Effects of Silver Nanoparticles on Lake Bacterioplankton

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Abstract

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Silver nanoparticles (AgNP) released into aquatic environments could threaten natural bacterial communities and ecosystem services they provide. We examined natural lake bacterioplankton communities' responses to different exposures (pulse vs chronic) and types (citrate and PVP) of AgNPs at realistic environmental conditions using a mesocosm study at the Experimental Lakes Area. An *in situ* bioassay examined interactions between AgNPs and phosphorus loading. Bacterial communities exposed to high AgNP concentrations regardless of exposure or capping agent type accumulated silver. We observed increases in community production during additions of polyvinylpyrrolidone (PVP) -capped AgNPs and that site and nutrient-specific conditions are important to AgNPs toxicology in aquatic systems. Toxicological effects of AgNP are attenuated in natural conditions and differ from results from laboratory studies of AgNP toxicity. Our results demonstrate more studies are needed to fully assess the risk posed by these novel chemicals to the environment. This work could be useful in forming risk assessment policies which are largely based on lab studies and typically demonstrate strong toxic effects.

Keywords: silver nanoparticles, natural bacterioplankton communities, bacterial production, mesocosms, nutrient bioassays, ecological stoichiometry, Experimental Lakes Area

This Thesis is dedicated to my Parents, Sister and my Grandad Fred

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Chapter 1: Introduction

1.1 Introduction

Nanomaterials are and rapidly developing form of technology. This field aims to exploit the unique nano-scale properties of nanomaterials for both commercial and industrial purposes. These industrial and commercial uses are expected to generate annual revenues of nearly a trillion U.S. dollars by 2015 (Bradford *et al*, 2009, www.nanotechproject.org). The increased and widespread use of nanomaterials is leading to their environmental introduction, including into natural surface waters and aquatic ecosystems (Gottschalk *et al*, 2009). Despite this potential threat, our understanding of how nanomaterials affect aquatic ecosystems remains incomplete. For example, there have been many studies of the anti-microbial properties of AgNPs, but most of these have been completed on a single species of bacteria under controlled laboratory conditions (Morones *et al*, 2005). How these observed effects will translate to natural aquatic environments is unknown (Fabrega *et al*, 2011).

1.2. Nanomaterials and Natural Bacterial Communities

With the expected increase in their use, nanomaterials have been the focus of research investigating their potential risk to biological organisms and the ecosystems they inhabit. The small size of nanoparticles (1-100 nm) confers on them unique properties (Moore, 2006), making them of interest to industry. This small size is similar to many biological macro molecules (proteins, DNA, and phospholipids), which contributes to their ability to cause disruptions at both the molecular and cellular level in many organisms (Feng *et al*, 2000). Such effects on organisms could affect aquatic food-webs

and ecosystem processes either by altering the metabolism of individual taxa or by changing the species composition of communities.

Das and colleagues (2012 b) demonstrated that four general AgNP exposure responses are elicited by a bacterial community: intolerant, impacted but recovering, tolerant and rare stimulated phylotypes. In another study (2012 a), they observed decreases in extracellular alkaline phosphatase affinity, and bacterial production in response to AgNP additions within the first few hours of AgNP exposure. They concluded that exposures in the low microgram per liter range would likely negatively impact natural aquatic bacterioplankton processes (Das *et al*, 2012 a).

1.3. Uses of Silver Nanoparticles

Silver had many uses throughout the course of human history. Ancient Chinese and Egypitan civilizations made use of its biocidal properties, which lent themselves well to water disinfection and the treatment of burn wounds (Li *et al*, 2008 & Trop *et al*, 2006). Now silver nanoparticles (AgNPs) are the most abundantly used nanomaterial as they account for 30% of commercialized products (www.nanotechproject.org). This partly reflects the rapid diversification of their applications and their amenability to commercial uses. A few examples of AgNPs as additives to commercial products include clothing (socks, underwear, T-shirts and athletic apparel) to prevent the growth of odour causing bacteria (Blaser *et al*, 2008; Walser *et al*, 2011) and food packaging products: Sunriver Industrial Nanosilver Fresh Food bags (Huang *et al*, 2011), and Freshbox Silver Nanoparticle Food Storage Containers® (Alfadul & Elneshwy, 2010). AgNPs also find themselves used in water treatment processes and surface coatings (Nowack *et al*, 2010; Li *et al*, 2008). AgNPs have even been used in electronics as electrodes for flexible

devices and as conducting films, while these applications do not often result in AgNP environmental introduction during their uses, such issues may arise during their disposal (Zeng *et al*, 2010).

The antimicrobial properties of silver have made AgNPs a subject of great interest to the medical industry. This is partly because their antimicrobial activity affects all strains of bacteria (Lansdown, 2004). AgNPs have been demonstrated to be effective antimicrobials against both gram positive and negative bacteria and even to antibiotic resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli (E. coli)* O157 and others (Lansdown, 2004). AgNPs are now a common addition to cleaning products, textiles, paints, food packaging and medical devices among many others. They are also used in conjunction with other nanomaterials to prevent the growth of biofilms (Cao *et al*, 2011). This variety of uses of many AgNP forms means, in a general sense, that there is a high probability that these materials will enter natural waters.

1.4. Silver Nanoparticles Characteristics and Behaviour

AgNPs gain many of their unique characteristics due to their small size, which gives them a large surface area to volume ratio. Similarly, their toxicity has been shown to be size dependent with smaller AgNPs more toxic than larger ones (Yen *et al*, 2009). These smaller particles are more likely to enter into cells and with an increased surface area the AgNPs dissolution to Ag^{+} ions is typically faster (Choi & Hu, 2008; Yang *et al*, 2012). The shape of the particle (spheres, rods, cones) has also been shown to be an important characteristic influencing AgNPs behavior (Pal *et al*, 2007).

AgNPs tend to be unstable in water suspensions where dissolution and agglomeration in short periods of time is commonplace (Stebounova *et al*, 2011). To increase their stability and maintain their function, AgNPs are usually synthesized with a coating or capping agent (Guo *et al*, 2013). These capping agents vary but two which are commonly used are citrate and polyvinylpyrrolidone (PVP). Each capping agent confers different properties to the particles, which in turn influences their dissolution rates to ionic silver (stability), interactions with biomolecules and their surface charge (Chappell *et al*, 2011; Choi & Hu, 2008; Guo *et al*, 2013). Surface charge or zeta potential can influence AgNPs interactions with living systems and thus their relative toxicity. Some studies have shown that the zeta potential of an AgNP has no impact on its toxicity (Yang *et al*, 2012) while others have found a correlation between cytotoxicity of dispersed AgNPs and their overall zeta potential whereby increasing zeta potentials resulted in higher rates of cellular death (Suresh *et al*, 2012). The stability of the AgNPs strongly influences toxicity, as it is a governing factor in an AgNPs dissolution rate to Ag^+ ions (Stebounova *et al*, 2011).

