNPs for 120 min are presented in Figures 9, 10, 11, and 12. Kreb’s cycle (citric acid cycle) is the base of energy of cellular metabolism in prokaryotic and eukaryotic cells. In eukaryotes this cycle is completed in the mitochondria; however in prokaryotes it is completed in cytoplasm as bacterial cells lack mitochondria. The MTT assay still is used to assess the integrity of energy cycle in bacterial cells. In general, with increasing exposure time, the MTT activity increased in laboratory isolates of Bacillus, Alcaligenes, and Pseudomonas and decreased in field isolate of Bacillus. The levels of MTT activity in Alcaligenes cells were relatively low compared to the other tested strains. Overall, CuNPs impact the energy pathway at different levels in each type of bacteria.

3.3.4. Release of Cu$^{2+}$ from CuNPs

Concentrations of Cu$^{2+}$ released from CuNPs in aqueous solution (0.5M PBS buffer) were measured under different conditions for exposure time of 120 min. The data shows no or insignificant concentrations of ions under all experimental conditions (Table 4). Even when exposure time was extended to 200 hours, no Cu$^{2+}$ was detected (Table 5); however, a light-blue precipitate was formed at the bottom of the solution (Fig. 13). The blue precipitate is Cu(OH)$_2$,
copper hydroxide, and they are formed when Cu\(^{2+}\) ions react with OH\(^-\) ions in water. The forming of Cu(OH)\(_2\) indicates that Cu\(^{2+}\) ions were released from CuNPs in aqueous solution; however, they are released in a slow rate and react with OH\(^-\) ions in water instantly which minimize their effects on bacterial inactivation.
Table 4: Cu\textsuperscript{2+} released from 50 & 100 nm CuNPs in 0.5M PBS under different conditions

<table>
<thead>
<tr>
<th>Contact Time (min)</th>
<th>pH 7.44 Cu\textsuperscript{2+} Concentration (M)</th>
<th>pH 6.5 Cu\textsuperscript{2+} Concentration (M)</th>
<th>pH 8.5 Cu\textsuperscript{2+} Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>6.9E-07</td>
<td>1.1E-06</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>7.9E-07</td>
<td>1.0E-06</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>7.9E-07</td>
<td>1.2E-06</td>
<td>NID</td>
</tr>
<tr>
<td>90</td>
<td>3.4E-07</td>
<td>7.9E-07</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>4.1E-08</td>
<td>NID</td>
<td>4.4E-07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contact Time (min)</th>
<th>pH 7.4 Cu\textsuperscript{2+} Concentration (M)</th>
<th>pH 6.5 Cu\textsuperscript{2+} Concentration (M)</th>
<th>pH 8.5 Cu\textsuperscript{2+} Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>NID</td>
<td>NID</td>
<td>NID</td>
</tr>
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<td>40</td>
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<tr>
<td>60</td>
<td>2.4E-07</td>
<td>4.9E-07</td>
<td>1.5E-06</td>
</tr>
<tr>
<td>90</td>
<td>4.9E-07</td>
<td>6.4E-07</td>
<td>1.4E-06</td>
</tr>
<tr>
<td>120</td>
<td>2.9E-07</td>
<td>8.4E-07</td>
<td>1.6E-06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contact Time (min)</th>
<th>pH 7.4 Cu\textsuperscript{2+} Concentration (M)</th>
<th>pH 6.5 Cu\textsuperscript{2+} Concentration (M)</th>
<th>pH 8.5 Cu\textsuperscript{2+} Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>0</td>
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</tr>
<tr>
<td>20</td>
<td>NID</td>
<td>NID</td>
<td>NID</td>
</tr>
<tr>
<td>40</td>
<td>3.5E-08</td>
<td>7.9E-07</td>
<td>2.1E-06</td>
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<tr>
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<td>4.9E-07</td>
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<td>90</td>
<td>4.9E-07</td>
<td>6.4E-07</td>
<td>1.4E-06</td>
</tr>
<tr>
<td>120</td>
<td>2.9E-07</td>
<td>8.4E-07</td>
<td>1.6E-06</td>
</tr>
</tbody>
</table>

* NID: No Ions Detected
Table 5: Cu$^{2+}$ released from 50 & 100 nm CuNPs in 0.5M PBS after 200 hours exposure time at 25°C, exposed to light

<table>
<thead>
<tr>
<th>Contact Time (hours)</th>
<th>pH 7.4</th>
<th>Cu$^{2+}$ Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CuNPs (nm)</td>
<td>Control</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 13. Light-blue precipitate after mixing 50 & 100 nm CuNPs in 0.5M PBS buffer for 200 hours.

