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The Tolerance of a *Rhodococcus* Drinking Water Isolate and *Zoogloea ramigera* to Silver Nanoparticles in Biofilm and Planktonic Cultures

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The Tolerance of a *Rhodococcus* Drinking Water Isolate and *Zoogloea ramigera* to Silver Nanoparticles in Biofilm and Planktonic Cultures

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Dedication

This thesis is dedicated to my parents, who taught me that even the most daunting tasks can be accomplished with patience and dedication.

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Abstract

The Tolerance of a *Rhodococcus* Drinking Water Isolate and *Zoogloea ramigera* to Silver Nanoparticles in Biofilm and Planktonic Cultures

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Spurred by a host of beneficial uses, the global use of nanoparticles is rapidly growing. Silver nanoparticles (Ag NPs) are used widely in consumer products, medicine, and the semiconductor industry. As nanoparticles become more commonly used, the transport of nanoparticles into the environment might negatively affect microorganisms in natural and engineered systems. The effects of Ag NPs on microorganisms have primarily been studied in planktonic or free-swimming cultures, but little work has been done to look at biofilm susceptibility to Ag NPs. This thesis describes bacterial tolerance, or the ability of an organism to survive exposure to an insult, to Ag NPs. The tolerance of planktonic and biofilm cells of the common wastewater treatment bacterium *Zoogloea ramigera* and a *Rhodococcus* strain isolated from drinking water was tested. These bacteria were exposed to different concentrations of Ag NPs, ranging from 0 to 25 mg/L,

for a period of 5 hours. Results showed decreased tolerance with increasing Ag NP concentrations for both bacterial species. *Z. ramigera* biofilm cells are slightly more tolerant to Ag NPs than are planktonic cells. On the other hand, *Rhodococcus* planktonic and biofilm cells exhibit similar tolerance. However, in both cases, biofilm cells do not exhibit a striking protective effect against Ag NPs as compared to planktonic cells. This study shows that even short-term insults with Ag NPs can affect bacteria in engineered systems. A preliminary study of the shedding of free silver ions as a possible mechanism of Ag NP toxicity demonstrated that free silver ions were toxic to *Escherichia coli* in a 0.14M chloride environment. The data suggest that free silver ions can be pulled into solution from Ag NPs in chloride environments via ligand-promoted dissolution. Further work is needed to examine the antibacterial mechanism of Ag NPs against planktonic and biofilm cells to better understand how the release of nanoparticles into the environment can affect microorganisms in natural and engineered water systems.

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Abbreviations

American Type Culture Collection	:	ATCC
Biologically Active Carbon	:	BAC
Colony Forming Units	:	CFU
Criteria Maximum Concentrations	:	CMCs
Detection Limit	:	DL
Distilled Deionized	:	DDI
Dynamic Light Scattering	:	DLS
Escherichia coli	:	*E. coli*
Ethylenediaminetetraacetic acid	:	EDTA
Extracellular Polymeric Substances	:	EPS
Inductively Coupled Plasma	:	ICP
Luria-Bertani	:	LB
Minimal Davis	:	MD
Phosphate-buffered saline	:	PBS
Pseudomonas aeruginosa	:	*P. aeruginosa*
Reactive Oxygen Species	:	ROS
Silver nanoparticle	:	Ag NP
Toxicity Characteristic Leaching Procedure	:	TCLP
Transmission Electron Microscope	:	TEM
United States Environmental Protection Agency	:	USEPA
Zoogloea ramigera	:	*Z. ramigera*

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Chapter 1: Introduction

1.1 BACKGROUND

The exponential growth in the production and usage of engineered nanoparticles across various technology sectors has prompted concerns over the release of these nanoparticles into the environment and their impacts on microorganisms in natural and built systems. Nanoparticles are defined by the *American Society of Testing Materials* as particles with at least two dimensions between 1 and 100 nm (ASTM, 2006). Nanoparticles have a high surface-to-volume ratio, giving them significantly distinct physical, chemical, and biological properties as compared to their bulk counterparts (Morones et al., 2005). Nanoparticles are used for a wide range of applications in numerous industries, including electronic, biomedical, pharmaceutical, textile, cosmetic, energy, environmental, and catalytic applications (Choi et al., 2003).

Due to the promising capabilities of nanotechnology, worldwide investment in nanotechnology research has increased exponentially. In fact, the nanotechnology sector is expected to grow to $1 trillion by 2015 (Aitken et al., 2006). This exponential growth of nanotechnologies may lead to contamination of aquatic and soil environments (Brar et al., 2010). Nanoparticles can be transported into the environment through the use of commercial products containing nanoparticles and the nanoparticle manufacturing process. Indeed, nanoparticles have been found in soil, the atmosphere, surface water, and wastewater (Tolocka et al., 2004; Biswa and Wu, 2005; Johansen et al., 2008; Brar et al., 2010). These environmental pathways allow for direct exposure of humans and other organisms to nanoparticles.

Nanoparticles have been found to be toxic to humans and other organisms. One study found that exposing human lung epithelial cells to silica nanoparticles containing

iron, cobalt, manganese, and titanium dioxide causes oxidative stress (Limbach et al., 2007). Another study determined that uncoated fullerenes can serve as effective carrier molecules in aquatic organisms, providing toxic chemicals direct access to the brain (Oberdörster et al., 2005). Nanoparticles also have been shown to negatively impact freshwater, marine, and soil organisms (Klaine et al., 2008).

Silver nanoparticles (Ag NPs) are used extensively as antibacterial agents. These nanoparticles have potential antimicrobial activity towards many pathogenic microorganisms (Panyala et al., 2008). Due to their low toxicity to human cells at low concentrations, high thermal stability, and low volatility, Ag NPs are utilized in burn treatment, surgical dressings, dental materials, stainless steel coatings, textile fabrics, wastewater and water treatment technologies, sunscreen lotions, and various other applications (Duran et al., 2007). The employment of Ag NPs in antimicrobial technologies is an emerging environmental research area due to the increase in microbial resistance to metal ions and antibiotics (Gong et al., 2007). More detailed studies are needed to understand the impact of burgeoning Ag NP technologies on microorganisms in natural and built environmental systems.

The effects of Ag NPs on microorganisms have primarily been studied in planktonic, or free-swimming, cells. Studies of the impact of Ag NPs on pure cultures of *Escherichia coli* show that these nanoparticles may adhere to the cell membrane, disrupting many important cellular functions; generate reactive oxygen species (ROS); and penetrate into the cell, causing DNA damage (Morones et al., 2005). Ag NPs have been shown to form pits in the membrane, allowing the nanoparticles to penetrate into the cell (Sondi et al., 2004). The release of free silver ions also is hypothesized to be a toxicity mechanism (Navarro et al., 2008; Miao et al., 2009; Sotirious and Pratsinis, 2010). However, little work has been done to look at biofilm susceptibility to Ag NPs.

Furthermore, most research has been done with common laboratory bacteria, and there is a lack of research carried out with bacterial species found in real treatment systems. To our knowledge, this the first study investigating the tolerance of bacteria from water and wastewater treatment systems. Furthermore, only a few studies have examined the antibacterial activity of free silver ions released from Ag NPs under real treatment system conditions.

This research examined the tolerance, or the ability of an organism to survive in the presence of a negative environmental factor, of planktonic and biofilm cells to Ag NPs. Tolerance assays were carried out with bacteria from engineered water systems, including *Zoogloea ramigera*, a common wastewater bacterium that is crucial for floc formation in activated sludge, and a *Rhodococcus* strain isolated from drinking water. This research also examined the potential for bacteria to develop an increased tolerance to Ag NP insults after previous exposure to a sub-lethal concentration of Ag NPs. Finally, this research included a preliminary study of the release of free silver ions as a toxicity mechanism of Ag NPs in the presence of chloride.

An understanding of the fate, behavior, and impact of Ag NPs on planktonic and biofilm microbial communities in wastewater and drinking water treatment systems is necessary. These studies are important to address how the growing use of nanoparticles in consumer goods can affect microorganisms in engineered environments. For instance, negative impacts of Ag NPs on activated sludge bacteria in wastewater treatment might necessitate the removal of these nanoparticles upstream of the activated sludge process, and, in drinking water systems, Ag NPs can be embedded in membranes to prevent biofouling. Furthermore, there exists a potential of water and wastewater treatment bacteria to gain increased tolerance to Ag NPs after pre-exposure to sub-lethal concentrations.

1.2 OBJECTIVES

The main objective of this research was to assess the tolerance of planktonic and biofilm microorganisms from engineered water treatment systems to Ag NPs at the laboratory-scale. The experiments tested the tolerance of *Z. ramigera*, a common wastewater bacterium, and a *Rhodococcus* isolate from drinking water in planktonic and biofilm cultures. This research also investigated the ability of these bacteria to develop increased tolerance after previous exposure to sub-lethal concentrations of Ag NPs. Lastly, this research examined silver ion shedding as a toxicity mechanism of Ag NPs toward microorganisms.

1.3 THESIS OVERVIEW

This thesis research focuses on the biocidal effect of Ag NPs on microorganisms that are found in engineered water systems. The common wastewater treatment bacterium *Z. ramigera* and a *Rhodococcus* strain isolated from drinking water were used as model microorganisms to examine the toxicity of Ag NPs. The toxic effects of Ag NP on planktonic and biofilm cells were characterized with tolerance experiments. Tolerance was investigated with and without previous exposure to Ag NPs at sub-lethal concentrations.

Chapter 2 reports the literature review for this research. Chapter 3 describes the materials and methods, including the synthesis of Ag NPs and the tolerance experiments. This chapter also describes the investigation of silver ion shedding from Ag NPs as a possible toxicity mechanism. Chapter 4 presents the results and discussion. Finally, Chapter 5 summarizes the conclusions and recommendations for future work.

Chapter 2: Literature Review

2.1 INTRODUCTION

This chapter presents the literature review on the effect of nanoparticles on microorganisms in planktonic and biofilm cultures. The following sections impart brief overviews of nanoparticles, their fate and transport in the environment, and their toxicity mechanisms on microorganisms. In particular, this literature review provides a summary of the current knowledge of the effect of silver nanoparticles (Ag NPs) on microorganisms in natural and engineered systems, including their fate and transport in the environment, and their mechanisms of toxicity. Lastly, this chapter presents a general review of the tolerances of planktonic and biofilm cells to antibiotics and heavy metals, including a discussion of increased tolerance after previous exposure to these agents at sub-lethal concentrations.

2.2 NANOPARTICLES

Nanoparticles are a subgroup of nanomaterials and are defined as materials with at least two dimensions between 1 and 100 nm (ASTM, 2006). Their unique physical and chemical properties have sparked the exponential production and usage of nanoparticles in various technology sectors, including medical, water, pharmaceutical, catalytic, electronic, textile, painting, and cosmetic industries. Silver and titanium dioxide nanoparticles are used extensively due to their antibacterial and antifungal properties (Mueller and Nowack, 2008). Carbon nanotubes have promising applications in the electronics and polymer industries and the energy sector (Koehler et al., 2008).

Nanoparticles have natural and anthropogenic sources. However, concentrations of naturally occurring nanoparticles are low compared to the concentrations of

manufactured nanoparticles that can be potentially released into the environment (Klaine et al., 2008). Maynard (2007) estimates that production of engineered nanoparticles will increase from 2,000 tons in 2004 to approximately 58,000 tons between 2011 and 2020. The exponential increase in nanoparticle production has prompted concerns over the release of these materials into the environment.

Figure 2.1 shows how engineered nanoparticles can enter the environment. In the manufacturing process, leakage and discharge from production, transportation, and storage result in the release of nanoparticles into the environment (Royal Society and Royal Academy of Engineering, 2004). Nanoparticles then have a potential to contaminate soil, air, surface waters, and groundwaters (Klaine et al., 2008).

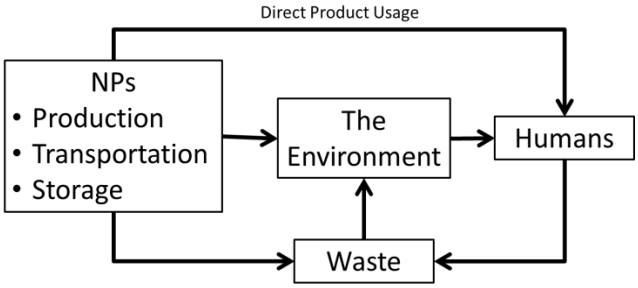


Figure 2.1: Nanoparticles (NPs) in the environment model (Royal Society and Royal Academy of Engineering, 2004)

Nanoparticles also can interact with the biota in the environment. Human exposure to nanoparticles results from drinking contaminated water, breathing contaminated air, and consuming food contaminated with nanoparticles. Humans also are exposed to nanoparticles through direct usage of consumer goods containing nanoparticles. Finally, nanoparticles are transported to wastewater treatment plants or landfills, where these particles can accumulate and/or be released back into the environment (Biswa and Wu, 2005).

The transport of nanoparticles through drinking water and wastewater treatment systems suggests the importance of understanding how these materials can affect the valuable functions of the microorganisms present in these systems. Synthetic Ag NPs can accumulate in the activated sludge of the wastewater treatment process (Benn and Westerhoff, 2008). Choi et al. (2008) observed that Ag NPs can adversely affect the growth of beneficial ammonia-oxidizing bacteria utilized in wastewater treatment. However, studies of the impacts of nanoparticles on microorganisms in natural and engineered systems and the mechanisms of nanoparticle toxicity toward microorganisms are still in their infancy.

2.2.1 Nanoparticle Toxicity

The unique properties of nanoparticles, due to their large surface area to volume ratio, make them beneficial for many technological applications. However, these properties also might cause some nanoparticles to pose a hazard to humans and the environment. Suspensions of titanium dioxide, silicon dioxide, and zinc oxides were found to be toxic to the model gram-negative bacterium, *Escherichia coli*, and gram-positive bacterium, *Bacillus subtilis* (Adams et al., 2006). This study also showed that antimicrobial activity increased with increasing concentrations of nanoparticles.

High concentrations of silver and molybdenum nanoparticles are toxic to rat liver cells, displaying cellular shrinkage and irregular shape after exposure (Hussain et al., 2005). Carbon nanotubes, zinc nanoparticles, and C_{60} fullerenes inhibit root growth and elongation in plants (Yang and Watts, 2005; Lin and Xing, 2007). C_{60} fullerenes damage cell membranes in human liver carcinoma cells through the release of reactive oxygen species (ROS) (Sayes et al., 2005). The toxicity of nanoparticles to bacteria is dependent

on the nanoparticle size, pH, surface area, surface charge, stability in aqueous systems, ionic strength of the background, and the presence of natural organic matter (Musee et al., 2011).

Nanoparticles exhibit several mechanisms of toxicity toward microorganisms. Nanoparticles can attach to the cell membrane, disrupt cellular functions at the cytoplasmic membrane, and prevent protein and nucleic acid synthesis (Morones et al., 2005). Disruption of the cell membrane may be caused by the generation of ROS from nanoparticles. These molecules can oxidize the cell membrane, causing the cell to become more permeable, which affects important respiration functions at the membrane (Klaine et al., 2008). Furthermore, ROS can disrupt protein synthesis through chemical oxidative interactions and physical electrostatic interactions (Imlay, 2003). The growing use of engineered molecules and their eventual release into the environment may cause harm to the microorganisms in natural and built systems.

2.2.2 Silver Nanoparticles

Ag NPs are widely used due to their antimicrobial properties (Maynard, 2007). These nanoparticles can be found in products used in medicine, food storage, textile coatings, and various other environmental applications (Abou El-Nour et al., 2009), including water filtration and disinfection (Jain and Pradeep, 2005).

Of the 500 tons of Ag NPs produced per year, natural waters receive the largest input of Ag NPs in the natural environment, and sewage treatment plants receive the largest flow of Ag NPs in the built environment (Mueller and Nowack, 2008). Gottschalk et al. (2009) estimated that the concentration of Ag NPs in the United States will increase from 0.7 to 2.2 μg/kg in sediments and from 2.3 to 7.4 μg/kg in sludge-amended soil

between 2008 and 2012. These simulations used a model developed by Limbach et al. (2008) showing 90.6 to 99.5% removal efficiency of Ag NPs in the wastewater treatment process. Gottschalk et al. (2009) also predicted that the average Ag NP concentration in a sewage treatment plant effluent in the United States is 21.0 ng/L.

2.2.3 Synthesis of Silver Nanoparticles

Ag NPs can be synthesized physically and chemically. The most common physical approach is an evaporation and condensation technique (Abou El-Nour et al., 2009). Ag NPs also can be synthesized with laser ablation of metallic bulk material in solution followed by coating with a surfactant (Abou El-Nour et al., 2009). Other physical approaches include ultraviolet (UV) photolysis and thermal decomposition (Isaeva et al., 2006; Tan et al., 2006; Balan et al., 2007; Lee et al., 2008).

Ag NPs are most frequently produced via chemical reduction of silver ions. Reduction agents most commonly used are borohydride, citrate, ascorbate, and elemental hydrogen (Abou El-Nour et al., 2009). Strong reductants, such as borohydride, are used to produce monodispersed small particles with narrow size distributions, while weaker reductants, such as citrate, result in a slower reduction rate and broader size distributions (Sharma et al., 2009). Ag NPs also can be produced inside a microemulsion (Krutyakov et al., 2008). Other chemical techniques are metal vapor deposition (Hozumi et al., 2006), sonochemical decomposition (Yang et al., 2008), electrochemical techniques (Richmonds and Sankaran, 2008), and microwave plasma synthesis (Chau et al., 2005).

Lastly, Ag NPs can be biologically produced via reduction of aqueous silver ions by microorganisms, such as *Bacillus licheniformis* (Kalishwaralal et al., 2008). Biosynthesis of Ag NPs is considered a green synthesis because it allows for more

environmentally friendly manufacturing, using extracts from microorganisms rather than the toxic chemicals required in some chemical and physical approaches (Sharma et al., 2009).

2.2.4 Antimicrobial Mechanisms of Silver Nanoparticles

Ag NPs have been shown to exhibit antimicrobial activity against planktonic cultures of *E. coli*, *Pseudomonas aeruginosa* (Morones et al., 2005), *Staphylococcus aureus* (Ayala-Núñez et al., 2009), *Bacillus subtilis* (Yoon et al., 2008), and *Enterococcus faecalis* (Panacek et al., 2006). Several mechanisms of Ag NP toxicity in bacteria are known. Ag NPs can adhere to the surface of the cell membrane, resulting in altered membrane properties that affect the permeability and the respiratory activity of the cell (Morones et al., 2005). Sondi and Salopek-Sondi (2004) hypothesized that Ag NPs can degrade lipopolysaccharide molecules on the membrane by producing ROS, resulting in a more permeable membrane. Ag NPs can penetrate inside bacteria and cause DNA damage due to silver's affinity to phosphorus (Morones et al., 2005).

The antimicrobial activity of Ag NPs is highly dependent on the size and shape of the nanoparticles (Rai et al., 2009). Ag NPs that are less than 10 nm are more toxic to *E. coli* and *P. aeruginosa* (Xu et al., 2006; Gopinath et al., 2006) compared to Ag NPs between 10 to 100 nm. In fact, Morones et al. (2005) showed that Ag NPs smaller than 10 nm can pass through the cell membrane and penetrate into the cell. Pal et al. (2007) showed that triangular or spherical Ag NPs exhibited antimicrobial activity at lower concentrations than did rod-shaped Ag NPs. However, it is important to note that antimicrobial activity varies with the type of bacteria, due to the differences in growth requirements and bacterial characteristics.

2.2.4.1 Adherence to Cell Membrane

One possible mechanism of Ag NP toxicity is adherence of nanoparticles to the cell membrane, thereby disrupting important cell functions and eventually leading to cell lysis and death (Morones et al., 2005). Similar to silver ions' strong affinity to phosphorus and sulfur compounds, Ag NPs are attracted to the sulfur-containing proteins at the cell membrane and thereby have the potential to denature these proteins (Feng et al., 2000). Once at the membrane, Ag NPs, similar to silver ions, have been found to interrupt the oxidative phosphorylation process in the respiratory chain, disrupt the proton-motive force across the cell membrane, and interact with thiol groups of membrane-bound enzymes (Holt and Bard, 2005). Feng et al. (2000) found the formation of a high-density region, rich with agglomerated DNA, at the center of an *E. coli* cell. This study suggests that the bacterium was aware of the presence of Ag NPs at the cell membrane and responded by concentrating its DNA at the center as a protective mechanism.