1.5. Mechanisms of Bacteriocidal Action

The exact mechanism of AgNP toxicity is unknown; there are several major functions of bacterial cells which have been proposed as being affected by AgNPs which result in impaired or reduced ATP generation and /or protein synthesis, as well as cell death (Jung *et al*, 2008; Shrivastava *et al*, 2007; Yamanaka *et al* 2005; Yang *et al*, 2009). It is important to understand the different mechanisms by which AgNP affects bacterial communities, as often it is not solely one mechanism at work, but a synergistic combination of several mechanisms. This multi-faceted ability gives AgNPs their greater toxicity when compared to just Ag^+ ions of the same concentrations (Li *et al*, 2008;

Samberg *et al*, 2011). It is also this multi-faceted effect which will be altered pending the site specific conditions which the AgNPs are released into.

Metallic silver (Ag (0)) atoms are found on the surface of AgNPs. In oxic (aerobic) conditions metallic silver can be oxidized to Ag_2O. Bacterial metabolism can create an acidic environment which facilitates the formation of Ag^+ from Ag_2O (AshaRani *et al*, 2009; Liu *et al*, 2010; Liu & Hurt, 2010). Ag^+ ions interact with bacterial cell walls, plasma membranes, bacterial DNA, proteins, and ribosomes, interfering with their function and leading to bacteriocidal effects (Jung *et al*, 2008; Shrivastava *et al*, 2007; Yamanaka *et al* 2005; Yang *et al*, 2009). Ag^+ ions can bind to the thiol (-SH) groups of enzymes and receptors found on bacterial cell walls containing peptidoglycan. This results in the misfolding of proteins, which can disable their function (Cho *et al*, 2005; Kim *et al*, 2007; Liau *et al*, 1997; Spacciapoli *et al*, 2001). It has been demonstrated that gram positive bacteria are less susceptible to AgNP than gram negative bacteria (Shrivastava *et al*, 2007). This difference likely results from the thick peptidoglycan layer found in gram positive bacteria, which prevents the penetration of Ag^+ ions to the inner parts of the cell wall. If Ag^+ ions are limited to interactions with the outer peptidoglycan layer they are unable to cause more significant adverse effects to the cell (Shrivastava *et al*, 2007).

Negatively charged DNA is also a target of Ag^+ ions. As Ag^+ ions diffuse into bacterial cells they can bind DNA bases which results in inhibition of replication and transcription (Feng *et al*, 2000; Yang *et al*, 2009). It has been demonstrated that a similar set of proteins to those employed during a "heat shock" response are deployed by stressed *E. coli* and *Staphylococcus aureus* cells as a mechanism to protect DNA from denaturing

in the presence of Ag^+ ions (Feng *et al*, 2000; Wong & Liu, 2010). It has also been shown that Ag^+ ions interfere with enzymes required in the functions of phosphorus, sulphur and nitrogen cycles of nitrifying bacteria (Lee *et al*, 2012).

Ag^+ ions localize around membranes and disrupt proton motive forces which are required for mitochondrial function and ATP production. Cao and colleagues (2010) demonstrated the localization of Ag^+ ions and subsequent disruption by showing proton depleted regions around AgNPs in and around bacterial cells. Another study by Xiu and colleagues (2012) controlled the synthesis of Ag^+ ions and found that AgNPs exerted no toxic effects on *E. coli* when metallic silver could not be oxidized and release toxic Ag^+ ions. They then found significant toxic effects under aerobic conditions (Xiu *et al*, 2012). Antibacterial activity caused by released Ag^+ ions is not the only explanation for the mechanism of AgNP toxicity; environmental conditions and the properties of the particles themselves also play an important role.

There is greater toxicity of AgNPs compared to just Ag^+ ions of the same concentration (mg/L) (Choi *et al*, 2008). The heightened antibacterial capabilities of AgNPs have led to the idea that AgNPs have intrinsic antibacterial capabilities besides the release of Ag^+ ions (Choi *et al*, 2008). AgNPs can bind to plasma membranes and gain entry to cells, their small size and large surface area allow AgNPs to make strong contact with bacterial cell membranes (Wong & Liu, 2010). Once inside or bound to the cell they can cause structural changes in the cell wall or internal proteins which can lead to their impaired function or cell death (apoptosis) (Raffi *et al*, 2008; Sondi & Sondi-Sapolek 2004). Choi and Hu (2008) showed that the inhibition of nitrifying bacteria was correlated with the fraction of silver less than 5 nm; this was more toxic than any other

form of silver (Ag^+ ions and AgCl colloids). Smaller silver nanoparticles may be more easily transported via cellular uptake processes due to their size. Ag^+ ions may also alter membrane permeability, which can lead to increased cellular penetration of the AgNPs. Common ligands chloride, sulfide, phosphate and organic acids have been shown to bind Ag^+ ions, possibly leading to precipitation and decreased bioavailability leading to buffered levels of AgNP toxicity (Choi *et al*, 2009; Xiu *et al*, 2011). Traditional approaches to modelling toxicity of metals in aquatic systems such as the biotic ligand model (BLM) or the free ion activity model (FIAM) cannot yet be applied to nanoparticles as fundamental assumptions of the models cannot be met, such as the dominance of free ions determining bioavailability, and that uptake across a membrane is the rate limiting step (Fabrega *et al*, 2011).

Nanomaterials have been shown to cause the production of reactive oxygen species (ROS) in biological systems. Excessive intracellular or extracellular ROS concentrations lead to the oxidative stress, which damages membranes and DNA (Liu *et al*, 2012; Thannickal & Fanburg, 2000). ROS can be produced by both Ag^+ ions and AgNPs. ROS interact with proteins, lipids and DNA causing significant antibacterial effects (He *et al*, 2012). It has been shown that intracellular generation of ROS was increased, and present at higher concentrations when bacterial cells were exposed to AgNPs than Ag^+ ions of the same concentrations (mg/L) (Choi & Hu, 2008).

1.6. Silver Nanoparticle Environmental Introduction

As products containing AgNPs become more prevalent economically their release as AgNPs, or other forms of silver to the environment is inevitable and a serious issue. Studies have been conducted assessing the release of AgNPs from several materials and

their environmental fate. Notable work modelled environmental concentrations of AgNPs in Europe and the United States (Gottschalk *et al* 2009). The researchers concluded that there are risks to aquatic organisms as AgNPs cannot be removed in sewage treatment effluents (Gottschalk *et al*, 2009). Blaser and colleagues (2008) predicted that AgNP concentrations in freshwater ecosystems may exceed the limit of "no effect concentration", where the concentration is above the concentration where adverse effects are expected to occur. A study examining AgNP release from a washing machine, showed the machine to release silver at an average concentration of 11 μg/L during its "nanowash" setting (Farkas *et al*, 2011). The machine continued to release silver even when the "nanowash" setting was disabled. Experiments assessing the toxicity of this "nanowash" effluent on natural fresh water communities have found dose dependent reductions of bacterial abundance of 60% at 2.5 μg/L, and 80% at 12.5 μg/L (Farkas *et al*, 2011), how such releases will affect natural bacterial communities remains largely unstudied.