3.3.5. Complex Ions Formation

Calibration of the (Cu$^{2+}$-ISE) is performed in nano-pure water by adding known concentrations of cupric ion standard and recording the corresponding mV readings. On the other hand, the experiments were conducted in 0.5M PBS buffer; therefore, another calibration was performed in a 0.5M PBS buffer solution to confirm that PBS buffer doesn’t induce copper ions complexation, which may result in false copper ions concentration readings. Comparative results are presented in Table 6 and plotted in Figure 14. The difference between the two
standard curves was insignificant which means PBS buffer does not form copper ion complex and the reaction is similar to the one seen in nano-pure water.

Table 6: Calibration curves Cu\(^{2+}\)-ISE in water vs. PBS

<table>
<thead>
<tr>
<th>Cu(^{2+}) Concentration (M)</th>
<th>mV</th>
<th>Nano-pure water</th>
<th>0.5M PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00E-04</td>
<td>104</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>1.00E-03</td>
<td>154</td>
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</tr>
<tr>
<td>1.00E-02</td>
<td>203</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 14. Calibration curves Cu\(^{2+}\)-ISE in water vs. PBS

3.4. Conclusions

CuNPs are involved in many technological applications and their use has expanded rapidly over the last decade; therefore, it is pertinent to investigate their fate in environment and their impact on environmental bacteria, which are the foundation of all biological processes. Most of the studies regarding CuNPs have focused on NPs impact on single laboratory strains of bacteria to study their environmental impact. In this study, a comparison between laboratory and wastewater isolates of *Bacillus* is reported as far as their response to CuNPs. Also,
the study found that laboratory and wastewater isolates respond similarly to different sizes of CuNPs. However, inter-species variations in response to CuNPs were recorded. The study identifies differences in the toxicity elicitation pathway of CuNPs in a variety of bacterial species; and further molecular studies are needed to confirm these finding by gene silencing studies, which were beyond the scope of this study.
CHAPTER 4

COMPARATIVE TOXICITY OF COPPER NANOPARTICLES AGAINST LABORATORY AND ENVIRONMENTAL ISOLATES OF PATHOGENIC AND NON-PATHOGENIC E. COLI

Abstract

Nanotechnology is a scientific field that has recently expanded due to its applications in pharmaceutical and personal care products, industry and agriculture. As result of this unprecedented growth, nanoparticles (NPs) have become a significant environmental contaminant, with potential to impact various forms of life in environment. Metal nanoparticles (mNPs) exhibit unique properties such as increased chemical reactivity due to high specific surface area to volume ratios.

In this study, laboratory and environmental isolates of pathogenic and non-pathogenic E. coli were exposed to 50 & 100 nm CuNPs in continuously mixing reaction tube. Bactericidal test, scanning electron microscopy (SEM) analyses, and probable toxicity pathways assays were performed. The results indicate that under continuous mixing conditions, CuNPs are effective in inactivation of the selected E. coli isolates. After a 2-hour contact time with CuNPs, laboratory and environmental isolates of non-pathogenic E. coli showed 7.22 and 6.47 log reduction for 50CuNPs, and 6.16 and 6.68 log reduction for 100CuNPs, respectively. In case of pathogenic isolate of E. coli, after 2 hr contact time, 5.24 log and 6.54 log reduction was recorded was recorded for with 50 & 100 nm CuNPs, respectively.
Based on this data it can be concluded that released NPs into wastewater treatment plants (WWTP) effluents and biosolids may have a similar negative impact on pathogenic and non-pathogenic isolates of *E. coli* in aquatic and terrestrial environments. These results point towards the need for an in depth investigation of the impact of NPs on the biological processes and long-term effect of high load of NPs on the stability of aquatic and terrestrial ecologies.
4.1. Introduction

*Escherichia coli* is a normal inhabitant of the digestive tract of both humans and animals and most strains are generally considered harmless (Bell and Kyriakides, 1998). They are the most widely used indicators of the microbial quality of water and foods. Pathogenic strains of *E. coli* are common in various environments and animals are the most common reservoirs of these isolates. In recent years, *E. coli* O157:H7 has been a growing problem in the United States since a meat-borne outbreak in 1982 (Jay et al., 2005). Pathogenic strains are highly infectious and ingestion of even low numbers of serotype O157:H7 may cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which may result in renal failure and death (WHO, 2007).