Electrostatic forces may cause surface interactions between Ag NPs and bacteria (Dror-Ehre et al., 2009). Depending on the mechanism of synthesis and background conditions, Ag NP surfaces can be either positively or negatively charged. Microorganisms with cell membranes that are negatively charged can attract the weak positive charge found in some Ag NPs (Kim et al., 2007). Furthermore, Dror-Ehre et al. (2009) found that the antimicrobial activity against bacteria is related to collision and attachment efficiencies between Ag NPs and bacteria.

2.2.4.2 Cellular Internalization

Another toxicity mechanism exhibited by Ag NPs is the ability to penetrate the cell. Sondi and Salopek-Sondi (2004) found damaged membranes in *E. coli* after

exposure to Ag NPs, resulting in increased permeability. This study hypothesized that the pits in the membrane were formed by Ag NP attachment and damage to lipopolysaccharide molecules on the membrane. Increased permeability also might result in the internalization of Ag NPs that are less than 10 nm (Morones et al., 2005). Ag NPs can penetrate into the cell and accumulate to toxic levels, eventually leading to cell death (Dror-Ehre et al., 2009). Xu et al. (2004) found that even large nanoparticles of up to 80 nm can accumulate inside *P. aeruginosa* cells.

2.2.4.3 ROS Generation

A third mechanism of toxicity is that Ag NPs can cause the formation and accumulation of intracellular ROS inside the cell membrane. These intracellular ROS can cause damage to cell components, including DNA and the cell membrane (Choi and Hu, 2008; Limbach et al., 2008; Nel et al., 2009). ROS generation can damage DNA, which can result in apoptosis, a cell suicide mechanism (Inoue et al., 2000; Bhakat et al., 2006).

2.2.4.4 Silver Ion Release

The release of free silver ions is a heavily debated mechanism of Ag NP toxicity. Silver cations have been used extensively for medical applications and are known to interfere with bacterial reproduction and growth (Ratte, 1999; Silver, 2003). Silver ions can bind to DNA and thiol groups in the cell, thereby inactivating vital components in bacterial reproduction and respiration (Holt and Bard, 2005).

There are several routes of silver internalization. Free silver ions can be taken up accidentally through the copper transport system, or other cation transport systems, such as those for sodium or potassium ions (Lee et al., 2009). Solioz and Odermatt (1995)

found silver cations being transported via the CopB-ATPase in membrane vesicles of *Enterococcus hirae*. Free silver ions and silver chloride complexes also can be transported via passive diffusion. Bury and Hogstrand (2002) found silver chloride complexes can be transported via passive diffusion across the gill cell membrane of Atlantic salmon and the yolk-sac fry of rainbow trout. Lastly, silver anion complexes, such as negatively charged silver-chloride or silver-thiosulfate complexes, can be transported via accidental anion transport in eukaryotes (Lee et al., 2009).

Navarro et al. (2008) and Miao et al. (2009) found that Ag NPs exhibit minimal toxicity against algae; however, the release of silver ions by the Ag NPs inhibits the algal cells. On the other hand, some studies show that the shedding of silver ions does not contribute to the toxicity of Ag NPs toward bacteria (Fabrega et al., 2009), fish embryos (Laban et al., 2010), and human cells (Kawata et al., 2009). Sotiriou and Pratsinis (2010) found that the dominant antibacterial activity of Ag NPs against *E. coli* is the release of silver ions when the nanoparticles are less than 10 nm. This study demonstrated that Ag NPs greater than 10 nm release less silver ions per Ag NP mass. Furthermore, Ag NPs greater than 10 nm contribute to the overall antimicrobial activity, unlike Ag NPs less than 10 nm, for which silver ions are the dominant antimicrobial agent.

The contradictory results from these studies suggest that the toxicity of Ag NPs is influenced by the method of nanoparticle synthesis, the microorganism studied, and the experimental conditions. For example, silver toxicity to microorganisms is dependent on the concentration of halide ions, such as chloride, in the surrounding environment (Silver, 2003). These halide ions can strongly bind silver ions and precipitate them out of solution (Silver, 2003). When the concentration of chloride ions is low (5 to 10 g/L NaCl), silver ions have a high affinity for bacterial cell surfaces. Moderate concentrations of chloride (10 to 20 g/L NaCl) can result in precipitation of silver chloride, and high concentrations

of chloride ions (20 to 30 g/L NaCl) result in the production of aqueous silver chloride, $AgCl_2^-$ (Gupta et al., 1998; Silver, 2003).

Lee et al. (2009) found uptake of free silver ions and silver chloride complexes decreased linearly with increasing concentrations chloride for the algae, *Chlamydomonas reinhardtii*. Bury and Hogstrand (2002) showed that silver uptake in fish increased linearly as a function of chloride concentration into the aqueous environment. The presence of 5 mM chloride resulted in a distribution of silver as 7% Ag^+, 31% $AgCl^0$, and 62% $AgCl_2^-$. Therefore, if silver ions are released from Ag NPs, the presence of chloride in the environment is an important factor to consider when investigating the toxicity of Ag NPs.

2.2.4.5 Ligand-Promoted Dissolution

We hypothesized that silver ion release from Ag NPs can be influenced by ligand-promoted dissolution in the presence of environmentally relevant aqueous inorganic ligands, such as sulfur, bromide, chloride, and iodide. The ligand-promoted dissolution model, developed by Stumm et al. (1990), shows a linear correlation between the dissolution rate of the core metal and the concentration of ligands at the surface. Although this model was developed for the weathering of minerals, ligand-promoted dissolution also can be applied to Ag NPs.

In the case of Ag NPs, as the concentration of ligands (such as chloride) increases, the dissolution rate of Ag NPs into solution as silver ions or silver complexes increases. Chappell et al. (2011) found that ethylenediaminetetraacetic acid (EDTA) promoted Ag NP dissolution to silver ions. Moreover, Wiley et al. (2004) found that 0.06 mM of chloride as NaCl and KCl promoted the dissolution of 20- to 80-nm Ag NPs to free silver

ions. In fact, Linnert et al. (1990) observed that ligands that coordinate more strongly with silver would induce oxidative dissolution of Ag NPs at much faster rates, even at very low ligand concentrations.

2.2.5 Silver Nanoparticles in Drinking Water Treatment

Ag NPs have the potential to revolutionize water treatment systems. Ag NPs are relatively inert in water, and they can function as excellent adsorbents and catalysts due to their high surface area to volume ratio (Li et al., 2010). The use of Ag NPs as a possible disinfectant in water treatment is spurred by weaknesses in current chemical disinfectants. Chlorination, chloramination, and ozonation result in the formation of toxic disinfection byproducts (Krasner et al., 2006). Interest in the use of Ag NPs for water treatment also has been sparked by the potential of drinking water pathogens to develop tolerance and resistance to current disinfectants. Shannon et al. (2008) proposed that Ag NPs can be used in drinking water treatment to avoid the problem of increased concentrations of disinfection byproducts, which is a result of raising the disinfectant dosage to combat increased pathogen resistance or tolerance.

One major drawback in the use of Ag NPs in water treatment is the danger of these extremely small particles escaping the treatment system and entering the distribution system, leading to human consumption. Another drawback is the need to separate, capture, and reuse these nanoparticles in a water treatment system; however, immobilization of these particles reduces their total surface area, and hence, their disinfection and treatment effectiveness (Li et al., 2008).

2.2.6 Silver Nanoparticles in Wastewater Treatment

Since most of the Ag NPs in consumer goods end up at wastewater treatment plants, the exponential usage of these consumer goods will result in accumulation in wastewater sludge and effluent (Benn and Westerhoff, 2008). In wastewater, Ag NPs can remain as nanoparticles (Blaser et al., 2008) or release silver ions (Benn and Westerhoff, 2008). Furthermore, Ag NPs can complex with ligands commonly found in wastewater, such as sulfate, sulfide, chloride, phosphate, and EDTA (Limbach et al., 2005). Ag NPs can agglomerate in wastewater, making them easier to remove in the treatment process (Zhang et al., 2007). Moreover, Ag NP aggregation is controlled by the pH, ionic strength, and the presence of organic material (Dunphy Guzman et al., 2006).

If Ag NPs are not removed in the wastewater treatment system, they can be released into the environment. Assuming removal efficiency of Ag NPs to be 95 percent in a typical wastewater treatment plant (typical removal of silver ions in wastewater), Benn and Westerhoff's model predicts that although the treated effluent does not exceed the United States Environmental Protection Agency (USEPA) secondary drinking water standard for silver (100 μg/L), the treated effluent does exceed the USEPA salt and freshwater Criteria Maximum Concentrations (CMCs). The model also estimates that the biosolids from wastewater treatment exceed the 5 mg/L USEPA Toxicity Characteristic Leaching Procedure (TCLP). Thus, the concentration of silver present in biosolids will prevent the use of wastewater biosolids as agricultural fertilizer (Benn and Westerhoff, 2008).

Due to their antimicrobial properties, Ag NPs can impact the biological treatment processes at wastewater treatment plants. Kim et al. (2010) found ellipsoidal-shaped Ag NPs present in sewage sludge, ranging from 5 to 20 nm. Ag NPs in a pilot wastewater plant adsorbed to wastewater biosolids and were chemically transformed into silver

sulfide nanoparticles (Kaegi et al., 2011). Choi et al. (2008) showed that Ag NPs can have detrimental effects on the beneficial nitrifying bacteria used in wastewater treatment. Ag NPs in wastewater may impact treatment performance by inhibition of bacteria in secondary treatment processes and by fouling membranes with the deposition of large aggregates (Brar et al., 2010). Additional work is needed to examine the impact of Ag NPs on microorganisms in wastewater treatment.

2.3 BIOFILMS

Biofilms are communities of microorganisms that are attached to a solid substrate and encased in a matrix of extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). Biofilms are the predominant form of bacteria in natural and built environmental systems. Some examples include dental plaque, biofilms formed on hulls of ships, and biofilms in drinking water distribution systems. The first step in the formation of biofilms starts with free-floating, or planktonic, bacteria encountering and attaching to a surface (Dunne, 2002). These attached bacteria then begin to colonize the surface and produce EPS. Biofilms can disperse to new locations through detachment of small or large clumps of cells or the dispersal of individual cells (Hall-Stoodley et al., 2004).

2.3.1 Biofilm Resistance and Tolerance

Biofilm and planktonic cells have been studied extensively for their resistance and tolerance to antibiotics and heavy metals. However, biofilm resistance and tolerance to Ag NPs have hardly been studied. Since Ag NPs possess antibacterial and metallic

properties, this literature review will include a general overview of the effects of antibiotics and heavy metals against planktonic and biofilm bacterial cells.

Tolerance is described as the ability of an organism to survive after exposure to an antimicrobial agent. Biofilms have been shown to be 10 to 1000 times more tolerant to antibiotics than are planktonic cells (Costerton et al., 1995; Ceri et al., 1999; Olson et al., 2002; Drenkard, 2003). Biofilms have been shown to have a 2- to 600-fold increase in tolerance to heavy metals as compared to planktonic cells (Teitzel and Parsek, 2003).

Resistance is defined as the ability of an organism to grow in the presence of an antimicrobial agent. Biofilms have been shown to possess greater resistance to antibiotics than do planktonic cells (McBain et al., 2003). *P. aeruginosa* biofilms have been shown to be 1000 times more resistant to antibiotics than are planktonic cells (Nickel et al., 1985). The higher resistance of biofilms to antibiotics is possibly due to slow diffusion or the prevention of antibiotic penetration into the biofilm; metabolic heterogeneity or zones of slow or non-growing cells resulting from a concentration gradient of metabolic substrates and products; an adaptive stress response by some cells of the biofilm; and generation of persister states within the biofilm (Stewart and Costerton, 2002).

2.3.1.1 Quorum Sensing

Quorum sensing cells mediate the regulation of changes in gene expression in response to fluctuations in cell-population density as reflected by the presence of signals that diffuse freely across cell membranes and between cells (Kjelleberg and Molin, 2002). Zhu and Mekalanos (2003) found that a quorum sensing system in *P. aeruginosa* was partly responsible for biofilm formation. Moreover, changes in physiological states of cells as a result of quorum sensing can alter the metal resistance and/or tolerance of the

entire biofilm population (Harrison et al., 2007). Quorum sensing has been shown to regulate the production of the extracellular matrix components that facilitate biosorption and immobilization of antimicrobial agents in the biofilm (Yarwood and Schlievert, 2003).

2.3.1.2 Metabolic Heterogeneity

Metabolite concentration gradients in biofilms result from the diffusion of nutrients, oxygen and metabolites throughout the attached bacterial community (Xu et al., 1998; Werner et al., 2004). These concentration gradients cause different microbial growth rates throughout the biofilm (Rani et al., 2007). Evans et al. (1991) showed that varying growth rates produce differences in the resistances of cells in a biofilm to antimicrobial agents. The study revealed that *P. aeruginosa* biofilm cells in the early stages of growth, exhibited a faster growth rate, and were more tolerant to ciprofloxacin than were planktonic cells. Borriello et al. (2004) found that in *P. aeruginosa* biofilms, slower-growing cells in the anoxic zone of the biofilm were inherently more tolerant to tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol, and tetracycline compared to the fast-growing cells in the aerobic zone of the biofilm. Tack et al. (1985) found that aminoglycoside antibiotics were more toxic to cells under aerobic conditions as compared to anaerobic conditions. Changes in a cell's physiology, which can be associated with the metabolic concentration gradients in a biofilm, produce cells with lower susceptibility to antimicrobial agents. In the case of heavy metal exposure, changes in the cell's physiology can decrease metabolic ROS production due to reduced metabolic activity (Harrison et al., 2007).

Cells embedded deep within the biofilm do not have easy access to nutrients, oxygen, and other metabolites as compared to those cells present at the surface of the biofilm. As a result, they are intrinsically slower-growing and less active metabolically (Walters et al., 2003). Furthermore, concentration gradients of waste metabolites in the biofilm causes changes in pH, which in turn can cause bacteria embedded within the biofilm to enter a slow or non-growing state (Zhang et al., 1995).

2.3.1.3 Slow Penetration into Biofilms

The biofilm can function as a barrier to antimicrobial agents. The EPS matrix may limit or prevent the transport of antimicrobial agents to the cells embedded in the biofilm (Mah and O'Toole, 2001). de Beer et al. (1996) found that the concentration of chlorine, a common disinfectant, inside a mixed biofilm of *Klebsiella pneumoniae* and *P. aeruginosa* was less than 20 percent of the bulk medium's concentration. Suci et al. (1994) found a slower rate of transport of an antibiotic, ciprofloxacin, within a *P. aeruginosa* biofilm as compared to a sterile surface. Furthermore, microbially produced enzymes, such as beta-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases, can inactivate certain antimicrobial agents as they slowly penetrate through a biofilm (Stewart and Costerton, 2001).

2.3.1.4 Immobilization by Biosorption

In biofilms, dead cells can serve as biosorptive sites for heavy metals, sequestering or precipitating reactive metal species, and in turn protecting the living cells within the biofilm (Harrison et al., 2007). Dead cells also can contribute to the metabolite gradients, such as pH, within a biofilm via the release of protons across dead cell

membranes (Hunter et al., 2005). Therefore, metal anions might bind more readily to dead cells. Metal ions can be sequestered and immobilized by sorbing to components of the biofilm, such as extracellular polymers, cell membranes, and cell walls (Harrison et al., 2007). Hence, the dead biomass and other components of a biofilm can help protect live cells by restricting diffusion of metals into the biofilm.

2.3.1.5 Adaptive Responses

Biofilm microorganisms exhibit the ability to adapt to environmental stress conditions, such as limited nutrients and oxygen availability, oxidative stress, presence of antimicrobial agents, and changes in pH and temperature (Stewart and Costerton, 2001). In fact, Sauer et al. (2002) found that biofilms have different gene-expression patterns than those of planktonic cells, giving biofilms better ability to adapt to negative environmental conditions and allowing them to have higher tolerance and resistance than do planktonic bacteria. Szomolay et al. (2005) hypothesize that cells embedded deep within the biofilm enter an adapted physiological state that is resistant to antimicrobial agents. This adaptive state is a result of being exposed to only low concentrations of the antimicrobial due to the antimicrobial's slow penetration into the biofilm.

2.3.1.6 Persister Cells

Persister cells are specialized cells that have entered a highly protected or even spore-like state when exposed to antimicrobial agents (Lewis, 2007). Spoering and Lewis (2001) hypothesize that biofilms may produce persister cells at a rate that is 100 to 10,000 times higher than do planktonic cells in the exponential growth phase. Therefore,

biofilms will inherently be more resistant and/or tolerant to an antimicrobial agent due to higher production of persister cells.

2.3.2 Diffusion of Nanoparticles in a Biofilm

Understanding the diffusion of nanoparticles in a biofilm is important in evaluating the impact of engineered nanoparticles on microbial communities found in natural and built environments. Peulen and Wilkinson (2011) show that Ag NPs greater than 50 nm are immobilized by extracellular components in a *Pseudomonas fluorescens* biofilm after 1.5 hours of Ag NP exposure. This study indicates that Ag NPs have a low diffusive flux into a biofilm and suggests that biofilms exhibit better protective abilities against nanoparticles than do planktonic cells. Results from these experiments also reveal that the effective pore size of the *Pseudomonas fluorescens* biofilm is about 50 nm for loose flocs but decreases to <10 nm for dense biofilms, where the biofilm density was controlled by the concentration of iron in the growth medium. In fact, nanoparticles that are greater than 50 nm demonstrated very little penetration into biofilms (Costerton et al., 1999). Therefore, better resistance and tolerance of biofilms toward antimicrobial agents can be attributed to hindered diffusion or retention in the outer biofilm layers (Costerton et al., 1999). However, further work is needed to fully understand the fate and transport of nanoparticles in biofilms.

2.4 Increased Tolerance Following Pre-Exposure

Bacteria can develop increased tolerance or resistance to antibiotics and heavy metals after a previous insult at a sub-lethal concentration. Díaz-Raviña and Baåth (1996) showed that bacterial communities in soil developed higher tolerance to Zn, Cd, Cu, and

Ni after previous exposure to concentrations greater than 2 mM. Braoudaki and Hilton (2004) found that *Salmonella enteric* and *E. coli* increased their tolerance after exposure to sub-inhibitory concentrations of antibiotics, including amoxicillin, gentamicin, and triclosan. Bacteria can develop cross-resistance or multidrug resistance, in which these microorganisms will acquire increased tolerance or resistance to one or multiple antibiotics after previous exposure to another antibiotic. Therefore, as Ag NPs are becoming more heavily used as an antimicrobial agent in many consumer products, it is important to understand the toxicity of these nanoparticles toward bacteria that have been previously exposed to sub-lethal concentrations.

Chapter 3: Materials and Methods

3.1 Introduction

This chapter details the synthesis of silver nanoparticles (Ag NPs), preparation of the growth media and spinning-disk reactor, and method for conducting the antimicrobial tolerance assays. The following sections also describe the silver ion shedding experiments used to evaluate silver ions as a possible Ag NP toxicity mechanism.

3.2 Silver Nanoparticle Synthesis

Ag NPs were synthesized by using sodium borohydride ($NaBH_4$) to reduce silver nitrate ($AgNO_3$), with mercaptosuccinic acid ($C_4H_6O_4S$) as a capping agent (Barron et al., 2009). Appropriate solutions were prepared for the Ag NP synthesis: 10.0 mg $AgNO_3$ in 20 mL of distilled deionized (DDI) water, 16.2 mg $C_4H_6O_4S$ in 20 mL of DDI water, and 15.0 mg $NaBH_4$ in 5 mL of ice cold DDI water.