AgNP behavior in the aquatic environment is complicated and difficult to predict due to the complex chemistry of nanoparticles, silver in aqueous solutions, and their relationship with physico-chemical characteristics of the environment (Liu & Hurt, 2010 & Lowry *et al*, 2012). As previously discussed, the stability of AgNPs influences their toxicity as Ag^{+} ions are a major contributor to their overall toxic effect. Environmental factors such as ionic strength, as well as pH, dissolved organic matter, dissolved oxygen concentration and temperature among others may all have as significant an impact on particle stability as the physical characteristics of the particles themselves (size, shape &

coating) (Chapell *et al*, 2011; Liu & Hurt, 2010; Suresh *et al*, 2012; Stebounova *et al*, 2011; Yang *et al*, 2010).

There are a number of studies which have attempted to assess the effects of AgNPs on microbial communities. For example, Bradford and colleagues (2009) looked at AgNP impact on bacterial assemblages in estuarine sediments and found minimal toxic effects, however this study was conducted over a relatively short term (20 days). Other studies have been made in carefully controlled laboratory environments (Morones *et al*, 2005), which exclude many of the possible influences outlined here. Interactions between AgNPs and chemical and biological processes are difficult to replicate at a bench scale and include: species interactions (predation and herbivory), lake mixing, gas exchange and sediment water exchanges. The physiological and ecological effects of AgNPs on organisms may also be dictated by other environmental variables such as nutrient conditions and light penetration.

1.7 Bacterial Nutrition and Silver Nanoparticle Toxicity

A bacterial community's nutritional state should play a substantial role in their response to the exposure to a contaminant such as AgNPs. Lessard & Frost (2012) and Hansen and colleagues (2008) demonstrated using *Daphnia magna*, that elemental food quality (Carbon (C), Nitrogen (N) and Phosphorus (P) content) is an important determinant in the toxicity of herbicides and fluoxetine. Under conditions of low nutrient supply (Low N & P), toxicity could be increased by reducing the ability for bacteria to repair cellular stresses or produce enzymes required to mitigate the negative effects caused by AgNPs. AgNPs could also affect cellular metabolism, slowing growth by binding critical proteins or enzymes, and inhibiting their functions or by disrupting the

proton motive gradients required for passive nutrient uptake and ATP generation (Cao *et al* 2011; Lok *et al*, 2006). AgNPs could also have an impact on the cellular demand for nutrients, possibly causing changes in the uptake and release of nutrients (Das *et al*, 2014). A study examining AgNPs and Ag^{+} ions have been shown to create leaky membranes, and this allows cytosolic contents to leak out of cells, which results in the loss of soluble P (Sondi & Sondi-Salopek, 2004; Schreurs & Rosenberg, 1982). It is suspected that AgNPs gain entry to cells via membrane bound transport proteins (Choi & Hu, 2008). It was demonstrated that AgNPs and P supply had an interactive effect decreasing sestonic C:P and N:P ratios while increasing C:N and cell bound Ag (Das, Metcalfe & Xenopoulos, 2014). Therefore during conditions of higher nutrient (P) availability, increased intracellular concentrations of AgNPs could be observed as a result of co-transport, we also could observe the inhibition of C and N uptake possibly leading to even higher rates of toxicity.

1.8 Experimental Rationale and Predictions

This project is a part of the Lake Ecosystem Nanosilver (LENS) project's mesocosm study conducted at the ELA. For more information on AgNPs speciation and fate within this study, consult Lindsay Furtado's MSc thesis entitled "Fate of Silver nanoparticles in Lake Mesocosms" as well as her publication Furtado et al, 2014. For information regarding AgNP effects on zooplankton and algae communities within this study please refer to Jennifer Vincent's MSc thesis entitled "Effects of silver nanoparticle exposure on community structure of natural lake phytoplankton and zooplankton" expected to be completed in the fall of 2014.

We conducted two studies to evaluate the effects of AgNPs on natural bacterial communities in aquatic ecosystems at the Experimental Lakes Area (ELA) in northern Ontario. We completed a six week field manipulation in large mesocosms. The assessment of AgNP's effects over a longer term (weeks) provided us with a timeline for quantifying bacterial community changes in response to AgNP addition over the summer season. We monitored changes in bacterial abundance, primary production, protein synthesis, bacterial-bound silver and community nutritional stoichiometry. During this experiment, we examined two different AgNP exposure scenarios: a chronic addition resulting in a concentration gradient ending at predetermined environmentally relevant concentrations (0-80 μg/L), a large one- time "pulse" addition, and a comparison of two commonly used capping agents (citrate and PVP). We expected bacterial communities to be negatively affected by the introduction of AgNPs. We predicted that differences in bacterial community responses would emerge between PVP and citrate capping agents; sterically stabilized PVP yielding stronger community responses (reductions in abundance and production/ changes in community stoichiometry) than charge stabilized citrate. We predicted bacterial abundance and production would decrease in a concentration dependent manner to continuous, increasing concentrations of PVP capped AgNPs. In contrast, recent evidence (Das *et al*, 2012 b) suggests that over time silver-tolerant/resistant taxa would become more prevalent and the bacterial pool would become increasingly less affected by AgNP exposure. In response to the pulse exposure, we expected a sharp drop off in abundance and productivity followed by a slow recovery in these responses.

Secondly we employed a three day *in situ* bag bioassay examining the interactive effects of P enrichment and AgNP exposure in two different boreal lakes. The bioassay was conducted in an attempt to observe differences in response between two unique communities' in response to P enrichment and AgNP exposure. We monitored bacterial abundance, bacterial-bound silver, and changes in nutritional stoichiometry. We predicted bacterial abundance would decrease with increasing concentration of AgNPs but that these effects would change with P supply such that P-limited communities would be more susceptible to AgNP toxicity. It was also expected that differences would emerge between the two lakes with bacteria in the relatively humic rich Lake 222 (L222) experiencing less toxicity as humic substances stabilize AgNPs preventing their dissolution to toxic Ag^{+} compared to their counterparts in a more algal-rich, Lake 114 (L114) (Huynh &Chen, 2011; Navarro *et al*,2008).

Chapter 2: Effects of Silver Nanoparticles on Bacterioplankton Communities in Aquatic Mesocosms

2.1. Introduction

Silver nanoparticles (AgNPs) are used widely for commercial and industrial purposes due to their antimicrobial, antifungal and antiviral capabilities (www.nanotechproject.org). The widespread use and diverse application of AgNPs mean that they may reach the aquatic environment both through wastewater discharge and through other indirect sources (Benn & Westerhoff, 2008; Blaser *et al.*, 2008; Gottschalk *et al.*, 2009). Considering their efficacy as antimicrobials, it is reasonable to expect that natural microbial communities would be negatively affected by exposure to AgNPs in the environment at relatively low concentrations (low µg/L; Das *et al.*, 2012a), and AgNPs could interfere with important ecosystem services provided by microbial communities, such as decomposition and nutrient cycling (Choi & Hu, 2008; Choi *et al.*, 2008).