The pathogenic and non-pathogenic isolates of *E. coli* have been shown to have different nutritional and growth requirements. In general *E. coli* has the capability of developing an increased adaptability to changing environments (Benjamin and Datta, 1995; Lin et al., 1996; Leyer et al., 1995; Buchanan et al., 1999). However, both types of isolates exhibit different stress response and toxicity elicitation pathways. For example, the *E. coli* O157:H7 has been reported to develop increased resistance (e-beam) after repeated exposure to sub-lethal doses of disinfectants.

The pathogenic and non-pathogenic isolates of *E. coli*, as pass through environmental phases, are exposed to an array of stresses, and NPs are one of the newest group of stressors. NPs possess unique physical and chemical properties, which make them useful in many industries and daily life products (Baccile,
2010; Theivasanthi & Alagar, 2011). As a result of their increased uses in industrial products, tremendous amounts of NPs expected to be released into the environment (Musee, 2011; Westerhoff et al., 2009). Since NPs fate and transport in the environment is not well studied, their toxicity impacts to the environment remain unpredictable. Therefore, NPs toxicity, recently, has gained the attention of the scientific community worldwide.

Most of mNPs, such as silver and copper NPS, are known for their antibacterial activities. Such NPs are incorporated in many daily life applications that end up discharged into the environment via effluent and biosolids of wastewater treatment processes (Aruoja et al. 2009; Kim et al., 2011). CuNPs are good candidate for studying the potential adverse effects of NP to the environment due to their existence in many industrial applications. Many previous studies have addressed the issue of NPs impact on different strains of bacteria; however, the methods applied for these studies were not consistent which may result in a false representation of NPs impact in the real environment. For example, in some studies bacterial cells were cultured on agar plates supplemented with NPs without mixing. (Brayner et al., 2006; Sondi & Salopek-Sondi, 2004).

The objective of this study was to investigate the effect of different sizes of CuNPs (50 & 100 nm) on the laboratory and environmental isolates of pathogenic and non-pathogenic strains of E. coli in continuously mixing aquatic conditions. In addition, to determine the pathways of CuNP toxicity elicitation in
the laboratory isolates of *E. coli*. This study is a continuation of a series of studies evaluating impact of CuNPs on a variety of bacterial cells.

4.2. Materials and Methods

4.2.1. Bacterial Strains

Laboratory and wastewater isolates of *E. coli* bacteria were tested in this study. Laboratory strains; *E. coli* (ATCC 25922) and *E. coli 0157* (ATCC 43888) were obtained from the American Type Culture Collection. Wastewater strain of *E. coli* was isolated from samples collected from reclaimed water ponds (Maricopa, AZ). Isolated colonies were identified by colony morphology on selective media (MacConkey agar) and verified by enzymatic tests (Colilert) and polymerase chain reaction (PCR).

4.2.2. Culture Preparation

Bacterial cultures were grown using trypticase soy broth (TSB) and harvested as previously described in chapter 3.

4.2.3. Exposure to Copper NPs

Experiments were performed in a similar manner as reported in Chapter 3.

4.2.4. Membrane Filtration

Membrane filtration was used to analyze the samples collected from different treatments as described in Chapter 3.

4.2.5. CuNP Toxicity Pathways

Toxicity pathways were assessed using LDH, GR, and MTT assays as described Chapter 3.
4.2.6. Scanning Electron Microscope

The physical impact of 50 & 100 nm CuNPs toxicity on the laboratory strain of E. coli was visualized using Scanning electron microscope (SEM) (XL30 Environmental FEG – FEI) (Hillsboro, OR). E. coli cells were washed with phosphate saline buffer (PBS). Separate aliquots of washed bacterial cells were exposed to 50 & 100 nm CuNPs. After a specified exposure time, bacterial cells were removed from reaction solution and placed on an aluminum stud and coated with gold by sputter coater (Denton Vacuum) (Moorestown, NJ). The prepared samples were observed under scanning electron microscope at 5-7 KV. As a control, non-exposed E. coli cells were also visualized using same protocol and experimental conditions. To determine whether CuNPs transported within the cell, exposed cells were washed 2 times by allowing CuNPs to settle down for 2 min and supernatant was transferred to a new tube in each wash. The washed cells were then observed under SEM.