Five mL of the $AgNO_3$ solution were mixed by vortexing and added to 5 mL of $C_4H_6O_4S$ in a 50-mL disposable conical tube. The resulting solution was well mixed with a Teflon-coated magnetic stir bar on a stirring plate and cooled in an ice bath. $NaBH_4$ was added drop-wise (about 10-20 drops) to the stirring solution until it turned a dark brown color, indicating formation of a suspension. Thirty mL of acetone, used as an anti-solvent to precipitate the nanoparticles before centrifugation, was added to the 10 mL suspension. The suspension was centrifuged (Beckman J2-21 centrifuge, USA) at 10,000xg (JA-10 rotor) for 15 minutes. Afterwards, the supernatant was decanted.

The Ag NPs were washed in DDI water. The Ag NPs were resuspended and vortexed in 5 mL DDI water at pH 9, adjusted by adding a small amount of diluted sodium hydroxide. Finally, the Ag NPs were suspended and dispersed uniformly in pH 9

DDI water by sonicating the suspension for 5 minutes in a bath sonicator (Branson 3510, Danbury, CT). The suspension was stored in the dark at room temperature for experimental use for up to 3 weeks. After 3 weeks, the Ag NPs began to aggregate. Ag NPs were disposed of through Environmental Health and Safety.

3.2.1 Concentration Determination

The total silver concentration of the synthesized Ag NPs was measured using an Inductively Coupled Plasma (ICP) Optical Emission Spectrometer (Varian 710-ES, Mulgrave, Victoria, Australia). The detection limit of silver for the Varian 710-ES ICP was 0.8 μg/L (Robinson and Calderon, 2010). A standard curve was prepared with known concentrations of silver, using silver nitrate. Ten mL of each Ag concentration, 0, 0.5, 1, 5, 10, 15 and 20 mg/L, were prepared in 15-mL BD Falcon™ conical tubes. These concentrations were prepared by diluting a stock solution of 1 g/L Ag. Each 10-mL dilution, including the blank, was acidified with concentrated nitric acid to achieve a 2% by volume nitric acid solution for the silver to remain dissolved in solution.

To measure the silver concentration present in the Ag NP solution, 1 mL of the Ag NP stock solution was digested with 3 mL of concentrated nitric acid. Six mL of DDI water was added to create a total solution volume of 10 mL. The particles were digested in acid for about 30 minutes before analysis. The solutions were analyzed by measuring the absorbance at wavelengths of 241, 328, and 338 nm with the ICP Optical Emission Spectrometer. Using the standard curve, the total silver concentration of the Ag NP stock solution was determined.

3.2.2 Size Determination

The size of the synthesized Ag NPs was characterized previously using Transmission Electron Microscopy (TEM), UV-vis spectrophotometry, and Dynamic Light Scattering (DLS) (Nguyen Phung-Ngoc, 2010).

3.3 MEDIA PREPARATION

The following sections describe the preparation of the media used for each step of the experimental process, bacteria culturing, and tolerance assays. For all media chosen for these tolerance experiments, there is a balance between using a medium with suitable buffering capacity and a medium that, due to its ionic strength, would increase aggregation and precipitation rates of Ag NPs.

3.3.1 Minimal Davis Medium

Minimal Davis (MD) medium for the culturing of the bacteria was made by reducing the potassium phosphate concentration of Davis media by 90 percent (Lyon et al., 2006). This medium was chosen because the lower phosphate concentrations prevented aggregation and precipitation of certain nanoparticles (Lyon et al., 2006).

The media was prepared by dissolving 0.7 g of K_2HPO_4, 0.2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate, and 0.1 g $MgSO_4 \cdot 7H_2O$ in 950 mL of DDI water. The solution was mixed well and autoclaved. After the autoclaved solution was cooled to room temperature, 50 mL of the glucose stock solution (10 g of glucose in 500 mL of DDI water) was added to give a final glucose concentration of 1 g/L in the MD medium. The glucose stock solution was mixed well and filtered through a 0.2-μm membrane to sterilize.

3.3.2 Phosphate Buffered Saline

Phosphate buffered saline (PBS) was the medium used in the Ag NPs tolerance assay. A 10X PBS solution was prepared by dissolving 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4 \cdot 7H_2O$, and 2 g KH_2PO_4 in 1L of DDI water (Ausubel et al., 2003). The pH of the solution was adjusted to 7.3. A 1X PBS solution was prepared by diluting 100 mL of the 10X PBS to 1 L using DDI water. These solutions were sterilized by autoclaving and were stored at room temperature. NaCl and KCl in the 1L 10X PBS solution were replaced with 118.738 g $NaNO_3$ for the silver ion tolerance assays.

3.3.3 Luria-Bertani Agar Plates

Luria-Bertani (LB) agar plates were utilized for plate counts. Twenty-five grams (25 g) of LB and 16 g of agar were dissolved in 1 L of DDI water. Miller's LB recipe is 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl. The solution was autoclaved and allowed to cool in a 60^{o}C water bath for 15 to 20 minutes before the plates were poured. The plates were dried overnight and stored at 4°C.

3.3.4 Nutrient Broth

Nutrient broth was used to culture the *Z. ramigera* from the freezer stock prior to inoculation of the reactor. Nutrient broth contains 5 g/L peptone, 3 g/L beef extract and 5 g/L NaCl.

3.4 SPINNING-DISK CHEMOSTAT REACTOR

The following sections describe the preparations for running the spinning-disk chemostat reactor. The spinning-disk chemostat reactor allows for the simultaneous

culture of planktonic and biofilm cells. Figure 3.1 shows a schematic of the spinning-disk chemostat reactor. The reactor system was autoclaved to sterilize the system prior to the addition of 750 mL of influent MD medium into a 2000-mL flask. A peristaltic pump pumped the medium into the chemostat reactor at a rate of 0.3 mL per minute. The chemostat reactor holds 250 mL of MD medium in which the planktonic and biofilm cells were cultured at room temperature, approximately 25˚C. Filters (0.2-µm) in the stoppers of the influent medium flask and chemostat reactor were used for sterile gas exchange. The biofilm cultures were grown using the spinning-disk.

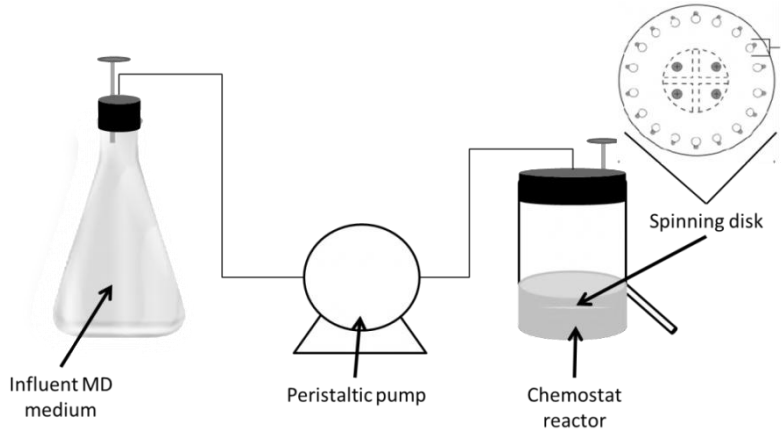


Figure 3.1: Schematic of spinning-disk reactor set-up

3.4.1 Spinning-Disk

Figure 3.2 shows a schematic of the spinning-disk. The polycarbonate spinning-disk contains 18 slots, into which 4-mm long, 3-mm wide, and 1.5-mm thick polycarbonate chips are placed and held in place by red silicone rubber tabs. The chips are the substrate onto which the biofilm cells attach. Prior to each use, the chips were

washed once with ethanol, 3 times with DDI water, and then fit into the spinning-disk. The spinning-disk was wrapped in foil, placed into a pipet tip box, and autoclaved.

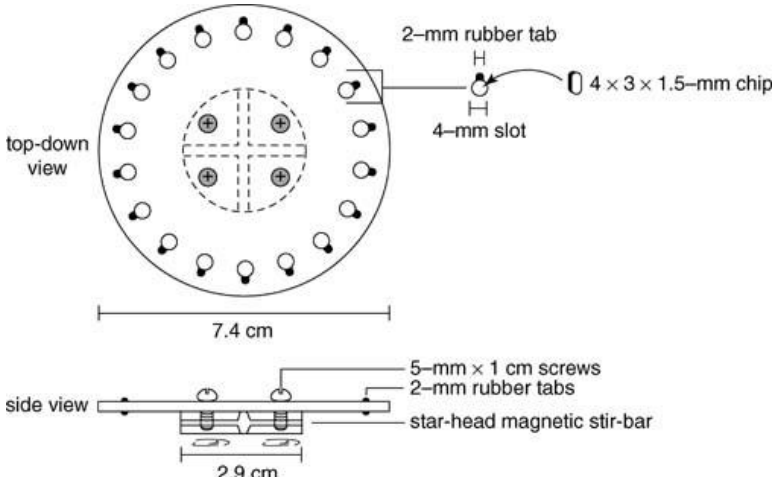


Figure 3.2: Schematic of spinning-disk

3.4.2 Biofilm and Planktonic Cell Culture Preparation

Two species of bacteria were used in these experiments: a gram-positive *Rhodococcus* isolate (strain B15) from previously operated biologically active carbon (BAC) filters treating tap water (Davidson et al., 2011) and a gram-negative *Zoogloea ramigera* strain (I-16-M) from the American Type Culture Collection (ATCC, 19623). Cells from the freezer stock of the *Rhodococcus* isolate or *Z. ramigera* were struck on LB plates to select an individual colony and to ensure there was no contamination in the freezer stock. A colony of the *Rhodococcus* isolate or *Z. ramigera* was used to inoculate a test tube with 5 mL of LB or Nutrient Broth medium, respectively, at 30°C. A 5-mL culture of the *Rhodococcus* isolate or *Z. ramigera* was allowed to grow aerobically overnight. One mL of the 5-mL culture was used to inoculate the batch reactor, which

contained 250 mL of MD medium. Thus, the reactor was first used to culture planktonic cells.

The solution in the batch reactor was well-mixed using a Teflon stir bar and allowed to grow overnight for 24 hours without turning on the pump. After 24 hours of growth, the pump was turned on, and the system became a chemostat. Planktonic cells grew in the chemostat overnight. The following day, the stir bar was aseptically removed and replaced with the spinning-disk. The spinning-disk allowed for the cultivation of biofilms on the 18 polycarbonate chips and also functioned to mix the solution. The biofilm and planktonic cells were allowed to grow for 24 hours before removal for the tolerance assays. A 1-mL sample of the planktonic culture and the spinning-disk (with biofilms attached to the chips) were taken out of the reactor.

3.5 TOLERANCE ASSAYS

Tolerance assays of planktonic and biofilm cultures were carried out for different Ag NP concentrations over an exposure period of 5 hours. A tolerance assay tests how many viable cells remain after exposure to Ag NPs. Tolerance assays were carried out in triplicate for each chemostat set-up. Each tolerance assay is comprised of duplicate planktonic and biofilm insults.

3.5.1 Silver Nanoparticle Concentrations

1X PBS was used to dilute the Ag NP stock concentration to 25000 μg/L, 12500 μg/L, 6250 μg/L, 3125 μg/L, 1563 μg/L, 781 μg/L, 391 μg/L, 195 μg/L, 97.7 μg/L, 48.8 μg/L, 24.4 μg/L, 12.2 μg/L, 6.10 μg/L, and 3.05 μg/L to test *Z. ramigera*. Ag NP concentrations used to test the *Rhodococcus* isolate were 25000 μg/L, 12500 μg/L, 6250

μg/L, 3125 μg/L, 1563 μg/L, 1000 μg/L, 781 μg/L, 500 μg/L, 391 μg/L, 250 μg/L, 125 μg/L, 62.5 μg/L, 31.25 μg/L, and 15.625 μg/L.

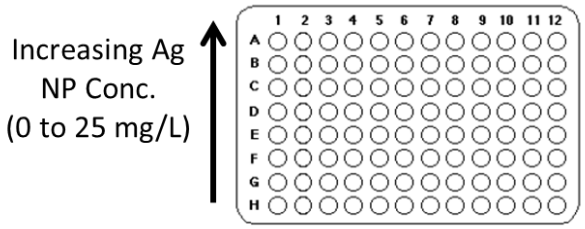


Figure 3.3: Microtiter plate used for Ag NP dilutions

The dilutions were prepared by adding 100 μL of 1X PBS into rows B through H for the first four columns of a 96-well microtiter plate (Figure 3.3). In row A, an appropriate amount of Ag NP stock suspension was diluted to achieve a concentration of 25000 μg/L or desired highest concentration for a total volume of 200 μL in the microtiter well. The wells in row A were mixed, and 100 μL from row A was transferred to the well in row B so each dilution resulted in one half of the previous concentration. These dilutions were carried out until row G. One hundred μL from row G were transferred to waste, and row H was left with 100 μL of 1X PBS only (0 μg/L Ag NPs) for the control.

3.5.2 Planktonic and Biofilm Exposure

To ensure that the same number of planktonic and biofilm cells were exposed to Ag NPs in order to have comparable results for the tolerance assays, the planktonic and biofilm cells were enumerated via plate counts. These experiments with the *Rhodococcus* isolate and *Z. ramigera* showed that the absolute number of biofilm cells on a chip and the number of planktonic cells in a 10-μL aliquot from the chemostat were the same.

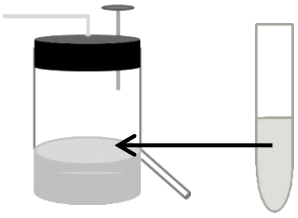
Exposure of the cells to each concentration of Ag NPs was carried out by adding 10 μL of the planktonic sample to each well in columns 1 and 2 of the microtiter plate, and adding a biofilm chip to each well in columns 4 and 5 of the microtiter plate (Figure 3.3); this resulted in duplicate tolerance assays at each Ag NP concentration.

The microtiter plate was incubated at 30°C for 5 hours. Afterwards, the chips were transferred into 1 mL of 1X PBS in eppendorf tubes, and 100 μL of planktonic cells from the microtiter plate were added to 900 μL of 1X PBS in eppendorf tubes. All the eppendorf tubes were sonicated for 10 minutes with a water bath sonicator (Branson 3510, Danbury, CT) and then briefly vortexed. This step is to remove biofilm cells from the chip and to resuspend the biofilm and planktonic cells in PBS.

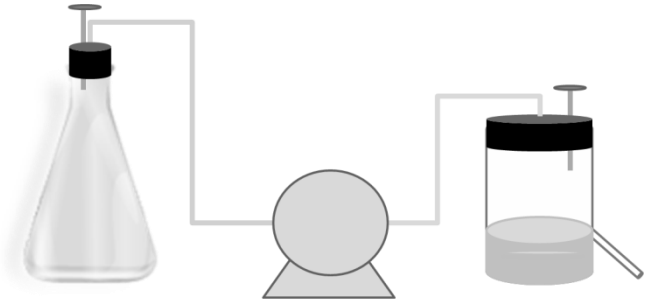
3.5.3 Dilutions and Plating

Cells from the 1-mL eppendorf tubes described above were used for plate counts. Ten μL of cells from an eppendorf tube were mixed with 90 μL of 1X PBS in a new microtiter plate, and this was serially diluted up to 10^{-5}. Ten microliter spots of each dilution (For biofilms, 10^{0} – 10^{-5} dilutions – cells from the microtiter plate; for planktonic cells, 10^{-1} – 10^{-6} dilutions – cells from the microtiter plate) were plated in duplicate on LB plates. The plates were sectioned into 6 areas so that duplicates of each dilution for a particular Ag NP concentration could be plated onto a single plate. The LB plates were incubated at 30°C for 36 hours before the colony forming units (CFU) were counted. The steps are summarized in Figure 3.4 below.

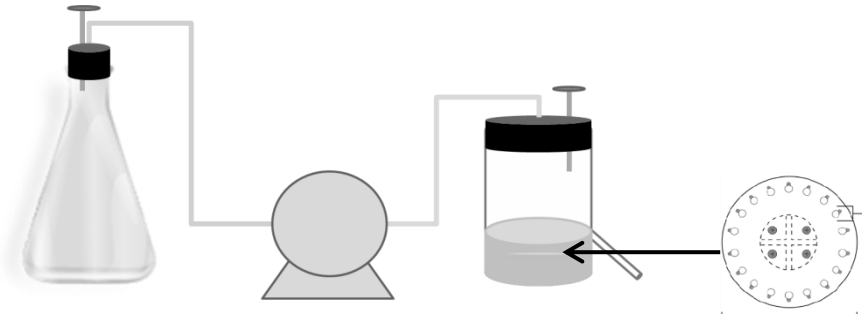
Day 1: **Inoculate chemostat reactor with 1mL bacteria from test tube**



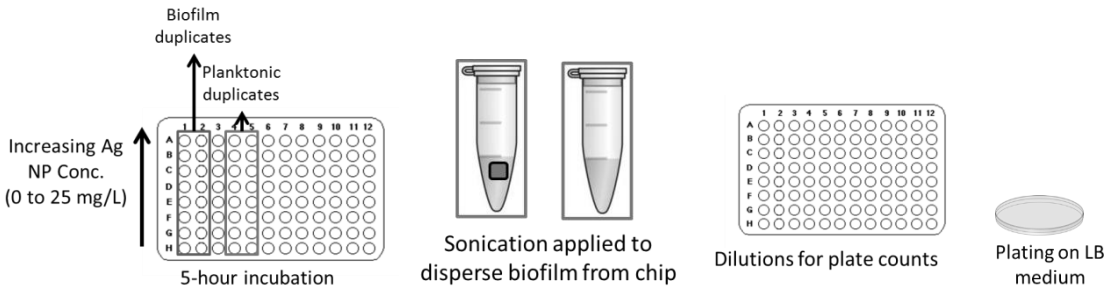
Day 2: **Start pump and chemostat reactor**



Day 3: **Insert spinning-disk**



Day 4: **Tolerance assays**



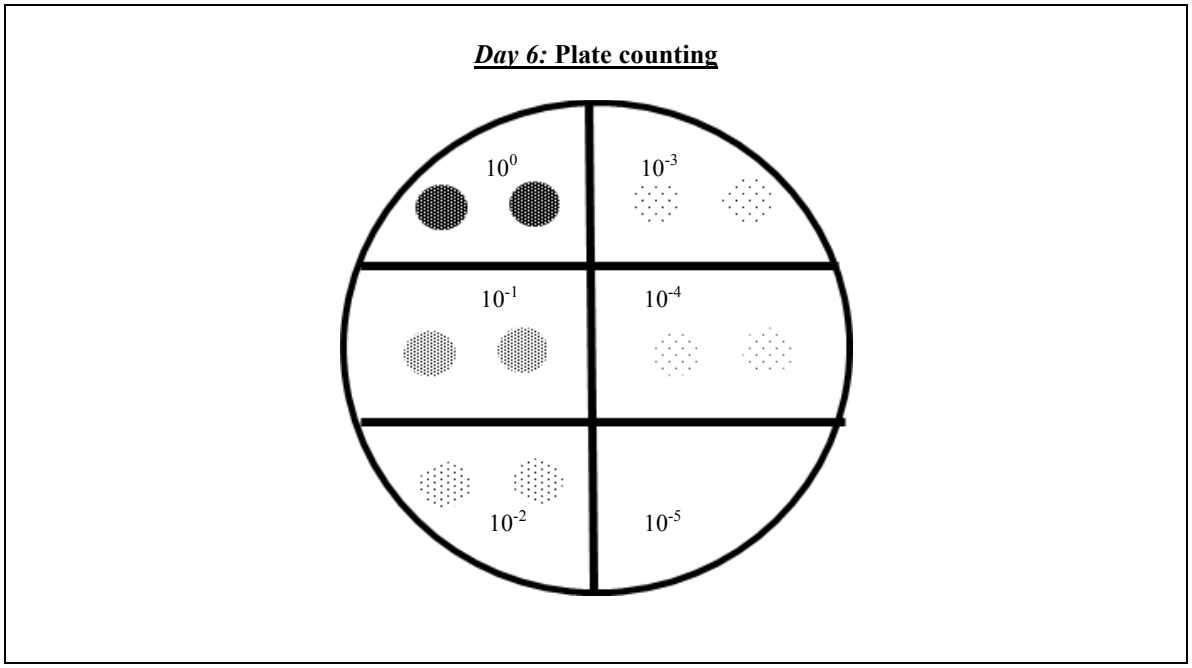


Figure 3.4: Summary of reactor set-up and tolerance assays

3.5.4 Germination with Silver Nanoparticles

To test whether the cells had increased tolerance after previous exposure to a sub-lethal Ag NP dose, planktonic cells of the *Rhodococcus* isolate and *Z. ramigera* were grown in the chemostat reactor containing a sub-lethal concentration of Ag NPs. The sub-lethal concentration was determined in the previous tolerance assays (Sections 3.5.1 - 3.5.3). In this research, a sub-lethal concentration was the concentration of Ag NPs that did not kill the cells after the 5-hour exposure period (as measured by plate counts). For the *Rhodococcus* isolate, the sub-lethal concentration was 391 μg/L. For *Z. ramigera*, the sub-lethal concentration was 3.05 μg/L. This concentration of Ag NPs was added to the influent MD medium in the spinning-disk reactor. The planktonic cells were grown in a batch reactor in the presence of the sub-lethal concentration before turning on the pump

for chemostat conditions and insertion of the spinning-disk. After growing biofilms and planktonic cells for 24 hours, the spinning-disk and an aliquot of planktonic cells were removed. The tolerance assays (Sections 3.5.1 - 3.5.3) were performed for these cells that were germinated in the presence of Ag NPs.