AgNPs may have diverse and negative effects on natural bacterial communities including the reduction of bacterial abundance, production, diversity and extracellular enzyme activity in both water and sediments (Wigginton *et al.*, 2010; Das *et al.*, 2012a, b; Doiron *et al*, 2012). However, apart from a few studies (Colman *et al.*, 2012; Lowry *et al.*, 2012), much of the work completed on AgNP toxicity to aquatic microbes has involved short-term (<3 days) controlled laboratory experiments and/or the use of laboratory-based bacterial cultures (Morones *et al.*, 2005; Yoon *et al.*, 2007; Li *et al.*, 2010). This work has been important in determining the mechanism(s) of AgNP antibacterial action, but is inadequate in considering how environmental variables (e.g., pH, ionic strength, the concentration and quality of both dissolved organic matter and inorganic ligands) may alter the toxicity of this emerging contaminant on multi-species

bacterioplankton communities (Fabrega *et al.*, 2009; Fabrega *et al.*, 2011; Das, Metcalfe & Xenopoulos, 2014). As environmental variables have large implications for the chemical behavior of AgNPs (Chapell *et al.*, 2011; Fabrega *et al.*, 2011; Liu & Hurt, 2010; Stebounova, Guio & Grassian, 2011; Suresh *et al.*, 2012; Yang *et al.*, 2012), there continues to be a need for *in situ* studies of AgNP effects on natural bacterioplankton under environmentally relevant conditions.

Nanoparticles can be highly chemically reactive in the absence of sufficient stabilization (Stebounova *et al.*, 2011). AgNPs often agglomerate with each other or adsorb to particulate organic matter, which can lessen their antibacterial properties and increase rates of sedimentary removal from the water column (Stebounova *et al.*, 2011). To overcome this, and allow for exploitation of their useful properties, AgNP solutions are stabilized through electrostatic repulsion and/or steric hindrance conferred to them by a capping agent (Tejamaya *et al.*, 2012). Two common capping agents of AgNPs are carboxy-functionalized charge-stabilized citrate (citrate) and sterically-stabilized polyvinylprrolidone (PVP). Chemical differences between capping agents affect the stability of these nanoparticles in the environment and alter their antimicrobial efficacy to natural bacterioplankton communities (Levard *et al.*, 2012; Guo *et al.*, 2013). However, there has been little work examining whether effects of AgNPs vary with capping agent under environmentally relevant conditions.

The mode of exposure also needs to be considered when assessing the toxicity of AgNPs in the environment. AgNPs could enter the environment as a rapid, one time, point source introduction such as what might be seen during an industrial spill. In a scenario such as this, the mobility and stability of the AgNPs will play a large role in

determining the effect on exposed biota. Upon the addition of a high concentration of AgNPs, the initial toxic response and the persistence/stability of the AgNPs will determine the rate at which the bacterial community is able to recover (Huynh & Chen, 2011; Guo *et al.*, 2013). Conversely, if the environmental introduction of AgNPs is chronic or continuous, from sources such as wastewater discharges, different toxic effects may ensue. The initial toxic response could be concentration dependent, and the stability (capping agent) of the AgNPs may play a significant role in the accumulation of AgNPs in the water column. While an increase in water column AgNPs should lead to larger antibacterial effects over time, this exposure regime could possibly lead to smaller effects due to changes in microbial communities from sensitive to resistant taxa (Das *et al.*, 2012b).

To examine the effects of AgNPs on natural bacterial communities, we conducted a six week aquatic mesocosm study at the Experimental Lakes Area (ELA) in northern Ontario during the summer of 2012. We examined responses to AgNPs stabilized with two different capping agents (citrate and PVP) and the effects of two different dosage regimes; i) a continuous "chronic" dose addition and ii) a one-time pulse high dose addition. We predicted bacterial abundance and production would decrease over time in a concentration dependent manner in response to chronic dosing at increasing concentrations of PVP and citrate capped AgNPs. In contrast, recent evidence presented by Das *et al.* (2012b) indicates that over time silver-tolerant/resistant taxa within the bacterial community become more prevalent and the bacterial pool becomes increasingly less affected during chronic AgNP exposure. In response to the pulse exposure, we expected a sharp decline in abundance and productivity followed by a slow recovery. It

was also expected that differences would emerge between the two capping agents, citrate and PVP, with bacterial communities exposed to the less stable and consequently less persistent citrate-capped AgNPs showing less severe toxic responses than bacteria exposed to the more stable PVP capped AgNPs (el Badawy *et al.*, 2011).

2.2. Methods

2.2.1. Location of Study

The study was carried out at the Experimental Lakes Area (ELA) in Lake 239 (L239), which is located in northwestern Ontario (49.660128, -93.714937). L239 receives water from an undeveloped watershed covered by boreal forest. This lake has been studied continuously since 1969 as a large part of a long-term ecological monitoring program. It is an oligotrophic lake characterized by relatively low total dissolved nitrogen and phosphorus concentrations (~250 µg/L and ~5 µg P/L, respectively), low primary productivity, and intermediate DOC concentrations (~7 mg/L).

2.2.2. Description of the mesocosms

We deployed twelve mesocosms in the southeastern bay of L239. This bay is relatively shallow (<3 m deep) and well-protected from wind-driven waves. To three floating docks anchored in the bay, we attached open-bottomed mesocosms that were sealed to the sandy lake bottom by sandbags (Figure 1).

Figure 1: Aerial view of the mesocosm study set up in L239 (left). Mesocosms attached to docks (right).

Mesocosms were ~2 m in diameter and ranged in depth between 1.5 m-2.0 m. Mesocosms were made out of polyethylene and supported by floating collars (Currie Industries, Winnipeg, MB, Canada), which were open at the water's surface. The volume of enclosure was ~4000 L as determined at the end of the experiment by the addition of 12.31 g of NaCl and measurements of resulting Cl^- concentrations using a chloride ion selective electrode. The experiment ran for six weeks from late June 2012 through mid-August 2012. Enclosures were randomly assigned to an experimental treatment with the condition that all treatment combinations were present on each side of the docks. Upon sealing of the bottom of the mesocosms, enclosures were left for three days to permit suspended sediments to settle. Over the course of the experiment, there were very few to no observable macrophytes within the mesocosms. Fish and leeches were removed from enclosures at the beginning of the experiment using nets and minnow traps.