4.3. Results and Discussion

4.3.1. Inactivation of Bacterial Cells by CuNPs

The bactericidal impacts of 50 & 100 nm CuNPs on the laboratory and wastewater isolates of pathogenic and non-pathogenic E. coli are presented in Figures 15, 16, and 17. For both laboratory and wastewater isolates of E. coli the CuNPs of 50 & 100 nm sized showed the highest level of bactericidal activity at 60 and 90 min, respectively. For pathogenic E. coli, the peak bactericidal effect of 50 & 100 nm CuNPs was noted at contact time of 60 and 120 min, respectively (Table 7). After 60-90 min of contact with 100CuNPs, a sudden decrease in the
inactivation of pathogenic and field isolates was noted (Fig. 16 & 17). Following this time frame, bactericidal activity resumed and lasted until the end of assay. For 50CuNPs, no such shift in bactericidal activity was recorded for any of the *E. coli* isolates (Fig. 15, 16, and 17).

![Fig. 15. Bactericidal Effects of 50 & 100 nm CuNPs on the Laboratory Isolate of *E. coli*. Note: Line graphs represent accumulative inactivation and bar graphs represent inactivation between time intervals.](image-url)
Fig. 16. Bactericidal Effects of 50 & 100 nm CuNPs on the field isolate of *E. coli*.
Note: Line graphs represent accumulative inactivation and column graphs represent inactivation between time intervals.

Fig. 17. Bactericidal Effects of 50 & 100 nm CuNPs on the pathogenic isolate of *E. coli*.
Note: Line graphs represent accumulative inactivation and column graphs represent inactivation between time intervals.
Table 7. Comparison of inactivation results of laboratory, wastewater, and pathogenic isolates of *E. coli*

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Highest Inactivation Rate Time (min)</th>
<th>Total Inactivation Log</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 nm</td>
<td>100 nm</td>
</tr>
<tr>
<td><em>E. coli</em> Laboratory Strain</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td><em>E. coli</em> (Wastewater Isolate)</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td><em>E. coli</em> 0157 (Pathogenic)</td>
<td>60</td>
<td>120</td>
</tr>
</tbody>
</table>

4.3.2. Toxicity Pathways

This section presents the results of LDH, GR and MTT assays performed on different bacterial strains after exposure to CuNPs. These enzymatic activities represent actions at three levels of energy cycle (Kreb’s cycle) in a normal bacterial cell (Fig. 8).

4.3.2.1. LDH Assay

The results of LDH assay for different isolates of *E. coli* after exposure to CuNPs for 120 min are presented in Figures 18, 19, and 20. The total LDH levels in the *E. coli* strains were inversely proportional to the inactivation effects. A decrease in LDH activity is associated with the increase in bacterial inactivation. Copper stress results in the expression of copper-binding proteins (CuBPs) on the surface of some bacterial species such as *Pseudomonas* and *Vibrio*; however, no such CuBPs are expressed on the surface of *E. coli*. Instead, *E. coli* uses different intracellular response strategies to handle copper stress. In addition, under environmental conditions, *E. coli* cells are reported to use multiple systems to handle copper toxicity (Rensing and Gross, 2003). For example, *E. coli* cells utilize a mechanism involving periplasmic proteins to pump out toxic copper ions.
Fig. 18. Enzymatic activities in the cells of the laboratory strain of *E. coli* after exposure to CuNPs for 120 min

Fig. 19. Enzymatic activities in the cells of the field strain of *E. coli* after exposure to CuNPs for 120 min
Fig. 20. Enzymatic activities in the cells of the pathogenic strain of *E. coli* after exposure to CuNPs for 120 min

4.3.2.2. GR Assay

The results of GR assay for all *E. coli* cells after exposure to CuNPs for 120 min are presented in Figures 18, 19, and 20. In the case of laboratory isolates of *E. coli*, GR activity levels were directly proportional to the inactivation levels. The level of GR activity increased as bacterial inactivation increased. The GR activity induced due to exposure to 50CuNPs was higher in magnitude than the GR activity due to 100CuNPs (Fig. 18). In contrast, field isolate of *E. coli* GR activity levels were inversely proportional to inactivation levels. Even though GR activity was noted at earlier stages of exposure time, the levels of activity were low—4.5 and 6.4 nmol/min/ml for 50CuNPs and 100CuNPs, respectively at 20 min, and disappeared at the end of the exposure time (120 min) (Fig. 19). No GR activity was induced due to exposure of pathogenic isolate of *E. coli* to CuNPs.