3.6 DOUBLING TIME DETERMINATION

The doubling time is the time required for a bacterial population to double during exponential growth. The doubling time for each bacterium was determined to ensure that the flow rate (equation 3.1) in the spinning-disk reactor was appropriate (i.e., the planktonic cells were not washing out of the reactor).

$$Q = D_{crit} \times V$$

Equation 3.1: Flow rate

where Q is the maximum flow rate allowable before the washing out of cells occurs, D_{crit} is the critical dilution rate, and V is the culture volume. The critical dilution rate (D_{crit}) at steady state is defined in equation 3.2.

$$D_{crit} = \frac{ln2}{t_d}$$

Equation 3.2: Dilution rate

where t_d is the doubling time.

To determine this doubling time, the cells were inoculated from freezer stock into sterile 250-mL flasks containing 50 mL of MD medium. The flasks were shaken at room temperature, approximately 25°C. Cell growth was measured via the absorbance at 600 nm (A600 nm) using a spectrophotometer. Measurements were taken every one or two

hours in triplicate. The natural log of the average absorbance was graphed as a function of time. The slope of the graph during the exponential growth phase was used to calculate the doubling time, using equation 3.3 (Tovey and Brouty-Boyé, 1979).

$$t_d = \frac{ln2}{\mu_{max}}$$

Equation 3.3: Doubling time

where t_d is the doubling time and μ_{max} is the maximum specific growth rate constant (slope of the plot of natural log of absorbance versus time). The doubling times for germination in the presence and absence of Ag NPs were determined and compared to see the impact of Ag NPs on bacterial growth.

3.7 SILVER ION SHEDDING EXPERIMENTS

To look at the toxicity of silver ions against *Escherichia coli* (ATCC, 25922), tolerance assays were carried out using a different PBS formula. The new PBS formula (called PBS-NO_3^-) replaced the chloride in standard PBS with nitrate, while still maintaining an ionic strength of 1.4 M. PBS-NO_3^- was used to prevent the precipitation and complexation of silver ions and chloride, so that any silver ions shed from the Ag NPs were readily available to attack the cells. Chloride forms a strong complex with silver, while nitrate does not. Therefore, in the presence of chloride, free silver ions can precipitate or complex with chloride, leaving few free silver ions available to interact with the bacteria. In the case of nitrate, most of the silver will remain as free silver ions, and still be available to interact with the bacteria. The components of standard PBS and PBS-NO_3^- are tabulated below in Table 3.1.

Table 3.1: 10X PBS using chloride or nitrate

	Standard 10X PBS	***10X PBS-NO_3^-***
Chemical	*Amount (g)*	*Amount (g)*
NaCl	80	0
KCl	2	0
$NaNO_3$	0	118.8
Na_2HPO_4 $7H_2O$	11.5	11.5
KH_2PO_4	2	2

In both PBS formulae, the pH was adjusted to 7.3. The 10X PBS was diluted to 1X PBS and autoclaved for used in the tolerance assays.

3.7.1 Bacterial Culture Preparation

A freezer stock of *E. coli* cells was streaked onto LB plates. An isolated colony was used to inoculate a test tube containing 5 mL MD medium. The planktonic cells were grown aerobically with shaking at 30°C. For these experiments with silver ions, only planktonic cells were used.

3.7.2 Organic Compound Removal

The impact of organic material in the MD medium on silver ion toxicity was tested. The planktonic cells from the liquid culture were washed twice with 1X standard PBS or PBS-NO_3^-. This was done by transferring 1mL of planktonic cells from the test tube to a sterile 1.5-mL eppendorf tube. This sample was centrifuged at 5000xg for 5 minutes. A pipette was used to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible. The cells were re-suspended and vortexed in 1000 μL of 1X standard PBS or PBS-NO_3^-. Another round of centrifugation and resuspension was carried out for the cells, and final resuspension was done in 500 μL of 1X standard PBS

or PBS-NO_3^-. Controls (without washing the cells in standard PBS or PBS-NO_3^-) also were prepared.

3.7.3 Tolerance Assays

The tolerance assays were carried out with the same method as described in Section 3.5, except that silver ions were dosed instead of Ag NPs. Also, both 1X standard PBS, containing chloride, and PBS-NO_3^- were used for the tolerance assays to look at the influence of free silver ions and aqueous silver chloride complexes on silver (dosed as Ag^+ or Ag NPs) toxicity.

Chapter 4: Results and Discussion

4.1 Introduction

The following sections present the results from the Inductively Coupled Plasma (ICP) analysis of silver nanoparticles (Ag NPs) and the Ag NP tolerance assays on a *Rhodococcus* isolate and *Zoogloea ramigera*. This chapter also presents results from the experiments investigating silver ion shedding as a possible toxicity mechanism of Ag NPs. Size characterization of the Ag NPs was done previously by the research group. Results from the size characterization studies will be used but will not be presented in depth in this thesis.

4.2 Silver Nanoparticle Size and Concentration

Table 4.1 shows a summary of Ag NP size in distilled deionized (DDI) water, Minimal Davis (MD) medium, phosphate buffered saline (PBS), and a mixture of PBS and MD medium as measured by transmission electron microscopy (TEM), UV-vis spectrophotometry, and dynamic light scattering (DLS) (Nguyen Phung-Ngoc, 2010).

Table 4.1: Size characterization of Ag NPs (Nguyen Phung-Ngoc, 2010)

Ag NPs Sample	TEM (size range, nm)	UV-vis Spectrophotometry (avg. size, nm)	DLS (avg. size, nm)
DDI water	3-10	5	9
MD medium	5-50	8	800
PBS	11-50	N/A	400
Standard PBS and MD medium	10-50	N/A	190

These data show that the range of Ag NP sizes is dependent on the medium in which the particles are suspended. They also show that the measured sizes of the NPs in solutions that might be expected to cause agglomeration (e.g., PBS) are inconsistent between measurement methods. These inconsistencies currently are being examined by the research group. We believe that the Ag NPs are agglomerating in MD medium and PBS, and an accurate size characterization is pending. Since nanoparticle size depends on background water chemistry, the extent to which each toxicity mechanism contributes to microbial inhibition or death likely also varies based on background water chemistry. Ag NP toxicity has been studied in all ranges of media, including DDI water, LB medium, and MD medium (Morones et al., 2005; Choi et al., 2008). Morones et al. (2005) found that Ag NP toxicity is highly dependent on the particle size. The dependency of Ag NP size on medium composition suggests that it is unwise to directly compare results of Ag NP toxicity from previous studies in different media.

Figure 4.1 (a) through (c) presents the standard curves for silver concentrations at wavelengths of 241, 328, and 338 nm, respectively, using the Inductively Coupled Plasma (ICP) Optical Emission Spectrometer. The abscissa is the absorbance measured at the corresponding wavelength, and the ordinate is the concentration of silver. Using the average of the total silver concentrations determined using the three standard curves, the silver concentrations of the stock Ag NPs was approximately 250 mg/L for each stock solution prepared. It is important to note here that the concentration determined using the ICP includes both the reduced silver found in the Ag NPs and any dissolved silver ions in the nanoparticle suspension.

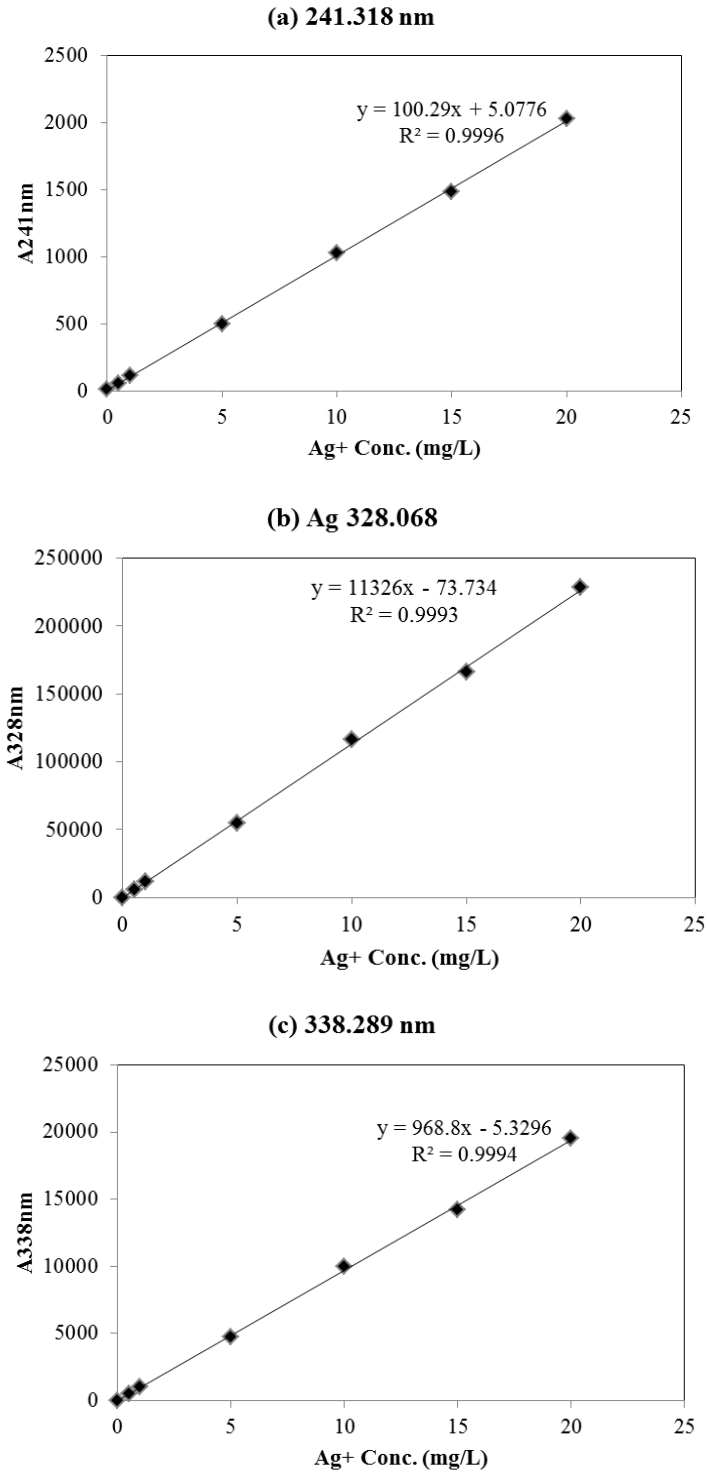


Figure 4.1: Standard curves for silver concentrations from ICP

4.3 DOUBLING TIME

This section reports the doubling times of the *Rhodococcus* isolate and *Z. ramigera* in MD medium at room temperature (~25°C) with and without the presence of a sub-lethal concentration of Ag NPs. Doublings times were measured to determine the appropriate flow rate for the spinning-disk bioreactor (Section 3.6). The sub-lethal concentrations of Ag NPs were determined from the tolerance assays (Section 3.5.4), and are defined as the highest concentration of Ag NPs at which the bacteria did not demonstrate growth inhibition. For the *Rhodococcus* isolate, the sub-lethal concentration is 391 μg/L. For *Z. ramigera*, the sub-lethal concentration is 3.05 μg/L.

4.3.1 Doubling Time of *Rhodococcus* Isolate without Ag NPs

Figure 4.2 shows a growth curve for the *Rhodococcus* isolate in the absence of Ag NPs. The slope in the exponential growth phase represents the maximum specific growth rate constant, μ_{max}. Therefore, using equation 3.3, the doubling time for the *Rhodococcus* isolate, determined from three growth curve replicates, is approximately 6.1 hours.

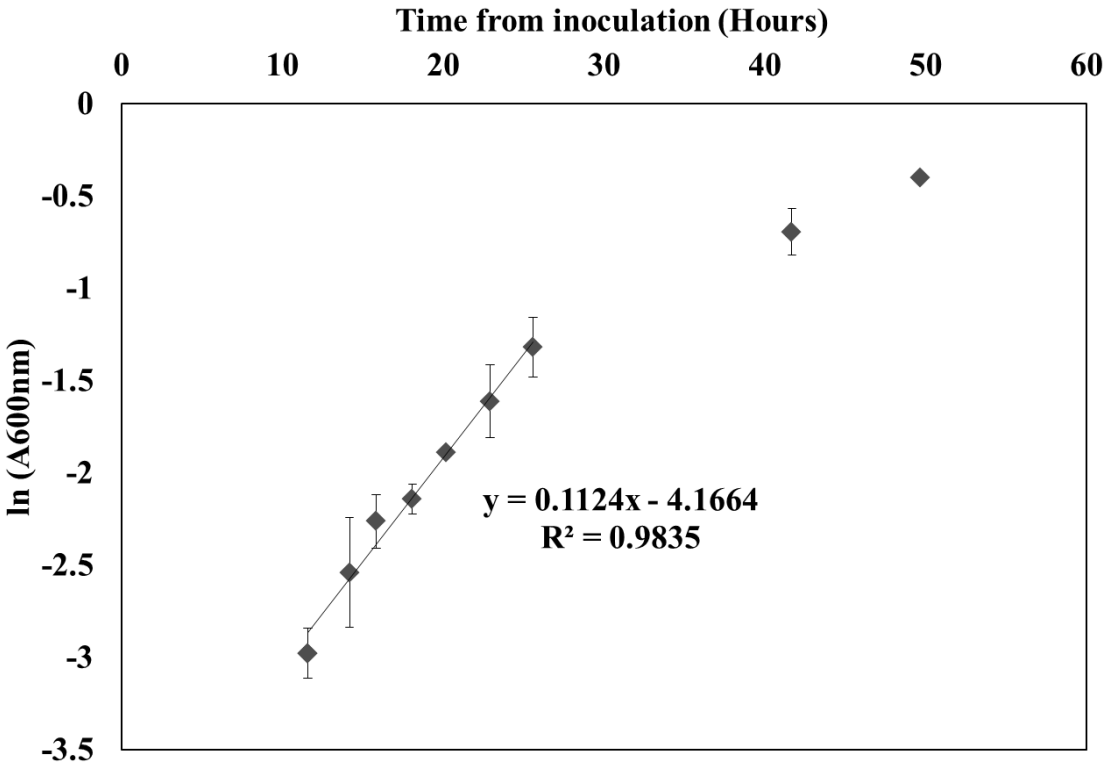


Figure 4.2: Growth curve of the *Rhodococcus* isolate in the absence of Ag NPs

4.3.2 Doubling Time of *Z. ramigera* without Ag NPs

Figure 4.3 shows the growth curve for *Z. ramigera* in the absence of Ag NPs. Using the slope in the exponential growth phase as the maximum specific growth rate constant, μ_{max}, and equation 3.3, the doubling time is approximately 4.4 hours.

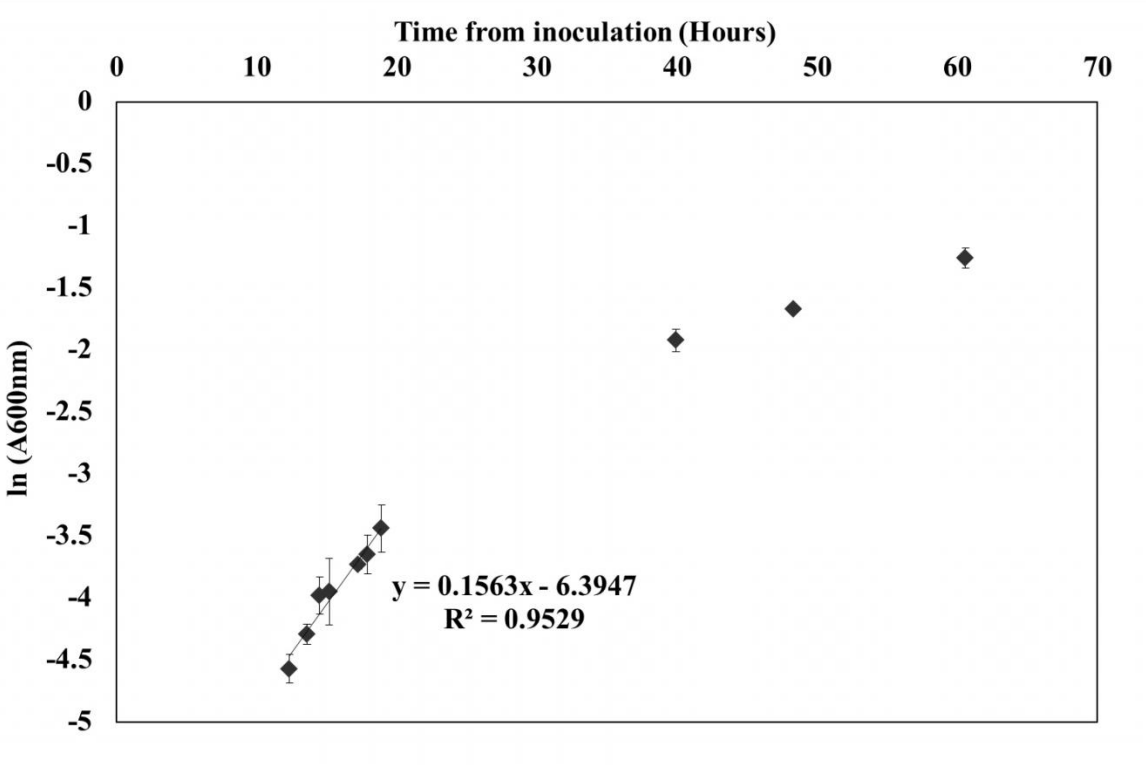


Figure 4.3: Growth curve of *Z. ramigera* in the absence of Ag NPs

4.3.3 Doubling Time of *Rhodococcus* Isolate in 391 µg/L Ag NPs

Figure 4.4 shows a growth curve for the *Rhodococcus* isolate in the presence of 391 µg/L Ag NPs. Using the slope as the maximum specific growth rate constant, μ_{max}, and equation 3.3, the doubling time for the *Rhodococcus* isolate growing in the presence of the sub-lethal concentration of Ag NPs is 4.4 hours. In the presence of Ag NPs, the *Rhodococcus* isolate's doubling time decreased by 1.7 hours compared to the isolate growing in the absence of Ag NPs. However, the lag phase increased significantly from 10 hours to about 30 hours.