2.2.3. Nanosilver

PVP capped and citrate capped BioPure nanosilver suspensions were purchased from nanoComposix, Inc (San Diego, CA, USA). They were provided in a milliQ water buffer and citrate buffer respectively. The capped nanoparticles were received as purified

monodispersent spheres at a nominal concentration of 1.0 g/L. Stock concentrations were confirmed by acid digestion and inductively coupled plasma mass spectrometry (ICP-MS). Transmission electron microscopy (TEM) determined the PVP capped particles to have a diameter of 48.3 nm. Dynamic Light Scattering (DLS) determined the PVP capped particles to have a hydrodynamic diameter of 56.3 nm and surface charge (zeta potential) of -33.9 mV. TEM determined the citrate capped particles to have a diameter of 49.1 nm. DLS determined a hydrodynamic diameter of 54.9 nm and a surface charge of -54.9 mV for the citrate capped particles. Once released in L239 waters the PVP capped AgNP was able to maintain a consistent hydrodynamic diameter of 50 nm for 7 days, after 25 days aged PVP capped AgNPs were 30 nm in hydrodynamic diameter, negligible Ag+ was detected (Furtado *et al.*, in press). This data was determined using single particle inductively coupled plasma mass spectrometry; hydrodynamic diameters were not assessed on the citrate particles in this congruent study.

2.2.4. Experimental Design

We used the 12 replicate enclosures to conduct three separate experiments simultaneously. For the "PVP chronic gradient" experiment, we added different volumes of PVP capped AgNPs stock (0, 0.89, 3.56 and 14.24 mL) every other day over the entire experimental duration of 39 days. Based on the 4000 L volume of the enclosures, this translated to a daily rate of addition of 0, 0.22, 0.89, and 3.56 µg Ag/L/day. 0 µg/L mesocosms were used as the reference for all three experiments and were mixed gently in the same manner as other chronic and pulsed enclosures. In the "capping agent chronic" experiment, we compared the chronic dosing of high concentrations of PVP with a similar dosing of citrate (14.24 mL added every second day). Finally, in our "pulse" experiment we added a single pulse of 240 mL PVP AgNP to achieve a starting

concentration of ~60 µg/L and then tracked the responses of exposed enclosures, see Table 1 for a summary of our experiments. Dosing of the citrate and PVP drip exposure mesocosms occurred every other day for 6 weeks starting from June 23rd, 2012. The pulse of PVP-AgNPs was added on July 11th, 2012. Each treatment combination described above was applied to two replicate mesocosms. After each addition of AgNPs, mesocosms were mixed gently to ensure distribution of the silver throughout the water column with care taken not to disturb the sediments.

Table 1: Summary of the mesocosm experimental treatments and their target concentrations based upon a 4000 L volume.

Experiment	Treatment	Target Concentration (µg/L)
PVP Chronic	Control	0
PVP Chronic	Low PVP	5
PVP Chronic	Medium PVP	20
PVP Chronic	High PVP	80
Citrate vs PVP	Control	0
Citrate vs PVP	High PVP	80
Citrate vs PVP	High Citrate	80
Pulse PVP	Control	0
Pulse PVP	Pulse PVP	60

2.2.5. Mesocosm sampling and water processing

We sampled all enclosures three days (June, 21st 2012) before the addition of the silver and then once a week for a total of six sampling dates throughout the course of the experiments until August 1st, 2012. Sampling began by collecting 8 L of water, which was screened through 35 µm mesh in order to remove large debris in the field and taken to the lab for further processing. This water was acidified with nitric acid and later analyzed for total silver (TAg) concentration.

A sample of the <35µm water was passed through a 20 µm mesh, preserved with 1% formaldehyde. This was later analyzed using flow cytometry for bacterial abundance

(TBACT; see below). Another water sample was taken from the <35 µm fraction and a leucine incorporation assay was conducted to determine protein synthesis (production). To further sample the size fraction which we called the bacterioplankton fraction (0.7-1.2 µm), screened water was passed through a GF/C (nominal pore size 1.2 µm; Whatman, NJ, USA) and subsequently a 1.2 µm polycarbonate filter. This filtrate was then sampled for particulate carbon (bact C), nitrogen (bact N), phosphorus (bact P), and chlorophyll a (bact chla). Subsamples of this filtrate were filtered through ashed GF/F (nominal pore size, 0.7 µm; Whatman, NJ, USA) filters in order to determine C and N content, and unashed GF/F filters in order to determine chlorophyll a content. An additional subsample was collected on a 0.8 µm polycarbonate filter (Isopore, Cork, Ireland) for bacterioplankton silver (bact Ag) analysis.

2.2.6. Carbon, Nitrogen, Phosphorus and Chlorophyll

Particulate C and N were analyzed in duplicate using a Vario EL III CN analyzer (Elementar, Hanau, Germany). Phosphorus analysis for particulate P was determined after persulfate digestion with the molydbate-blue reaction (APHA, 1992). For bacterio-chlorophyll, filters were stored frozen (-20ºC) until extraction. A 24 h dark ethanol extraction (EPA method 446.0) was performed and absorbance was measured at 885 nm on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, CA, USA) (Arar, 1997).

2.2.7. Bacterial production and abundance (TBACT)

Bacterial production was measured from protein synthesis using ^{3}H-leucine incorporation following the standard centrifugation method (Xenopoulos & Bird, 1997). Four 1 mL subsamples from each mesocosm were incubated for one hour with 50 nmol ^{3}H-leucine/L (29.4 nmol leucine/µCi; PerkinElmer Inc., MA, USA). Incubations were

terminated by the addition of formaldehyde to reach a 1% final concentration. Bacterial cells were harvested by centrifugation and proteins were precipitated by repeated washing with 5% tricholoracetic acid. Radioactivity of each sample was determined using a liquid scintillation counter (Tri-Carb Model 1600CA, PerkinElmer Inc., MA, USA) and counts were converted to µg C/L/d following Kirchman (2001). TBACT was enumerated on a flow cytometer by staining with SYBR Green Stain I (Invitrogen-Thermo Fisher Scientific, MA, USA). A Cytonomics FC 500 flow cytometer with CXP data processing software (Beckman Coulter) was used for all measurements (Marie *et al*, 1997).

2.2.8. Silver (TAg & Bact Ag)

Silver concentrations were determined after digestion with 4% HNO_3 in a heat block at 70ºC and were measured by inductively coupled plasma-mass spectrometry (ICPMS; Das *et al.*, 2012a). Indium was added to the samples as an internal standard and samples were run on ICPMS using a Varian 820 instrument (Varian Canada Inc., QC, Canada) using analytical methods similar to those described by Das *et al.* (2012a). Standard solutions were prepared from 1000 mg/L Ag in 2% HNO_3 in milliQ H_2O. The quadrupole was operated in peak hopping scan mode with a dwell time of 10 ms for monitoring ^{107}Ag and ^{115}In. The method parameters (ion optics, nebulizer and plasma perimeters) were auto-optimized by Varian expert Software (Varian Canada INC, QC, Canada) using 5 mg/L tuning solution of Be (beryllium), In, and Th (thorium).

2.2.9. Statistical Analysis

Statistical analysis was carried out using SAS 9.2 (SAS Institute Inc, NC, USA). A 2-way Repeated Measures ANOVA was used to test for differences and interactions

between treatments and experimental weeks. Where significance was found, differences between weeks and treatments were examined using a Holm-Sidak post hoc test ($p<0.05$).