Copper is known for decreasing the cellular levels of nicotinamide adenine dinucleotide phosphate (NADP), an enzyme in Kreb cycle. The GR assay is used...
to assess the levels of NADPH (reduced form of NADP). After exposure to CuNPs, different levels of GR activity were detected in pathogenic and non-pathogenic strain of *E. coli*. Similarly, laboratory and field isolates of *E. coli* showed opposite trend of in GR activity after exposure to CuNPs. This divergence in the response of different *E. coli* strains to CuNPs suggests that these strains use different pathways to handle CuNPs toxicity.

4.3.2.3. MTT Assay

The results of MTT assay for all *E. coli* isolates after exposure to CuNPs for 120 min are presented in Figures 18, 19, and 20. In all bacterial cells, MTT activity levels were inversely proportional to the inactivation levels. MTT activity decreased as bacterial inactivation increased. The MTT assay was used to assess the effects of CuNPs on energy pathways in bacteria. The greater the difference between MTT values of control and sample, the greater the impact of CuNPs on the energy pathway of bacteria. For laboratory isolates of *E. coli* (pathogenic and non-pathogenic), MTT reduction reached up to 92-93% of control after 120 min of exposure to 50 & 100 nm CuNPs. In contrast, MTT reduction was less in the environmental isolate of *E. coli* (70% of control) (Table 8).

<table>
<thead>
<tr>
<th></th>
<th>50 nm</th>
<th>100 nm</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20 min</td>
<td>120 min</td>
</tr>
<tr>
<td><em>E. coli</em> (Laboratory Strain)</td>
<td>15</td>
<td>93</td>
</tr>
<tr>
<td><em>E. coli</em> (Wastewater Isolate)</td>
<td>61</td>
<td>70</td>
</tr>
<tr>
<td><em>E. coli</em> 0157 (Pathogenic)</td>
<td>7</td>
<td>92</td>
</tr>
</tbody>
</table>
4.3.3. *Scanning Electron Microscopy (SEM)*

A scanning electron microscope (SEM) was used to obtain optical images of CuNPs and *E. coli* cells mixture to visually investigate the exposure scenarios of single and/or cluster forms, and to confirm the size of CuNPs used in this study. The electron micrographs were analyzed for potential surface changes of exposed *E. coli* cells. Figures 21 and 22 show respectively the impacted *E. coli* cells by 50 & 100 nm CuNPs. They also clearly show that CuNPs can attach to each cell either individually as single particles or grouped in clusters form and bound together to the cell. Formation of CuNPs clusters (agglomeration) might be because of the fusing of individual CuNPs when they are free to contact each other due to attractive van der Waals forces.
Fig. 21: Electron micrographs of *E. coli* laboratory strain exposed to 50CuNPs. The CuNPs appeared as clusters and single particles attached to the cells’ surface. The diameter confirmed at 50 nm. Arrows point to the CuNPs.
Electron micrographs of *E. coli* laboratory strain exposed to 100CuNPs. The CuNPs appeared as clusters and single particles attached to the cells’ surface. The diameter confirmed at 100 nm. Arrows point to the CuNPs.

### 4.4. Conclusions

CuNPs used in this study exhibit high bactericidal activity against a variety of bacterial strains. Initiation of bactericidal activity of CuNPs is size-dependant as faster inactivation occurs when bacteria are exposed to smaller size of CuNPs. However, 50 & 100 nm CuNPs reached similar inactivation log values by the end of the exposure time.

The results of enzymatic measurements (LDH, MTT, and GR) showed that CuNPs might impact bacterial cells in different strain-specific pathways. It should be noticed that a direct proof of a specific toxicity mechanism of CuNPs on bacteria was not accomplished in this study. Variations of enzymatic activities indicate that there could be a variety of factors that affect toxicity mechanisms
and pathways. Further molecular work is necessary to determine such mechanisms and pathways; however, this work can be used as an outline for the direction that must be followed in order to specifically determine exact toxicity pathways.

Toxicity of CuNPs to bacteria may lead to harmful impact on aquatic ecosystems and possibly compromise the health of many organisms in the environment. Most of CuNPs end up in wastewater treatment plants (WWTP) in which they are either removed by WWTP processes or discharged into surface water from the effluents. The removed portion will be contained in the biomass of the sludge, which will eventually end up in the environment via agricultural land applications and runoffs.
REFERENCES