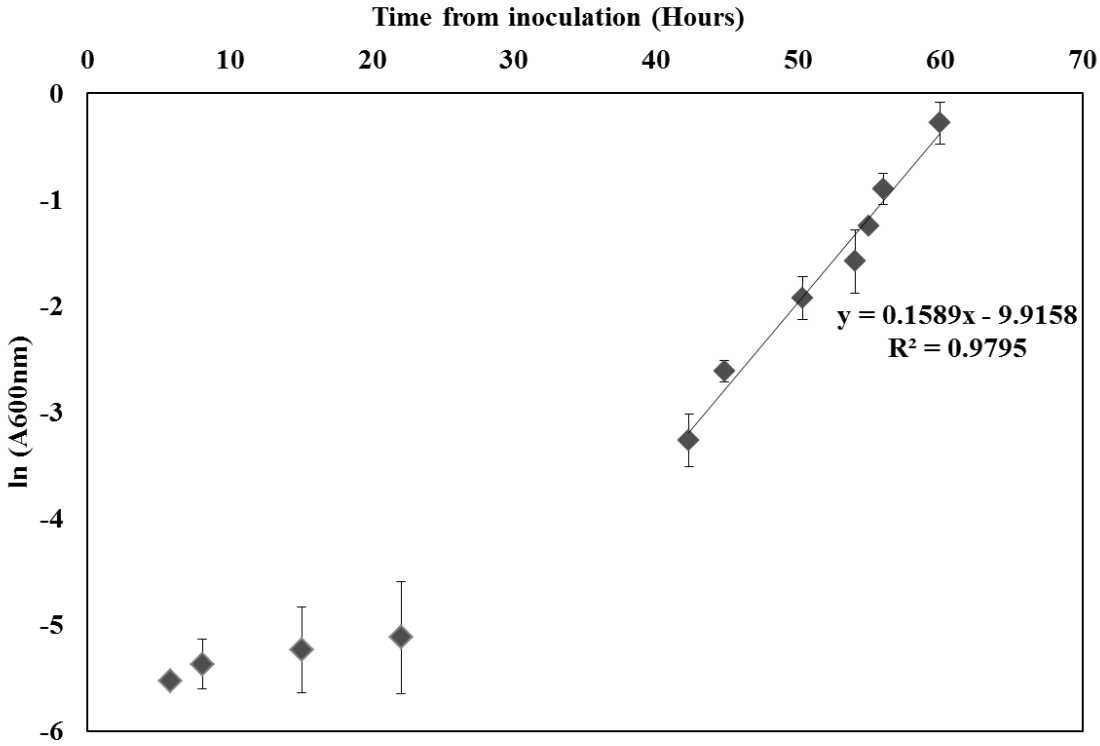


Figure 4.4: Growth curve of the *Rhodococcus* isolate in the presence of 391 µg/L Ag NPs

4.3.4 Doubling Time of *Z. ramigera* in 3.05 µg/L Ag NPs

Figure 4.5 shows the growth curve for *Z. ramigera* growing in the presence of 3.05 µg/L Ag NPs, the sub-lethal concentration. Using the slope as the maximum specific growth rate constant, μ_{max}, and equation 3.3, the doubling time is approximately 7.8 hours. The doubling time of *Z. ramigera* growing in the presence of Ag NPs increased from the doubling time in the absence of Ag NPs (4.4 hours). The lag phase for *Z. ramigera* also increased significantly from approximately 10 hours to 30 hours when grown in the presence of the sub-lethal concentration of Ag NPs.

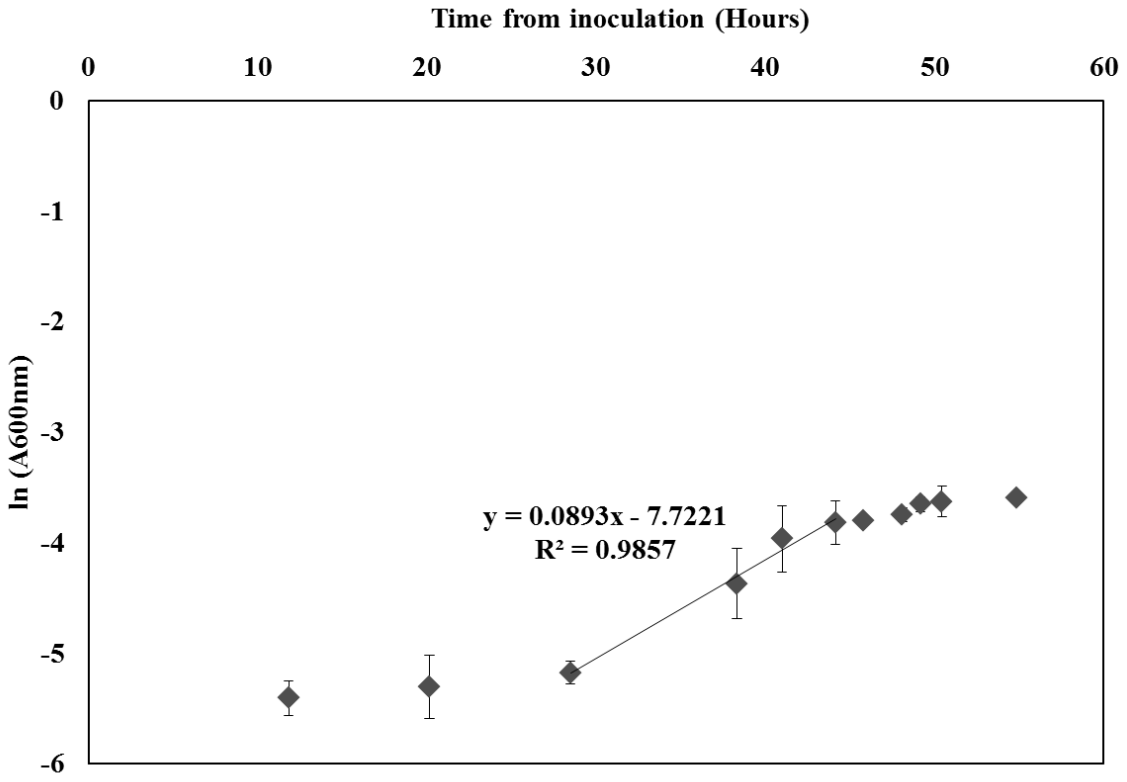


Figure 4.5: Growth curve of *Z. ramigera* in the presence of 3.05 µg/L Ag NPs

4.4 TOLERANCE ASSAYS

This section presents the data from the tolerance assays of biofilm and planktonic cultures of the *Rhodococcus* isolate and *Z. ramigera*. Each data set and the standard deviation error bars were produced by running the spinning-disk chemostat reactor twice (biological duplicates). Three tolerance assays were carried out from each biological duplicate, producing six replicates in total for each bacterium. This section is split into two parts: the first part presents tolerance data without previous exposure to a sub-lethal concentration of Ag NPs; the second part presents tolerance data with previous exposure to a sub-lethal concentration of Ag NPs.

Below, the detection limits (DL) for colony forming units (CFU) for biofilm (equation 4.1) and planktonic cells (equation 4.2) are defined. Data that fell below the DL were plotted on the graphs as half of the DL.

$$DL_{biofilms} = \frac{1\ CFU}{V_1} \times \frac{V_o}{DF} = \frac{1\ CFU}{10\ \mu L \times 10^0} \times 1000\ \mu L = 100\ CFU$$

Equation 4.1: Detection limit of biofilm CFU

$$DL_{planktonic} = \frac{1\ CFU}{V_1} \times \frac{V_o}{DF} = \frac{1\ CFU}{10\ \mu L \times (10^{-1})} \times 110\ \mu L = 110\ CFU$$

Equation 4.2: Detection limit of planktonic CFU

where 1 CFU/ V_1 represents the minimum number of CFU (1) that can be detected per volume of diluted sample that is plated (10 µL); V_o is the volume of PBS in which the cells are re-suspended after exposure to Ag NPs (1000 µL for biofilm cells and 110 µL for planktonic cells); and DF is the smallest dilution factor of plated cells The chip containing the biofilm cells is added to 1000 µL of PBS, yielding a DF of 10^0. One hundred µL of the planktonic sample is added to 900 µL of PBS, yielding a DF of 10^{-1}.

4.4.1 Tolerance of *Rhodococcus* Isolate

Figure 4.6 shows the tolerance of planktonic and biofilm cells of the *Rhodococcus* isolate to Ag NPs. The figure suggests that as the Ag NP concentration increases from 0 to 25000 μg/L, the ability of the *Rhodococcus* isolate to survive the 5-hour Ag NP insult decreases such that the viable cells remaining decrease about two orders of magnitude from 10^5 CFU to 10^3 CFU. Therefore, planktonic and biofilm cells of the *Rhodococcus* isolate decrease in tolerance with increasing concentrations of Ag NPs.

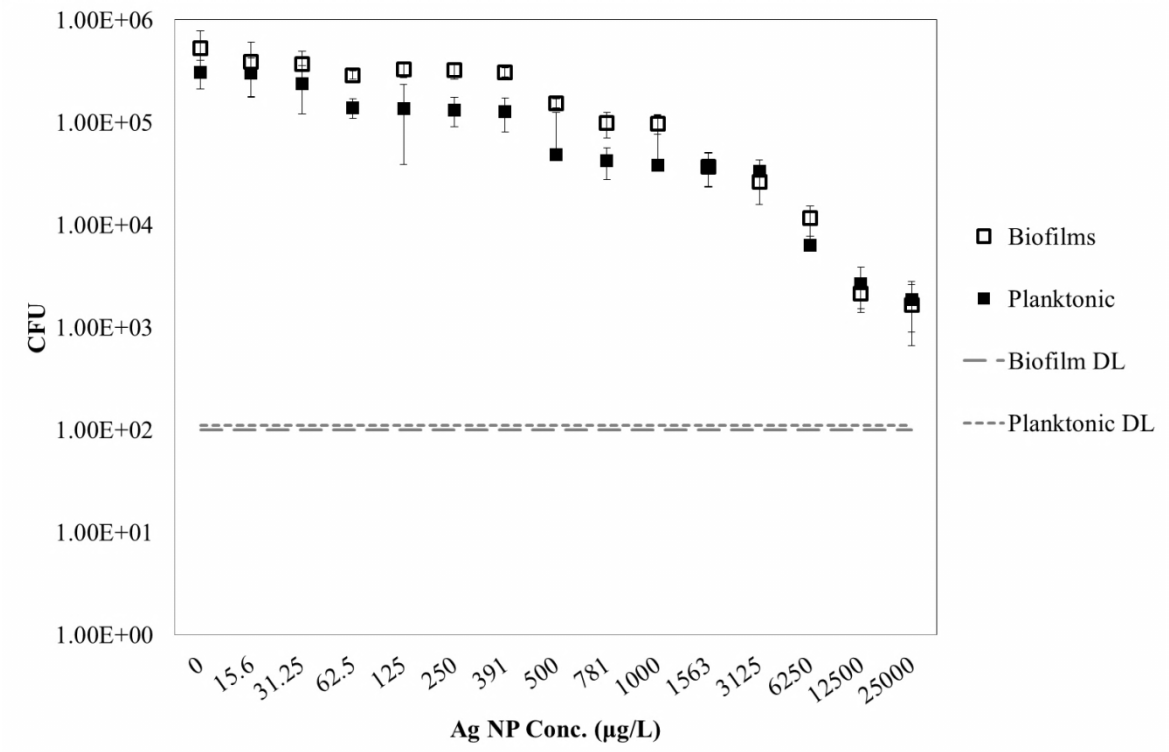


Figure 4.6: Tolerance of *Rhodococcus* isolate planktonic and biofilm cells to Ag NPs (Ag NP concentration represents the dosed concentration)

The figure indicates that the *Rhodococcus* isolate biofilm cells exhibited only a slightly higher tolerance to Ag NPs than did the planktonic cells, which is likely due to the slightly higher initial concentration of biofilm cells as compared to planktonic cells in the assay. These results show that there is no striking protective effect of biofilms as compared to planktonic cells, in contrast to what we expected based on the much higher tolerance and resistance of biofilms to antibiotics and dissolved heavy metals (Nickel et al., 1985; Stewart and Costerton, 2001). From the figure, the sub-lethal concentration, defined as the highest Ag NP concentration that did not reduce growth by the cells after the 5-hour insult with Ag NPs, was 391 μg/L.

These results from a drinking water isolate of *Rhodococcus* suggest that Ag NPs can affect bacteria in drinking water treatment systems. However, a significant decrease in tolerance for the *Rhodococcus* isolate occurred at 500 μg/L of Ag NPs, which is a concentration that is much higher than the expected background concentration in a drinking water system. The United States Environmental Protection Agency (USEPA) secondary drinking water standard for silver is 100 μg/L.

4.4.2 Tolerance of *Z. ramigera*

Figure 4.7 shows the tolerance of planktonic and biofilm cells of *Z. ramigera*, a common floc-forming bacterium in activated sludge, to Ag NPs. The figure shows that as the Ag NP concentration increases from 0 to 25000 μg/L, the ability of *Z. ramigera* to survive the 5-hour Ag NP insult decreases such that the viable cells remaining decrease by several orders of magnitude, from 10^5 CFU to below the DL. Therefore, planktonic and biofilm cells of *Z. ramigera* decrease in tolerance with increasing concentrations of Ag NPs. In fact, *Z. ramigera* reaches the DL at about 781 μg/L Ag NPs.

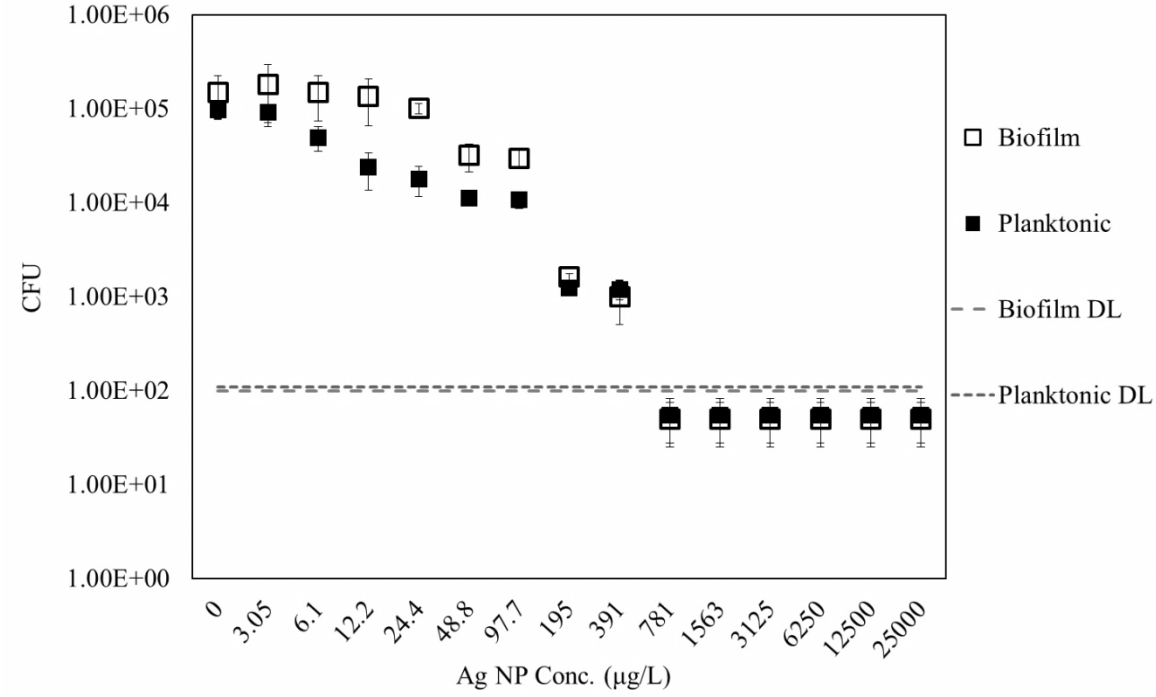


Figure 4.7: Tolerance of *Z. ramigera* planktonic and biofilm cells to Ag NPs (Ag NP concentration represents the dosed concentration)

The decrease in tolerance of *Z. ramigera* with increasing concentrations of Ag NPs suggest that Ag NPs might impact *Z. ramigera* in activated sludge and potentially affect their treatment capabilities in the activated sludge process. Hu et al. (2010) found that a shock loading experiment using a simulated activated sludge model resulted in a peak total silver, including silver ions and Ag NPs, of 0.75 mg/L. Examining this concentration (0.75 mg/L) in Figure 4.7, *Z. ramigera* is already below the DL. Furthermore, Hu et al. (2010) used this model to show that total silver, including Ag NPs, can be washed out of the activated sludge process after 25 days; however, Benn and Westerhoff (2008) reported that Ag NPs can easily accumulate in activated sludge at wastewater treatment plants. Thus, while Figure 4.7 shows the tolerance of bacteria after

only 5 hours of exposure, bacterial exposure to Ag NPs might be longer in a real system and the impact of Ag NPs on *Z. ramigera* might be more detrimental.

Figure 4.7 shows that at Ag NP concentrations between 3.05 and 195 μg/L, biofilm cells of *Z. ramigera* demonstrated a slightly higher tolerance to Ag NPs as compared to planktonic cells. However, these results show that there is no striking protective effect of biofilms as compared to planktonic cells, in contrast to what we expected based on the much higher tolerance and resistance of biofilms to antibiotics and dissolved heavy metals (Nickel et al., 1985; Stewart and Costerton, 2001). One potential explanation for these results is that *Z. ramigera* is a floc-forming bacterium. When the planktonic cells were exposed to Ag NPs in these experiments, they were present as flocs and not individual cells. Flocs and biofilms share similar properties, including the presence of extracellular polymeric substances (EPS) (Wingender and Flemmig, 1999). Moreover, Zhoa and Bai (2009) found that yeast flocs exhibited higher tolerance when subjected to ethanol shock treatment compared to single cells.

Figure 4.7 indicates that 3.05 μg/L is a sub-lethal Ag NP concentration for *Z. ramigera*. *Z. ramigera* demonstrates a decrease in tolerance at much lower Ag NP concentrations than observed with the *Rhodococcus* isolate (Section 4.4.1) and *E. coli* and *P. aeruginosa* (Nguyen Phung-Ngoc, 2010). However, the initial biomass in the tolerance assay for *Z. ramigera* in the absence of Ag NPs was 10^5 CFU, which is about 10 and 100 times lower than those in the tolerance assays for *E. coli* and *P. aeruginosa*, respectively.

4.4.3 Tolerance of *Rhodococcus* Isolate after Previous Exposure to 391 µg/L Ag NPs

Figure 4.8 shows the tolerance of planktonic and biofilm cells of the *Rhodococcus* isolate after being cultured in the spinning-disk reactor in the presence of the sub-lethal Ag NP concentration of 391 µg/L for 6 to 7 doubling times.