2.3. Results

2.3.1. Effects of chronic PVP exposure

TAg concentrations in the chronic-PVP mesocosms increased over time especially at the medium and high doses (Figure 2). There was no significant difference between the TAg concentrations between low dose and the zero-added enclosures. Low level Ag contamination occurred in no Ag added enclosures due to the unplanned addition of a small volume of water from high dose enclosures from the release of the dead volume within an autosampler. In this experiment, bact Ag concentrations increased over weeks 1-5, particularly in high and medium doses, with a period of no change or decline (weeks 5-7). Bact Ag accounted for 0.4-1.5% of the total silver we sampled in the chronic treatments and 4% in the pulse treatment (See Appendix, Table 2, 3 & Figure 2 for more information on bacterial silver accumulation and fate). While there were temporal changes over the study, we found no effect of chonic PVP-AgNP exposure on TBACT and bacterioplankton carbon (Figure 2). For bacterial production, we observed an increase in production in weeks 5 and 6 in enclosures receiving the highest doses but no effects at lower doses (Figure 2). There was also no consistent effect or interactive effect with time of PVP-AgNP exposure on bact chla (Figure 2). We also found no significant treatment effects on bacterioplankton C:N, C:P and N:P ratios (Table 2).

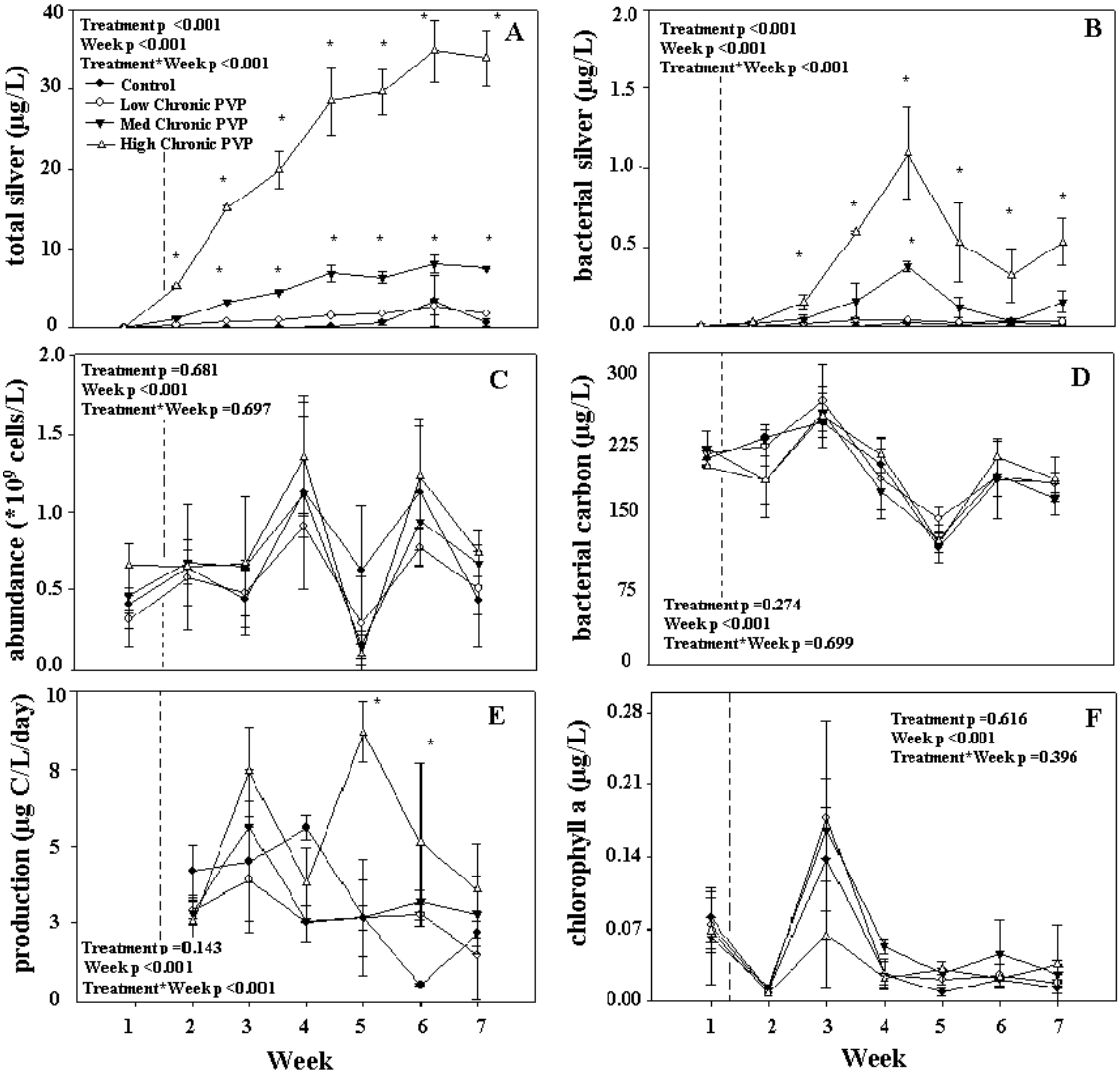

Figure 2: Silver and bacterioplankton responses to the continuous addition of different doses of PVP-capped AgNPs: A) total silver, B) bacterioplankton silver (0.8-1.2 µm), C) bacterial abundance, D) bacterioplankton (0.7-1.2 µm) carbon, E) bacterial production, and F) bacterioplankton (0.7-1.2 µm) chlorophyll a.

Vertical dotted lines denote the start of the PVP capped nanosilver dosing regimen (June 23rd, 2012). Shown are means and standard deviation of the 2 replicate enclosures. Also included on each panel are the p-values for the main and interaction effects of AgNP concentrations with time. * indicates significant differences between exposed and the zero enclosure on a given experimental week ($p < 0.05$). Note no production measurements were taken week 1.

Table 2: Main and interactive effects of chronic PVP, chronic citrate, and pulsed PVP exposure (Treatment) with sampling week (Week) on bacterioplankton C:N, C:P, and N:P ratios.

Presented are p-values computed from a two-way repeated measures ANOVA ($p<0.05$). We have noted the only significantly marginal effect with a *, see Appendix Figure 1 for more information.

Stoichiometric Ratio (mol)		PVP Chronic	Citrate Chronic	PVP Pulse
C:N	Treatment	0.940	0.775	0.209
	Week	0.655	0.574	0.247
	Treatment*Week	0.458	0.885	0.604
C:P	Treatment	0.768	0.682	0.799
	Week	0.165	0.057*	0.307
	Treatment*Week	0.413	0.196	0.978
N:P	Treatment	0.905	0.809	0.598
	Week	0.791	0.641	0.308
	Treatment*Week	0.609	0.599	0.971

2.3.2. Effects of chronic citrate exposure

TAg and bact Ag concentrations increased at similar rates in the high dose citrate and PVP mesocosms over the course of the experiment (Figure 3). Bact Ag in the citrate AgNP enclosures showed a temporal pattern of early increase and late stasis similar to that seen in the PVP-AgNP enclosures (Figure 3). There was also no difference in TBACT and bacterioplankton carbon between the PVP and citrate-AgNP enclosures. While there was a time-related dynamic in bacterial abundance and biomass, but no interactive effect or main AgNP treatment effect was detected (Figure 3). Bacterial production was higher in the chronic PVP-AgNP enclosures than the citrate-AgNP enclosures receiving approximately the same quantity of AgNP (Figure 3). As for many of the other variables, there was no difference between PVP- and citrate-AgNP on bact chla (Figure 3). Bacterioplankton C:N, C:P and N:P ratios were also not significantly

affected by citrate-AgNP additions (Table 1). In the high continuous treatments with citrate and PVP capped AgNPs, there was a marginally significant ($p=0.057$) effect of time, as in week 5 the bacterioplankton C:P ratios decreased, but there were no significant differences in ratios observed between any of the treatments or in any other weeks.

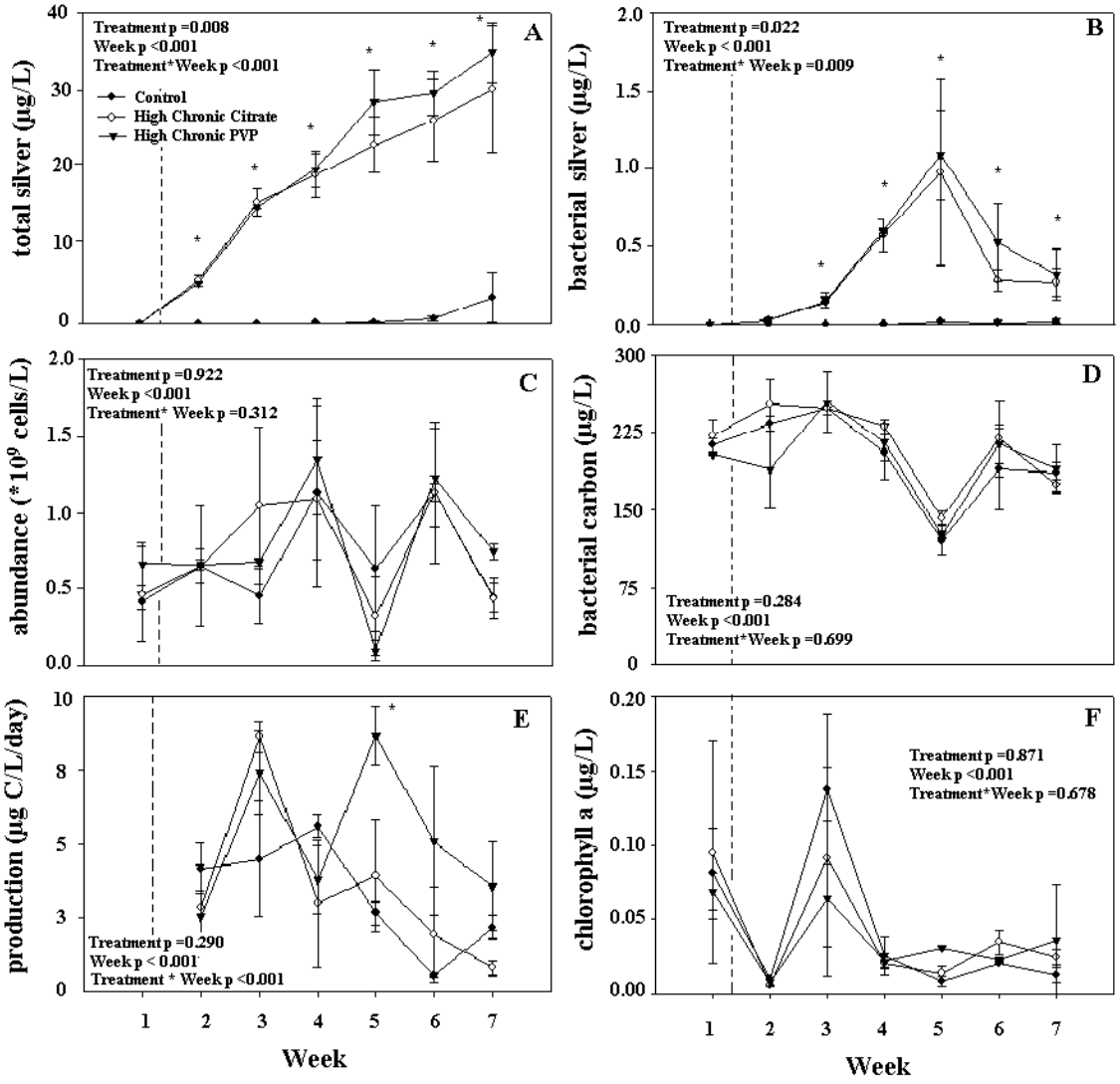

Figure 3: Silver and bacterioplankton responses to the continuous addition of citrate-capped and PVP-capped AgNPs: A) total silver, B) bacterioplankton silver (0.8-1.2 µm), C) bacterial abundance, D) bacterioplankton (0.7-1.2 µm) carbon, E) bacterial production, and F) bacterioplankton (0.7-1.2 µm) chlorophyll a.

Vertical dotted lines denote the start of the PVP capped nanosilver dosing regimen (June 23rd, 2012). Shown are means and standard deviations of the 2 replicate enclosures. Also included on each panel are the p-values for the main and interaction effects of AgNP concentrations with time. * indicates significant differences between exposed and the zero enclosure on a given experimental week ($p < 0.05$). Note no production measurements were taken week 1.

2.3.3. Effects of pulsed PVP exposure

In the pulse experiment, TAg declined over time (Figure 4), with a half-life for the decline in TAg concentration after the initial spike of approximately 20 days (Furtado *et al*., in press). A portion of this Ag in pulse addition was transferred to the bacterial fraction as we observed an immediate increase in bact Ag and then subsequent decreases in this Ag pool (Figure 4b, Appendix Table 2). TBACT decreased following the pulse Ag addition in week 5 (Figure 4), but this coincided with similar reductions in bacterial population in no silver Ag added enclosures. While bacterial abundance declined, we found the opposite for bacterial production with increases in the week following the large single dose of PVP capped AgNP. This significance persisted until the end of the experiment (Figure 4). No effects of treatment on bact chla were observed in the enclosures receiving the pulse Ag addition (Figure 4) nor did it affect bacterial elemental composition (Table 1).