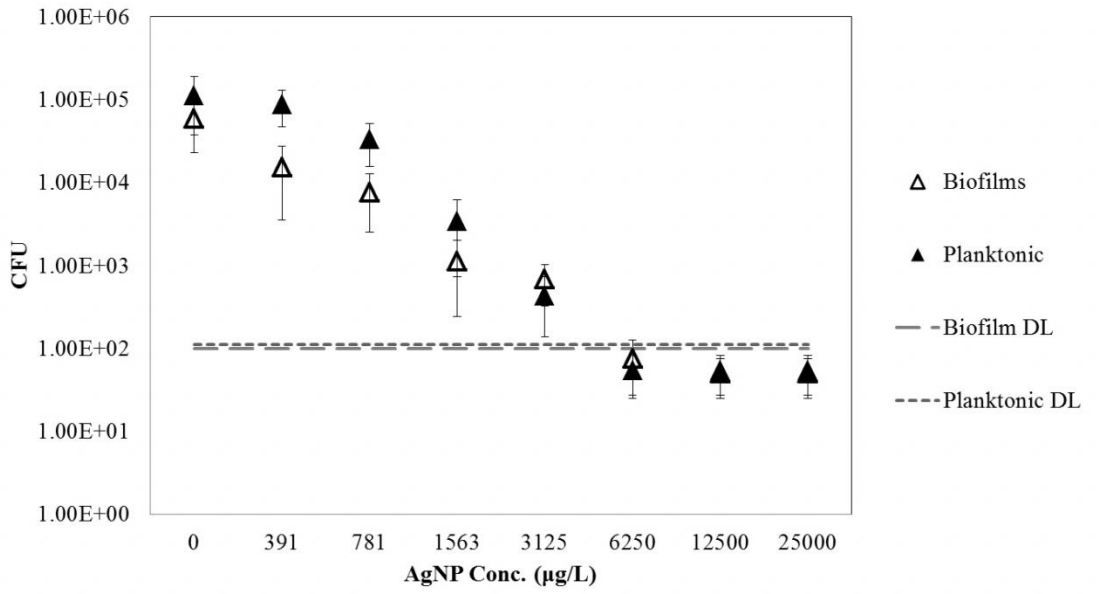


Figure 4.8: Tolerance of *Rhodococcus* isolate planktonic and biofilm cells to Ag NPs with previous exposure to sub-lethal Ag NP concentration of 391 µg/L

(Ag NP concentration represents the dosed concentration)

The figure shows that as the Ag NP concentration increases from 0 to 25000 µg/L, the ability of the pre-exposed *Rhodococcus* isolate to survive the 5-hour Ag NP insult decreases such that the viable cells remaining decrease several orders of magnitude, from 10^5 CFU to below the DL. Therefore, planktonic and biofilm cells of the *Rhodococcus* isolate decrease in tolerance with increasing concentrations of Ag NPs. As discussed next, these data differ from the tolerance without previous exposure to a sub-lethal Ag NP concentration. The planktonic cells are slightly more tolerant to Ag NPs than are biofilm cells after previous exposure to 391 µg/L of Ag NPs. Without previous

exposure to a sub-lethal Ag NP concentration, biofilm cells exhibited only a slightly higher tolerance to Ag NPs than planktonic cells. These results show that there is no striking protective effect of biofilms as compared to planktonic cells.

Figure 4.9 compares the tolerance of the *Rhodococcus* isolate planktonic cells to Ag NPs when the cells are cultured in the absence of Ag NPs to when they are cultured in the presence of a sub-lethal Ag NP concentration of 391 µg/L for 6 to 7 doubling times. The figure suggests that the tolerance of the *Rhodococcus* isolate planktonic cells to Ag NPs decreases after being cultured at the sub-lethal Ag NP concentration of 391 µg/L. Prolonged stressed caused by Ag NPs might have caused the *Rhodococcus* isolate planktonic cells to decrease their ability to survive exposure to higher concentrations of Ag NPs.

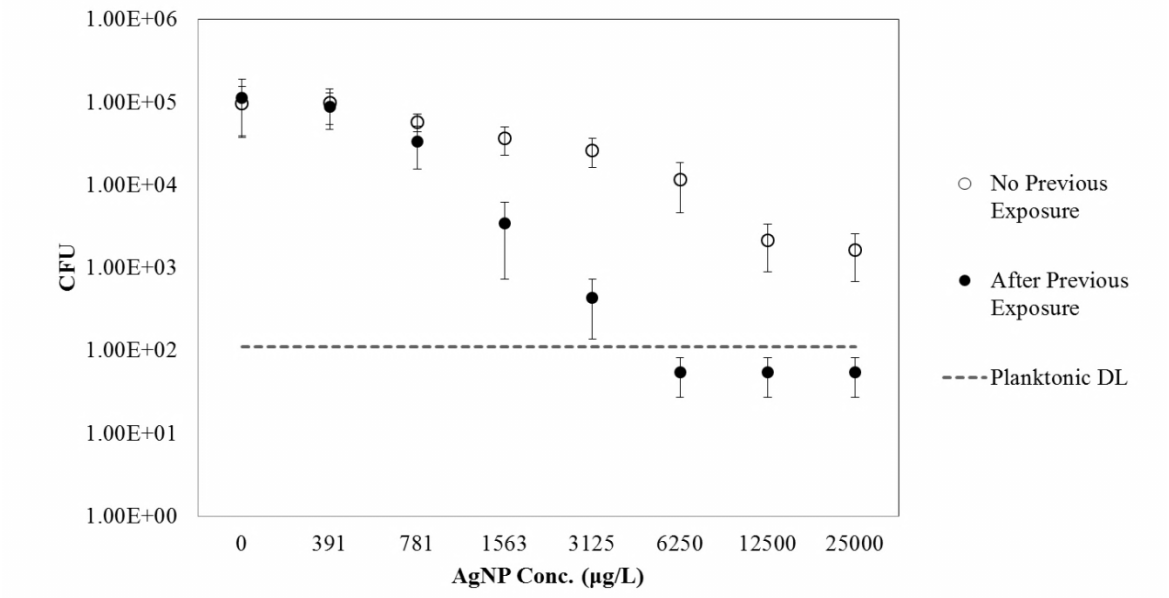


Figure 4.9: Comparison between the tolerances of *Rhodococcus* isolate planktonic cells, with and without previous exposure to a sub-lethal Ag NP concentration of 391 µg/L

(Ag NP concentration represents the dosed concentration)

Figure 4.10 compares the tolerance of the *Rhodococcus* isolate biofilm cells to Ag NPs when the cells are cultured in the absence of Ag NPs to when they are cultured in the presence of a sub-lethal Ag NP concentration of 391 µg/L for 6 to 7 doubling times.

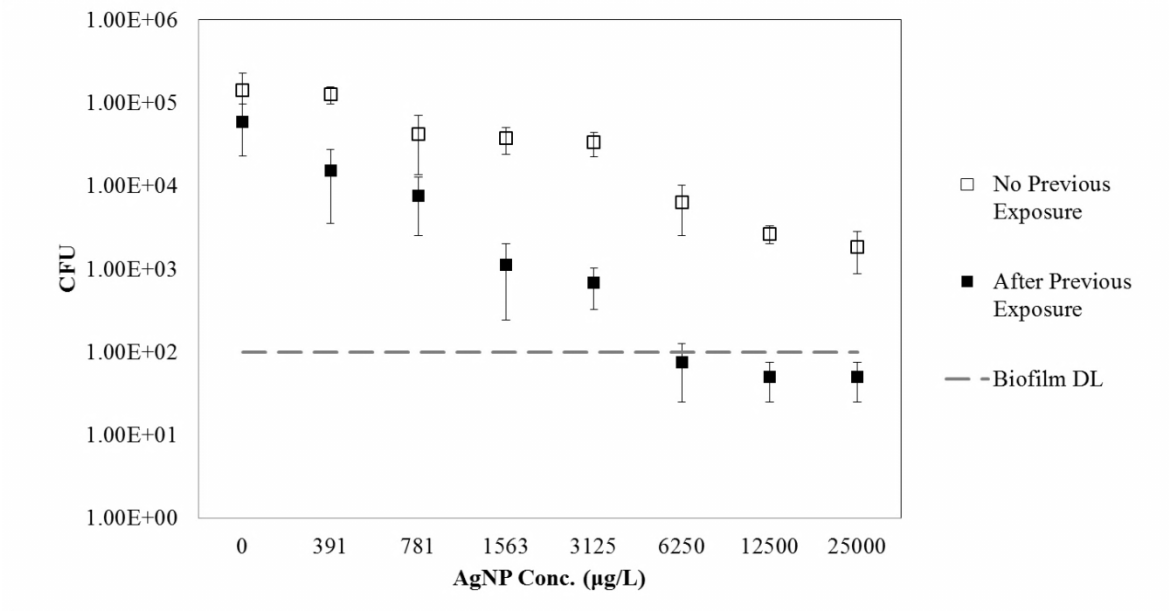


Figure 4.10: Comparison between the tolerances of *Rhodococcus* isolate biofilm cells, with and without previous exposure to a sub-lethal Ag NP concentration of 391 µg/L

(Ag NP concentration represents the dosed concentration)

The figure shows that the tolerance of the *Rhodococcus* isolate biofilm cells to Ag NPs also decreases after being cultured at the sub-lethal Ag NP concentration of 391 µg/L. Similar to the planktonic cells, prolonged stressed caused by Ag NPs might have caused the *Rhodococcus* isolate biofilm cells to decrease their ability to survive exposure to higher concentrations of Ag NPs.

4.4.4 Tolerance of *Z. ramigera* after Exposure to 3.05 µg/L Ag NPs

Figure 4.11 shows the tolerance of planktonic and biofilm cells of *Z. ramigera* after being cultured in the spinning-disk reactor in the presence of the sub-lethal Ag NP concentration of 3.05 µg/L for 6 to 7 doubling times.

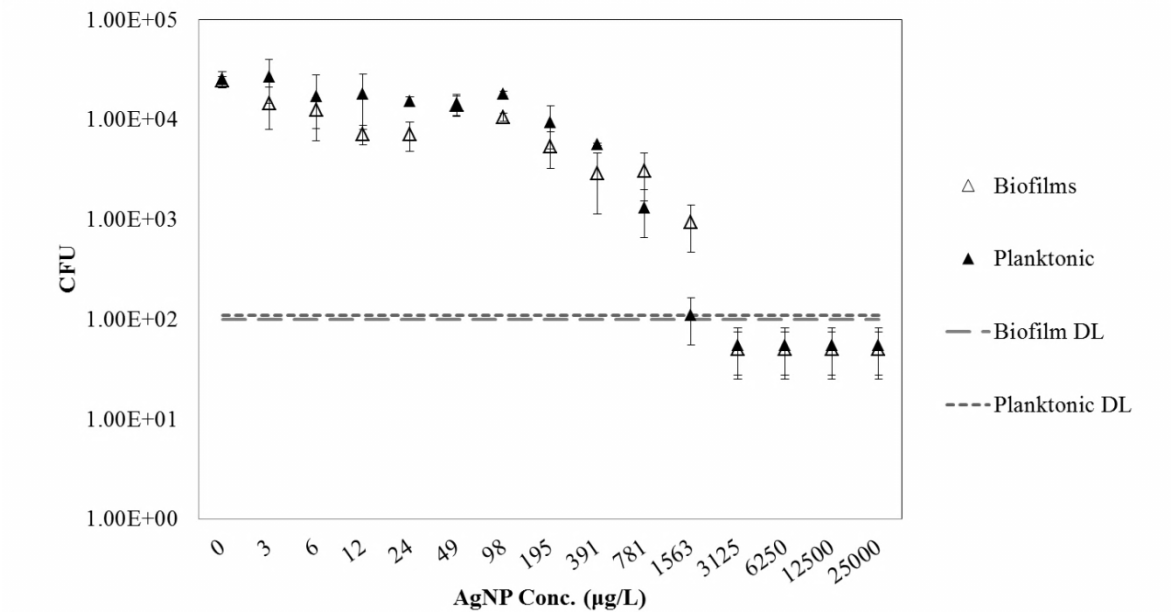


Figure 4.11: Tolerance of *Z. ramigera* planktonic and biofilm cells to Ag NPs with previous exposure to sub-lethal Ag NP concentration of 3.05 µg/L

(Ag NP concentration represents the dosed concentration)

The figure shows that as the Ag NP concentration increases from 0 to 25000 µg/L, the ability of *Z. ramigera* biofilm and planktonic cells to survive the 5-hour Ag NP insult decreases such that the viable cells remaining decrease several orders of magnitude, from 10^4 CFU to below the DL. Therefore, planktonic and biofilm cells of the *Z. ramigera* decrease in tolerance with increasing concentrations of Ag NPs.

Figure 4.12 compares the tolerance of *Z. ramigera* planktonic cells to Ag NPs when the cells are cultured in the absence of Ag NPs to when they are cultured in the

presence of a sub-lethal Ag NP concentration of 3.05 μg/L for 6 to 7 doubling times. The figure suggests that the tolerance of *Z. ramigera* planktonic cells to Ag NPs changes slightly after being cultured at the sub-lethal Ag NP concentration of 3.05 μg/L. The data also show that at Ag NP concentrations of about 195 μg/L to 781 μg/L, planktonic cells after previous exposure are slightly more tolerant to Ag NPs. Hence, after previous exposure to a sub-lethal concentration of Ag NPs, the planktonic cells are tolerant of higher Ag NP concentrations than are planktonic cells without previous exposure to Ag NPs.

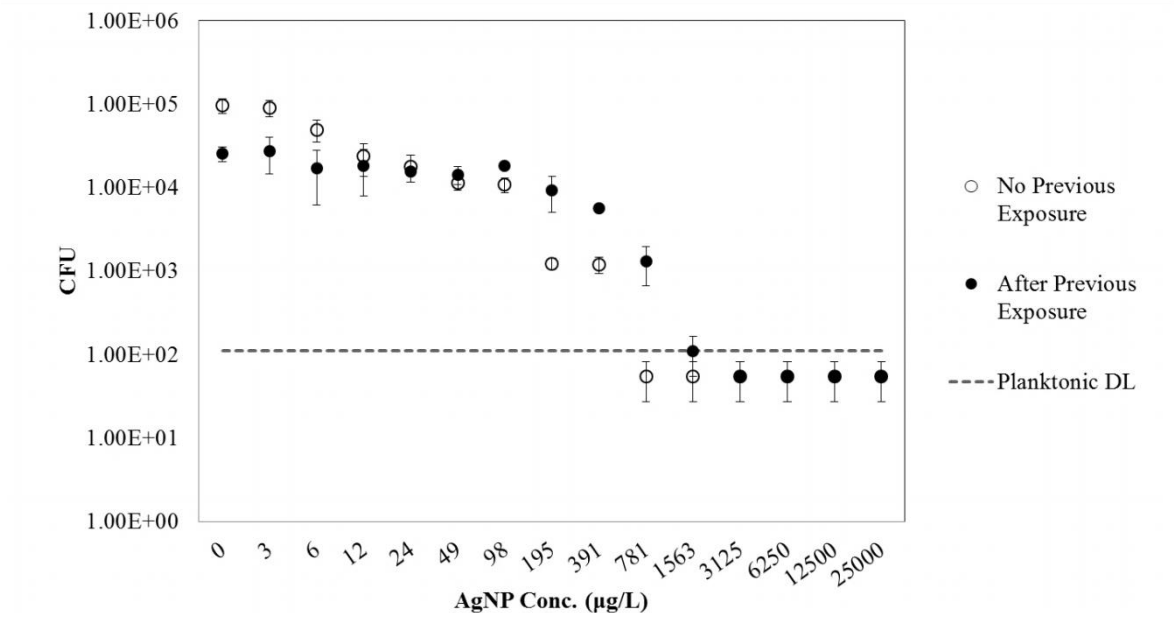


Figure 4.12: Comparison between the tolerances of *Z. ramigera* planktonic cells, with and without previous exposure to a sub-lethal Ag NP concentration of 3.05 μg/L

(Ag NP concentration represents the dosed concentration)

Figure 4.13 compares the tolerance of *Z. ramigera* biofilm cells to Ag NPs when the cells are cultured in the absence of Ag NPs to when they are cultured in the presence of a sub-lethal Ag NP concentration of 3.05 μg/L for 6 to 7 doubling times. The figure shows that the tolerance of *Z. ramigera* biofilm cells to Ag NPs also changes slightly after being cultured at the sub-lethal Ag NP concentration of 3.05 μg/L. The data show that at Ag NP concentrations of about 195 μg/L to 1563 μg/L, biofilms after previous exposure are slightly more tolerant to Ag NPs. Hence, after previous exposure to a sub-lethal concentration of Ag NPs, the biofilm cells are tolerant of higher Ag NP concentrations than are biofilm cells without previous exposure to Ag NPs.

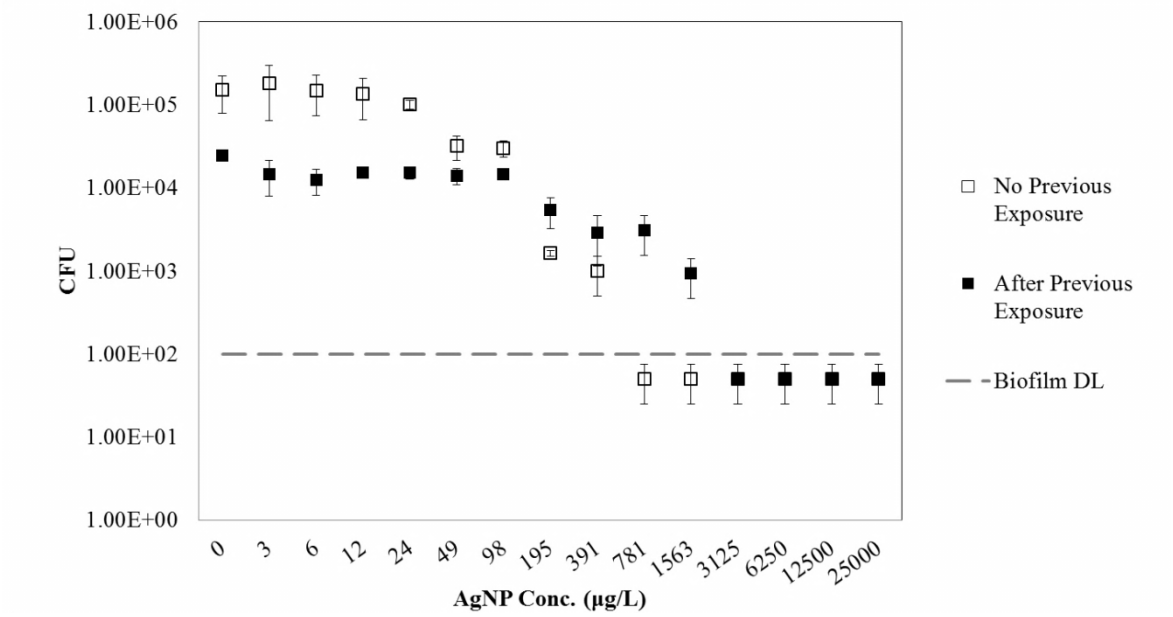


Figure 4.13: Comparison between the tolerances of *Z. ramigera* biofilm cells, with and without previous exposure to a sub-lethal Ag NP concentration of 3.05 μg/L

(Ag NP concentration represents the dosed concentration)

4.5 Effect of Silver Ion Complexes on Tolerance Assays

This section shows results from the silver ions (Ag^+) tolerance assays of biofilm and planktonic cultures of the *Rhodococcus* isolate. The first part includes an analysis of the effect of the removal of organics from the growth medium prior to the tolerance assays. This is followed by results on how the presence of different ligands, such as chloride (Cl^-) and nitrate (NO_3^-), affects the tolerance of *E. coli* planktonic cells toward silver dosed as Ag^+. Chloride forms several strong aqueous complexes with Ag^+, while nitrate does not form strong complexes with silver and is highly soluble in water. Since the formation of solid and aqueous complexes depend on the solubility product, these behaviors of silver chloride and silver nitrate can be attributed to their different solubility products, presented in Table 4.2.

Table 4.2: Silver solubility products (Weast, 1968)

Silver Compound	Solubility Product (M)
Silver Chloride (AgCl)	1.6×10^{-10} (at 25°C)
Silver Nitrate ($AgNO_3$)	0.7 (at 0°C)

Using the solubility product of silver chloride, at the highest experimental concentration of Ag^+ (1000 μg/L or 9.3×10^{-6} M) and 0.14 M chloride, the calculated ion product (1.3×10^{-6}) is greater than the solubility product. Therefore, silver chloride precipitation will be formed under these conditions. When the concentration of Ag^+ is less than 0.12 μg/L, the ion product will be less than the solubility product, and hence, no precipitation will be formed. For silver nitrate, the concentration of Ag^+ must exceed 46.4 mg/L for a silver nitrate precipitate to form.

Table 4.3 shows the stability constants of silver chloride species.

Table 4.3: Silver chloride species stability constants (Morel and Hering, 1993)

Silver Compound	Stability Constants (log β)
$AgCl^0$	3.3
$AgCl_2^-$	5.3
$AgCl_3^{2-}$	6.4

Using the stability constants and solubility product of silver chloride, the distribution of silver chloride species can be calculated as a function of chloride concentration. This equilibrium distribution is presented in Figure 4.14, which shows the calculated distribution of silver chloride complexes as a function of the chloride concentration (pCl or – log [Cl^-]). The figure shows that at the studied concentration of 0.14 M chloride or pCl of 0.85 in 1X PBS, silver species exist as 0.009% free silver ions (Ag^+), 2.5% silver chloride ($AgCl^0$), 35.3% $AgCl_2^-$, and 62.2% $AgCl_3^{2-}$. Free silver ions are the dominant species only greater than a pCl of 3.5 ($< 3.0 \times 10^{-4}$ M).

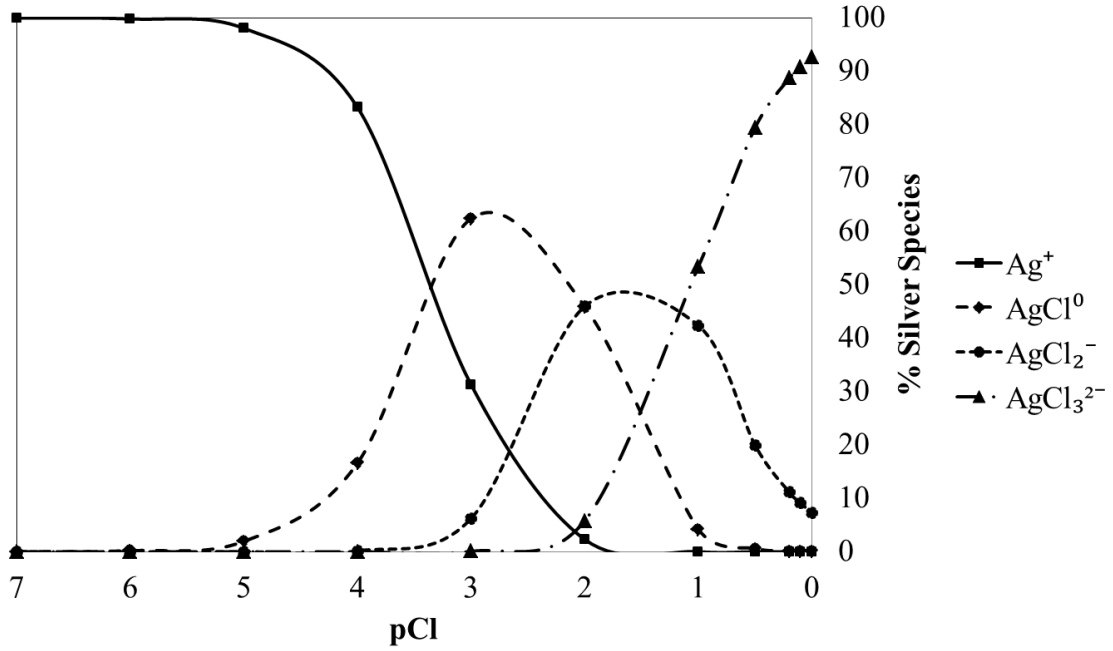


Figure 4.14: Silver species distribution as a function of chloride concentration

In the case of nitrate, most of the Ag^+ will still be present as Ag^+, as indicated by the high solubility product, and will remain bioavailable. The following sections include results that show how the presence of chloride and nitrate affects the tolerance of *E. coli* planktonic cells to Ag NP.

4.5.1 *Rhodococcus* Isolate Tolerance to Silver Ions

Figure 4.15 shows the tolerance assay of biofilm and planktonic cells of the *Rhodococcus* isolate to Ag^+ using standard IX PBS, which contains chloride. Biofilm and planktonic cells of the *Rhodococcus* isolate appeared highly tolerant of the Ag^+ dose, with the number of biofilm and planktonic cells remaining unchanged after a 5-hour insult with 0 to 1 mg/L Ag^+. These results indicate that Ag^+ does not have any effect on biofilm and planktonic cultures under the experimental conditions. However, Ag^+ is

known to be toxic to bacteria (Silver, 2003). These results led to several hypotheses. The first one is that the organic compounds from the culturing medium may affect the silver ion toxicity. Bury et al. (2009) found that increasing organic matter concentration decreased silver toxicity in rainbow trout and fathead minnows. Second, the concentration of chloride in the 1X PBS in which the tolerance assays were carried out may have complexed strongly with Ag^{+}, making them unavailable to interact with the bacteria. These hypotheses were tested in more detail with *E. coli* planktonic cells.

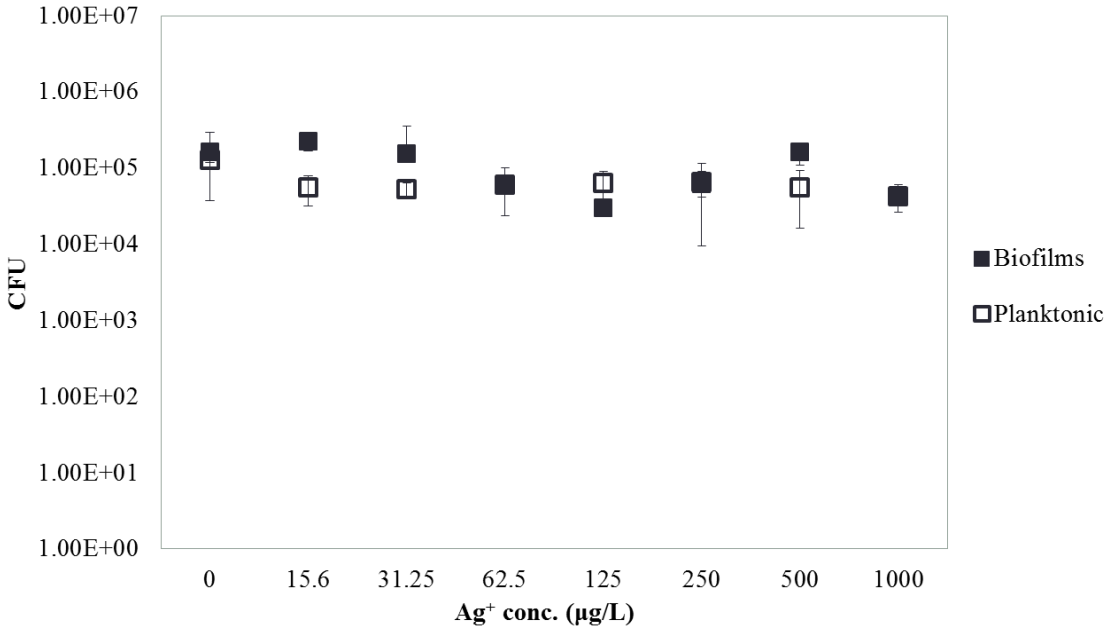


Figure 4.15: Tolerance of *Rhodococcus* isolate planktonic and biofilm cells to Ag^{+} in standard PBS, containing chloride

(Ag^{+} concentration represents the dosed concentration)

These results indicate that the effect of silver is highly dependent on the water chemistry of the medium, in which the experiments were run (e.g., formation of strong

and weak complexes). These results underline a need to test how the water chemistry of the medium can change the toxicity of silver (e.g., effect of organics and presence of ligands), and provide an idea on how to test Ag^+ shedding from Ag NPs as a toxicity mechanism.

4.5.2 Effect of Organics Removal

Figures 4.16 and 4.17 show the effect of the removal of organic material from the Minimal Davis (MD) medium on the tolerance of *E. coli* to Ag^+ in standard PBS and PBS-NO_3^-), respectively. These results indicate that the removal of organics does not significantly affect the silver ion tolerance assays.

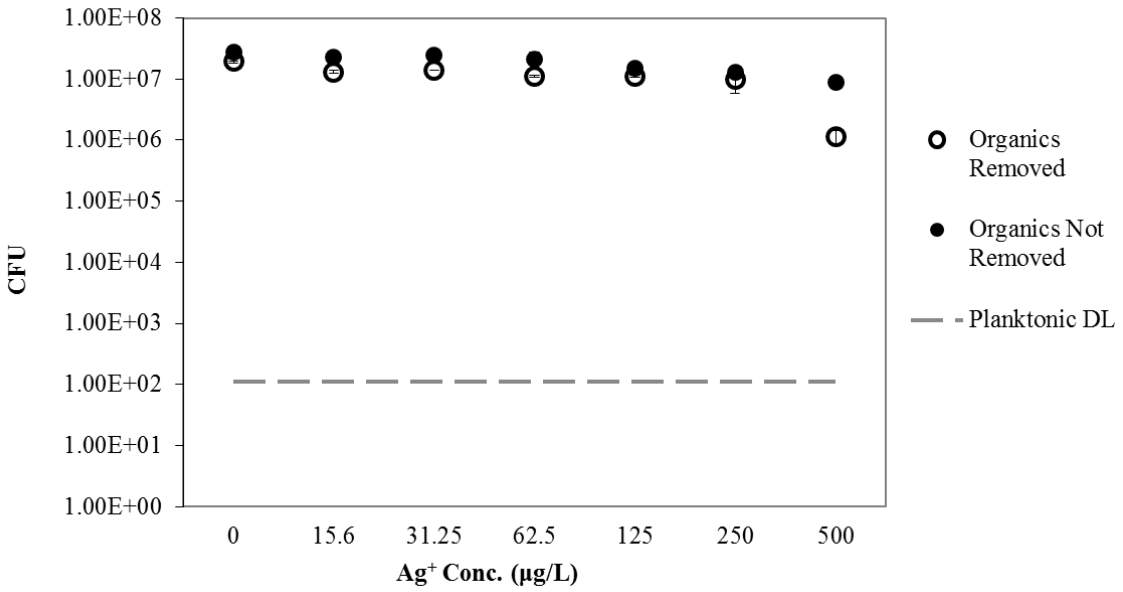


Figure 4.16: Effect of organics removal on the tolerance of planktonic *E. coli* cells to Ag^+ in standard PBS, containing chloride

(Ag^+ concentration represents the dosed concentration)

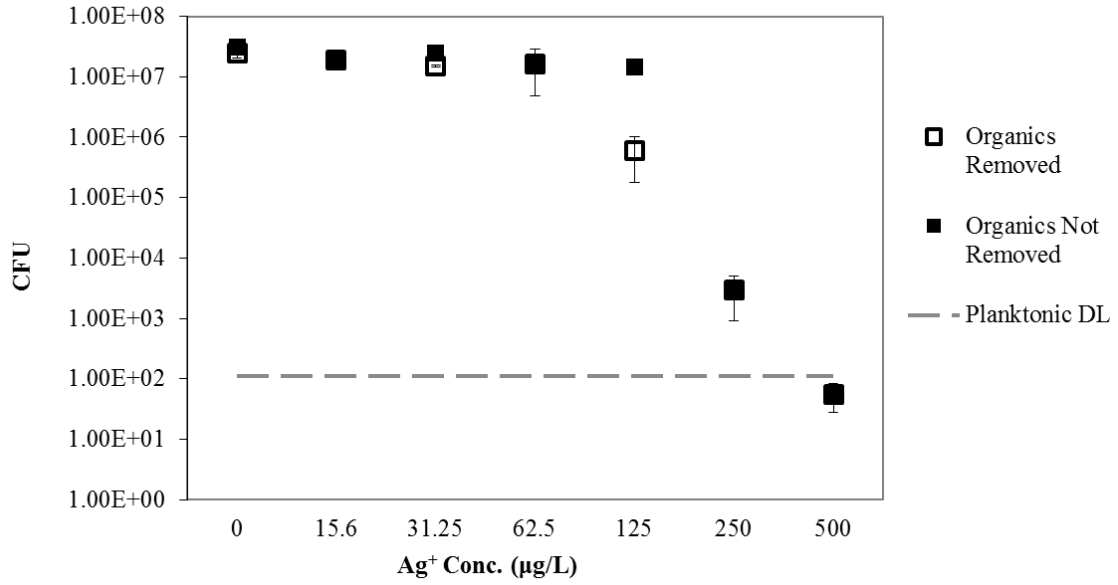


Figure 4.17: Effect of organics removal on the tolerance of planktonic *E. coli* cells to Ag^+ in PBS-NO_3^-

(Ag^+ concentration represents the dosed concentration)

Please note that the open squares are behind the filled squares for several data points, such that they cannot be seen

Similar results with the effect of organics removal are obtained in the Ag NP tolerance assays. Figures 4.18 and 4.19 show the effect of the removal of organics from the MD medium on the tolerance of *E. coli* to Ag NPs in standard PBS and PBS-NO_3^-, respectively. Once again, the results show that the removal of organics does not significantly affect the Ag NP tolerance assays.

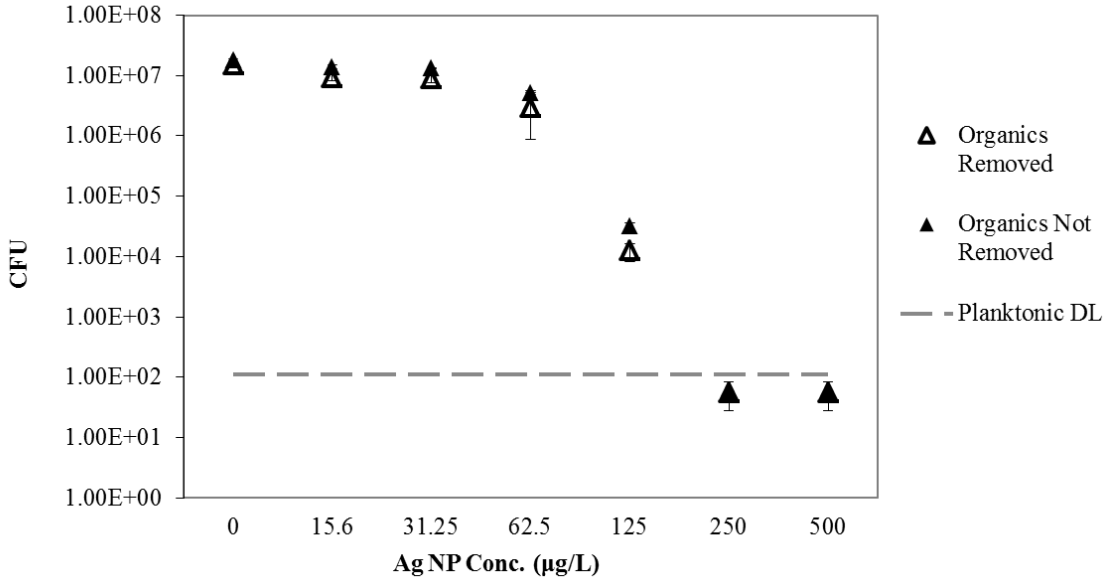


Figure 4.18: Effect of organics removal on the tolerance of *E. coli* planktonic cells to Ag NPs in standard PBS, containing chloride

(Ag NP concentration represents the dosed concentration)

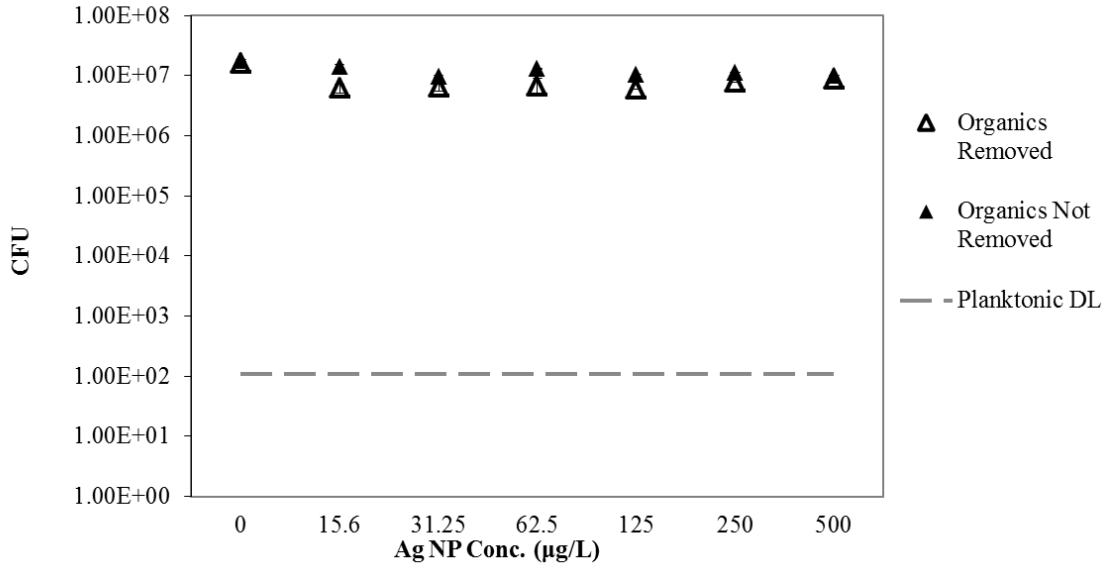


Figure 4.19: Effect of organics removal on the tolerance of *E. coli* planktonic cells to Ag NPs in PBS-NO_3^-

(Ag NP concentration represents the dosed concentration)

4.5.3 *E. coli* Tolerance Assays with Ag^+ in Presence of Chloride or Nitrate

Figure 4.20 shows the effect of the presence of 0.14 M chloride (standard PBS) or 0.14 M nitrate (PBS-NO_3^-) on the tolerance of planktonic *E. coli* cells to Ag^+. In the presence of chloride, there is no change in the tolerance with increasing silver ion dosage. In contrast, in the presence of nitrate, there is a decrease in tolerance with increasing silver ion dosage. Thus, Ag^+ are toxic to *E. coli*. These data are consistent with the hypothesis that in the presence of chloride, a strong ligand of silver, most of the silver will be complexed with chloride, and become less toxic towards bacteria. Figure 4.14 shows that at 0.14 M chloride, silver species exist at 0.009% free silver ions, and the remaining species are silver chloride complexes. However, in the presence of nitrate, a weaker ligand of silver, most of the silver will still be present as Ag^+, which is toxic to bacteria (Figure 4.20).

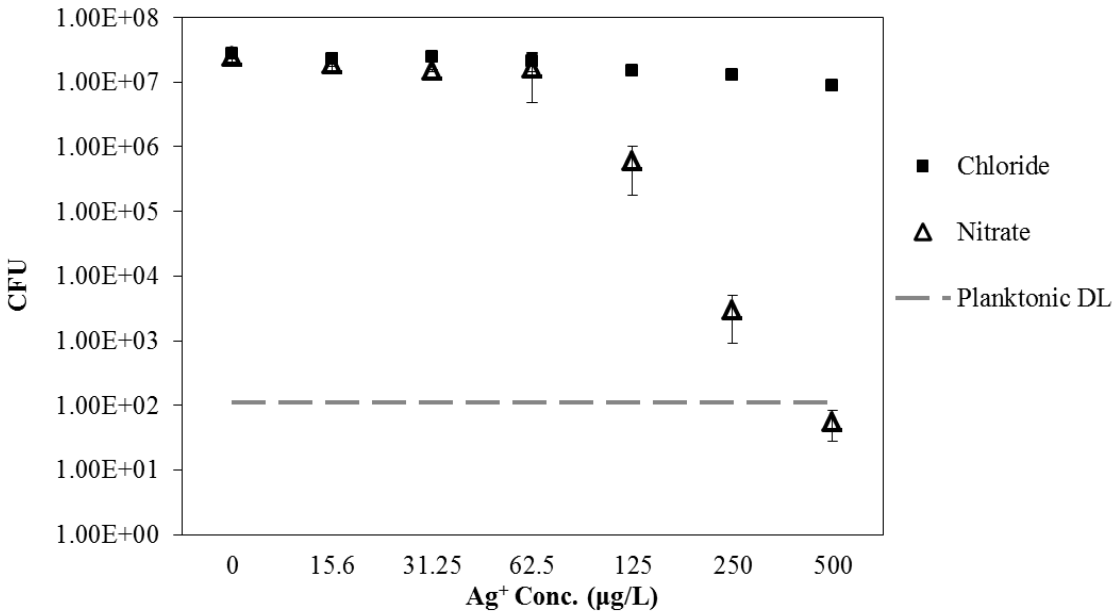


Figure 4.20: Effect of chloride and nitrate on the tolerance of *E. coli* to Ag^+ (Ag^+ concentration represents the dosed concentration)

These results are consistent with findings from Silver (2003), who found that silver toxicity to microorganisms is dependent on the concentration of halide ions, such as chloride, present in the surrounding environment. He found that chloride, present at moderate concentrations, can strongly bind to Ag^+, leaving silver unavailable to attack the cell membranes of bacteria (Silver, 2003). Therefore, the background water chemistry in which these tolerance assays are carried out can significantly affect the magnitude of silver toxicity.

Ligands present in wastewater treatment, including sulfate, sulfide, chloride, phosphate and EDTA can impact how Ag^+ affects bacteria in these engineered water systems (Hu et al., 2010). Silver toxicity is typically not observed in natural and engineered water systems because the aqueous concentrations of Ag^+ are generally low due to strong complexes formed with various ligands such as chloride, sulfide, thiosulfate, and dissolved organic carbon (Herrin et al., 2001; Choi et al., 2009). Such can be the case of these results showing low toxicity of Ag^+ in the presence of 0.14 M chloride (Figure 4.20). However, studying the toxicity of Ag^+ in the presence of different ligands is important in understanding a commonly hypothesized toxicity mechanism of Ag NPs, which states that in the presence of certain ligands, Ag^+ can be shed from the nanoparticles and be toxic to bacteria.

4.5.4 *E. coli* Tolerance Assays with Ag NPs in Presence of Chloride or Nitrate

Figure 4.21 shows the effect of the presence of 0.14 M chloride or 0.14 M nitrate on the tolerance assay of planktonic cells of *E. coli* against Ag NPs. These data show a completely opposite effect than that seen with Ag^+. In the presence of nitrate, there is no change in the tolerance with increasing Ag NP dosage. On the other hand, in the presence

of chloride, there is a decrease in tolerance with increasing Ag NP dosage. These results suggest that due to the different water chemistry and surface chemistry properties of Ag NPs compared to Ag^+, the presence of different ligands in the exposure medium can significantly impact the tolerance assays.

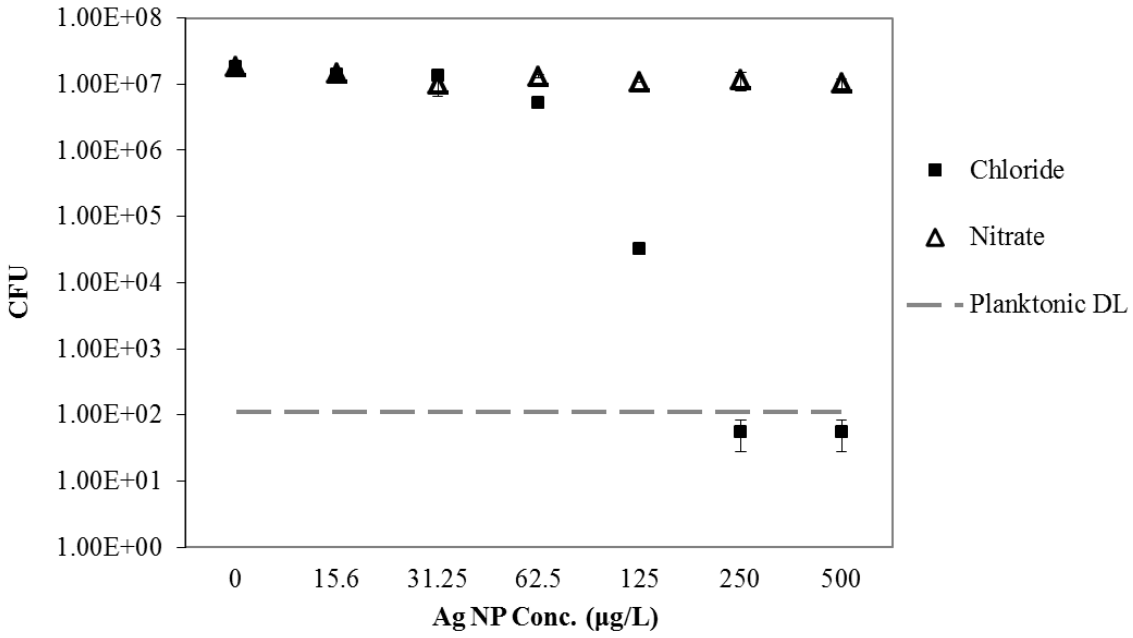


Figure 4.21: Effect of chloride and nitrate on the tolerance of *E. coli* to Ag NPs (Ag NP concentration represents the dosed concentration)

In a 0.14 M chloride environment, Ag^+ can complex strongly with chloride; the binding of free Ag^+ to chloride causes the toxic Ag^+ to become less bioavailable (Silver, 2003). However, in this same 0.14 M chloride environment, Ag^+ can be brought into solution via ligand-promoted dissolution of Ag NPs. Strong ligands of silver, such as

chloride, can pull the zero-valent Ag NPs into solution as Ag^+. On the other hand, weaker ligands, such as nitrate, cannot promote the dissolution of Ag NPs into Ag^+.

These results are verified by the color change that occurred when Ag NPs were added to a 0.14 M chloride solution. These color changes, presented in Figure 4.22, indicate that yellow suspensions of Ag NPs become colorless after exposure to 0.14M chloride after about 10 minutes, while the color does not change in the presence of 0.14 M nitrate. The initial yellow Ag NP solution becoming colorless in the presence of chloride suggests that Ag NPs are pulled into solution as Ag^+, thus resulting in a lower concentration of Ag NPs and higher concentration of Ag^+.

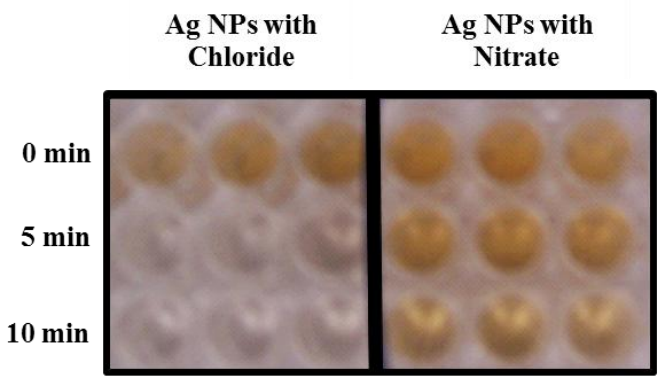


Figure 4.22: Color changes of Ag NPs in chloride and nitrate solutions

These results are similar to a Wiley et al. (2004) study, which found that 0.06 mM of chloride as NaCl and KCl promoted the dissolution of 20- to 80-nm Ag NPs to free silver ions. Therefore, at higher chloride concentrations, such as 0.14 M used in these experiments, dissolution of Ag NPs to free silver ions was likely to have occurred. Furthermore, Chappell et al. (2011) found that EDTA-promoted Ag NP dissolution to Ag^+ occurs through chelation of dissolved silver at low ionic strengths (1 mM). Choi et al. (2009) also found that the inhibition of Ag NPs to *E. coli* did not correlate linearly

with total silver concentrations. These data suggest that Ag^+ can complex with chloride ions present in wastewater and become ineffective towards bacteria.

4.5.5 Silver Ions as Toxicity Mechanism of Ag NPs

Figure 4.23 presents the summary of the effect of chloride and nitrate on silver ion and Ag NP toxicity. In the presence of 0.14 M chloride, dosing Ag^+ does not impact the viability of *E. coli*, but Ag NPs negatively impact *E. coli* viability under these experimental conditions. On the other hand, in the presence of 0.14 M nitrate, the dosing of Ag^+ negatively impacts *E. coli* viability while Ag NPs do not. Therefore, the inactivation of cells by Ag^+ and Ag NPs depends on presence of chloride in the experimental system.

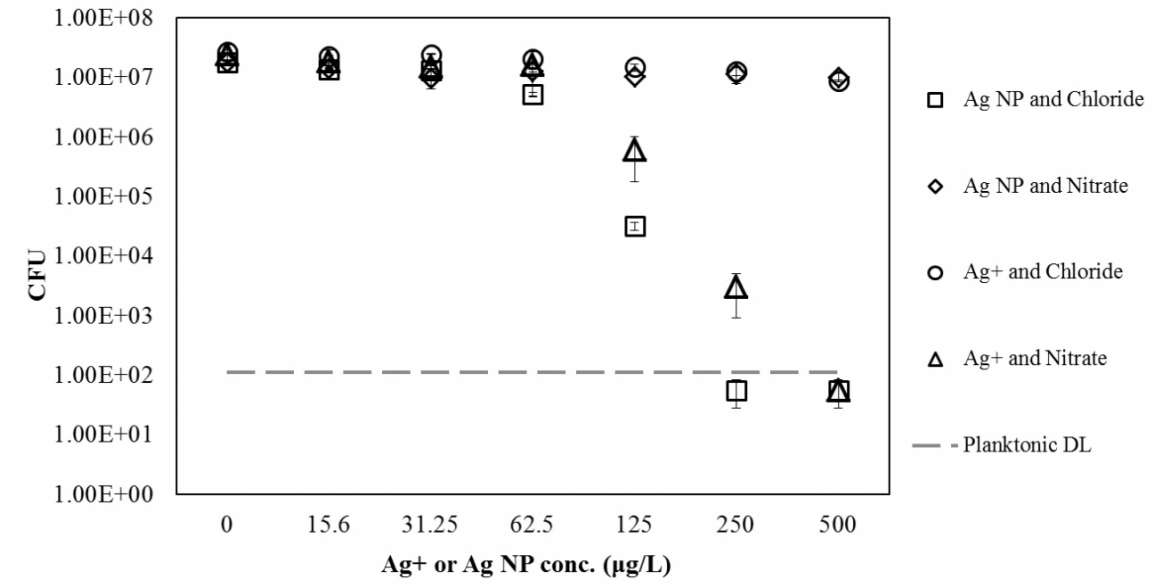


Figure 4.23: Summary of effect of chloride and nitrate on Ag^+ and Ag NP toxicity to planktonic *E. coli*

(Ag NP or Ag^+ concentration represents the dosed concentration)

Choi et al. (2008) found that Ag^+ were more toxic than Ag NPs in the presence of nitrate because Ag^+ can generate more reactive oxygen species (ROS) in the cell than Ag NPs. Increased ROS levels can then lead to increased damage to cell structures, stress on the bacterial population, and reduced growth rates. These results are consistent with these data, which show that in the presence of 0.14 M nitrate, Ag^+ are more toxic than are Ag NPs.

Sotiriou and Pratsinis (2010) showed that for Ag NPs less than 10 nm, the dominant mechanism of antimicrobial toxicity is the release of Ag^+ in media with 0.17 M chloride. Figure 4.23 suggests that Ag^+ is a possible mechanism of toxicity. Nonetheless, these results also indicate that the main toxicity mechanism of Ag NPs may be different in various media. The ability of Ag NPs to release Ag^+ and the speciations of dissolved silver are highly dependent on the background water chemistry of the medium.

Li et al. (2011) found that the antibacterial toxicity of zinc oxide (ZnO) nanoparticles depends on their complexation species and bioavailability, which changes with the aqueous medium in which the nanoparticles are suspended. The study found that in aqueous media, such as MD, LB, and PBS, free zinc ions are the dominant mechanism of toxicity of zinc oxide nanoparticles. Furthermore, the study showed that medium components such as phosphate, citrate, and organic matter affect the toxicity by influencing the dissolution of the nanoparticles and changing the chemical species of zinc. Therefore, further work is needed to understand the effects of water chemistry on the physiochemical properties and antimicrobial activity of nanoparticles.

Chapter 5: Conclusions and Future Work

5.1 Summary of Research Findings

This research examined the tolerance of microorganisms to silver nanoparticles (Ag NPs) in planktonic and biofilm cultures relevant to engineered water systems. To our knowledge, no research has been done to look at tolerance of *Zoogloea ramigera*, a common wastewater floc-forming bacterium, and *Rhodococcus*, found in drinking water, to Ag NPs. The results showed that *Z. ramigera* and the *Rhodococcus* isolate decreased in tolerance with increasing Ag NP concentrations (0 to 25000 μg/L) in the presence of 0.14 M chloride. *Z. ramigera* exhibited a lower sub-lethal concentration of Ag NPs than did the *Rhodococcus* isolate, suggesting that Ag NPs might be more toxic to *Z. ramigera* than to the *Rhodococcus* isolate.

The results showed that there was no striking protective effect of biofilm cells in either bacterium as compared to planktonic cells, in contrast to previous studies of microbial tolerance to antibiotics and heavy metals. In the case of the *Rhodococcus* isolate, biofilms did not demonstrate a difference in tolerance to Ag NPs compared to planktonic cells. For *Z. ramigera*, biofilms demonstrated a slightly higher tolerance to Ag NPs as compared to planktonic cells at Ag NP concentrations between 3.05 and 195 μg/L. From 195 to 25000 μg/L, *Z. ramigera* biofilm and planktonic cells exhibited equal tolerances.

The results of this work have many implications for bacteria in engineered water systems. For a drinking water treatment system, Ag NPs might inactivate desirable biofilms in drinking water treatment processes. The data suggest that the accumulation of Ag NPs may cause detrimental effects on essential microbial populations (e.g., *Z. ramigera*) in wastewater treatment plants. Hence, removal of Ag NPs upstream of activated sludge may be required. For these engineered water systems, Ag NP toxicity

against bacteria in these environments suggest that in the future, more stringent regulations may be needed to protect the integrity of these microbial systems from the harmful effects of Ag NPs.

This project also aimed to investigate the ability of these treatment bacteria to develop increased tolerance after previous exposure to sub-lethal concentrations of Ag NPs. Results showed that the *Rhodococcus* isolate had decreased tolerance to Ag NP as a result of previous exposure to a sub-lethal concentration of Ag NPs. These result indicated that Ag NPs can be an effective antimicrobial agent for the *Rhodococcus* isolate. However, *Z. ramigera* had increased tolerance to Ag NPs as a result of previous exposure to a sub-lethal concentration of Ag NPs.

Lastly, this research presented experiments and results detailing the possibility of silver ion shedding as a toxicity mechanism of Ag NPs in the presence of a chloride. Under these experimental conditions, it appears that 0.14 M chloride had the ability to promote the dissolution of Ag NPs into free silver ions: a process known as ligand-promoted dissolution. These results were consistent with current literature stating that the antibacterial activity and mechanisms of Ag NP toxicity are highly dependent on the water chemistry of the surrounding environment. The chemical properties of the Ag NP suspension can change the physiochemical properties of the Ag NPs, and thus alter their antibacterial activity. Further work is needed to fully understand how the properties of Ag NPs are altered in real water treatment systems.

5.2 FUTURE WORK

This section identifies the future work of this research, including a discussion on how background water chemistry can lead to physiochemical changes of Ag NPs in laboratory-scale methods to investigate the toxicity of Ag NPs toward organisms and their fate in the environment.

5.2.1 Water Chemistry and Silver Nanoparticles

Further work is needed to understand the toxicity mechanisms of Ag NPs against bacteria. Results from this research reveal that silver ions are a possible mechanism of Ag NP toxicity against *E. coli* via ligand-promoted dissolution in environments containing 0.14 M chloride, but not in 0.14 M nitrate environments. Further investigation is needed to identify ligand-promoted dissolution of silver ions as a possible mechanism of Ag NP toxicity at different concentrations of chloride. For example, typical chloride concentrations at wastewater treatment plants are approximately 9.87 mM, but can range up to 2.14 M, depending on the source of wastewater (Ucisik and Henze, 2004; Renato et al., 2009). In drinking water, the United States Environmental Protection Agency (USEPA) Secondary Drinking Water Regulations recommend a maximum concentration of 250 mg/L of chloride ions or 7.05 mM. Testing Ag NP toxicity in these ranges of chloride will provide more information on the fate, transport and toxicity mechanisms of Ag NPs in real engineered treatment systems.

Due to the antibacterial activity of Ag NPs being highly dependent on the size and shape of the nanoparticles, as well as the chemical properties of the medium in which the Ag NP toxicity studies are carried out, it is important to standardize the protocols for Ag NP synthesis and dispersion. Standardization will allow for comparable results across all research studies on Ag NP toxicity. For instance, Kim et al. (2007) found that gram-

positive bacteria were more resistant than gram-negative bacteria to 12-nm Ag NPs, while Yoon et al. (2008) found the opposite effect for 40-nm Ag NPs. Therefore, additional research is needed to fully understand how different ligands and other ionic species present in experimental aqueous solutions can alter the physiochemical properties, such as size, shape, surface charges, and silver ion shedding, and antimicrobial properties of differently synthesized Ag NPs.

5.2.2 Electrostatic Interactions between Bacteria and Nanoparticles

Zeta potentials, described as the electrostatic potential at the shear plane between the charged surface and the electrolyte solution or buffer, have been shown to be a feasible tool for understanding the interaction between cells and nanoparticles (Zhang et al., 2008). Zeta potentials on the cell surfaces indicate changes in cell and nanoparticle surface charges during interactions between cells and nanoparticles. Therefore, by measuring Zeta potential as a function of exposure time to Ag NPs, the mechanism of Ag NP toxicity may reveal how different species of bacteria vary in their interactions with nanoparticles. Furthermore, biofilm cells exhibit different cell surface charges and Zeta potential as compared to planktonic cells (De Carvalho et al., 2009). Measuring the Zeta potential changes on these two different types of cells would provide important information regarding how biofilm cells and planktonic cells interact with Ag NPs.

5.2.3 Silver Nanoparticles in Natural Mixed Microbial Communities

Little work has been done to look at the impact of Ag NPs on mixed microbial communities, such as those found in drinking water, wastewater, and water reuse systems. Future work should be carried out to understand how Ag NPs can change the

microbial communities in these systems. This work is significant because changes in the microbial community in water treatment processes can potentially break down the existing biological structure essential for these treatment processes.

5.2.4 Synergistic Effects of Silver Nanoparticles

Little work has been done on the synergistic effects of nanoparticles and other antibacterial agents on microbial toxicity. Li et al. (2005) and Fayaz et al. (2010) found that after exposure to Ag NPs, gram-positive and gram-negative planktonic bacteria were more susceptible to antibiotics. Ruden et al. (2009) showed synergistic effects of Ag NPs with antimicrobial peptides against planktonic gram-negative bacteria. Wu et al. (2009) found that copper nanoparticles enhanced the antimicrobial activity of titanium dioxide nanoparticles, which have a relatively low toxicity in the presence of no sunlight. To our knowledge, no research has been carried out on the synergistic effects of Ag NPs and other antimicrobial agents on biofilm cells. These studies are necessary to understanding the synergistic effects of nanoparticles and other antimicrobial agents on the microbial ecology in natural and built environmental systems.

Appendices

Appendix A List of Materials

Description	**Item No.**	**Vendor**
$AgNO_3$ (99.99%)	7761888	Sigma-Aldrich
Mercaptosuccinic acid ($C_4H_6O_4S$)	88450	Sigma-Aldrich
0.22-μm nylon membrane	R92755507	Fisher Scientific
Toluene	AC17716-0025	Fisher Scientific
Eppendorf tubes	5408133	Fisher Scientific
0.22-μm cellulose acetate membrane	430626	Corning, Inc.
$MgSO_4.7H_2O$	R00252852	Fisher Scientific
Spinning-Disk and chips		Fabricated at the University of Iowa by Mike Neville
Chemostat reactor		Fabricated at the University of Texas at Austin by Michael J. Ronalter,

Appendix B List of Instruments

Instrument	**Brand**	**Location**
TEM	FEI Tecnai Spirit Biotwin, Hillsboro, Oregon	Institute for Cellular and Molecular Biology, UT-Austin
ICP	Varian 710-ES, Mulgrave, Victoria, Australia	8th floor, ECJ building, UT-Austin
Bath sonicator	Branson 3510, Danbury, CT	Dr. Kirisits's lab, ECJ building, UT-Austin
Centrifuge	Beckman J2-21 centrifuge, USA	Dr. Speitel's lab, ECJ building, UT-Austin

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Vita

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