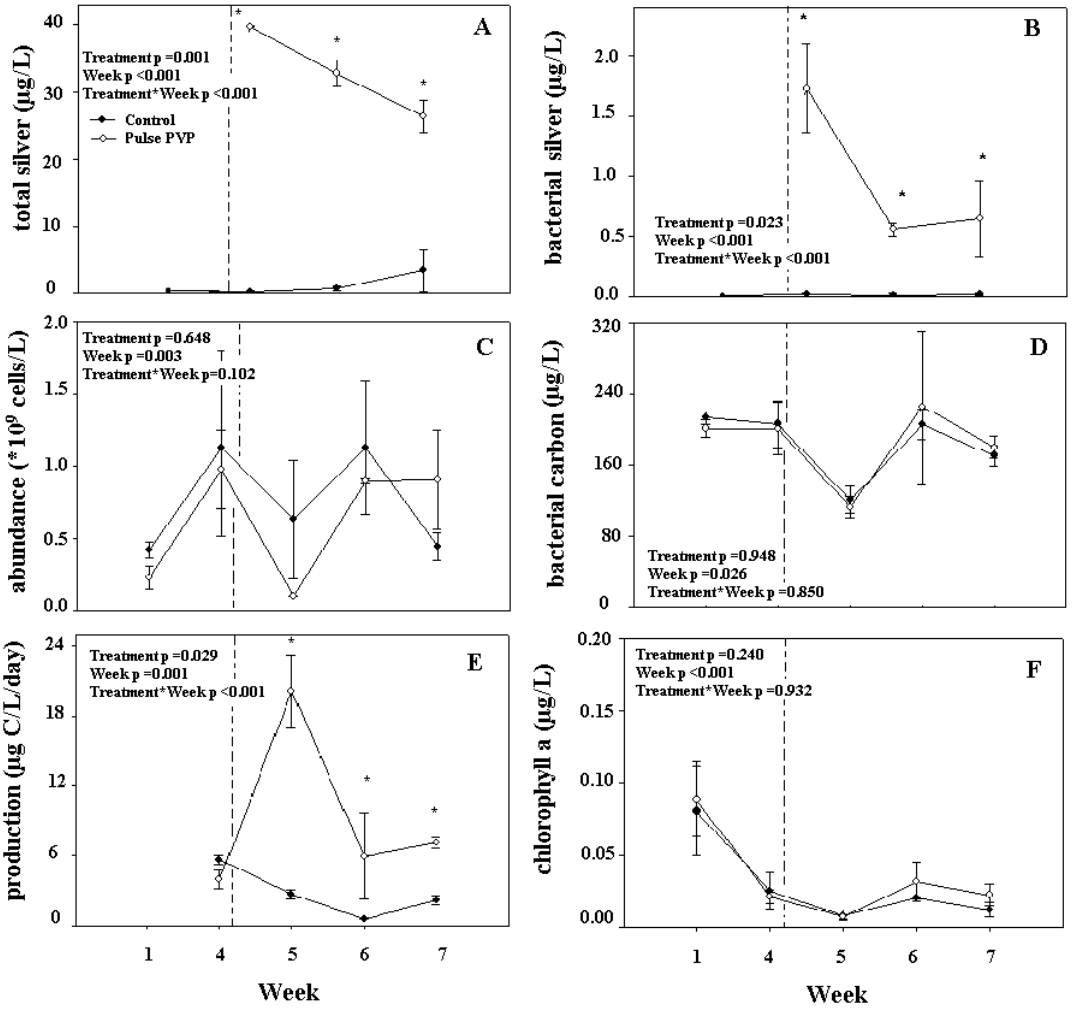

Figure 4: Silver and bacterioplankton responses to the pulsed addition of PVP-capped AgNPs: A) total silver, B) bacterioplankton silver (0.8-1.2 µm), C) bacterial abundance, D) bacterioplankton (0.7-1.2 µm) carbon, E) bacterial production, and F) bacterioplankton (0.7-1.2 µm) chlorophyll a.

Vertical dotted lines denote the start of the PVP capped nanosilver dosing regimen (July 11th, 2012). Shown are means and standard deviations of the 2 replicate enclosures. Also included on each panel are the p-values for the main and interaction effects of AgNP concentrations with time. * indicates significant differences between exposed and the zero enclosure on a given experimental week ($p < 0.05$). Note no production measurements were taken week 1.

2.4. Discussion

We monitored responses of natural bacterial communities to the addition of AgNPs in lake mesocosms. Our results show that PVP and citrate capped AgNPs generally produce little response in the abundance, biomass and elemental composition of natural bacterioplankton. These observations are quite different from laboratory studies where the inhibition of bacterial assemblage function and reductions in their abundance are commonly observed at AgNP concentrations of <100 µg/L (Morones *et al.*, 2005; Choi *et al.*, 2008; Li *et al.*, 2010). However, similar to our results, minimal impacts on bacteria were reported by others (Bradford *et al.*, 2009; Gao *et al.*, 2011; Colman *et al.*, 2012) from *in situ* studies of estuaries, sediments and wetlands. We found evidence that AgNP exposure increased bacterial production although the mechanisms for this increase were not clear. Consequently, our results indicate that AgNP toxicity will differ in natural environments perhaps due to the multi-species composition of bacterial communities and chemical interactions between AgNPs and surface waters. Our results thus add to a growing understanding of AgNPs toxicity on bacterial communities in natural aquatic environments.

2.4.2. Measured silver in the environment and bacteria

Our results show that Ag accumulated in the water column in enclosures and in the bacterioplankton over the course of the experiment. This increase in total and bacterial Ag demonstrates that we successfully altered the exposure of bacterioplankton to Ag largely as planned. While we found increasing total Ag within enclosures, dissolved Ag^+ was at or below the limits of detection (Furtado, MSc Thesis, 2014). This absence of dissolved Ag^+ likely reflects its rapid binding by DOM (Gao *et al.*, 2009) and may explain the absence of toxicity in the mesocosms. Other ligands (e.g. sulfur-

containing molecules) can also precipitate or flocculate AgNP, which would reduce the exposure to biota and negative antimicrobial effects (Navarro *et al.*, 2008; Stebounova *et al.*, 2011; Xiu, Ma & Alvarez, 2011; McTeer *et al.*, 2014). Here, AgNP and Ag^{+} ions may have formed complexes with sulfur-containing molecules in DOM (Choi *et al.*, 2009; el Badawy *et al.*, 2010; Unrine *et al.*, 2012), which increases the stability of the AgNP and reduces its toxicity (Chappell *et al.*, 2011; Bone *et al.*, 2012; Unrine *et al.*, 2012; Guo *et al.*, 2013). Consequently, we raised total Ag concentrations within the enclosures and exposed bacterioplankton to different types and concentrations of AgNP, but the toxicity of these materials may have been reduced by DOM and other ligands. In the chronic PVP-AgNP experiment, we continuously added this type of nanoparticle at different concentrations to mesocosms for seven weeks. For most variables, we found limited or no toxicity and no obvious relationship between our primary response variables and AgNP concentration. During the experiment, we observed bacterial Ag to increase proportionally with the quantity of AgNP added, but this was only detectable amount at the medium and highest doses. Even at these higher doses, we found no strong toxic effects on bacterioplankton abundance or carbon. Instead, the synchronous changes in these variables are strongly indicative of external forces (e.g., temperature, nutrients, and predators) of controlling the bacterial community.

2.4.3 Comparison of citrate and PVP capped AgNPs

The chronic effects of nanosilver could also depend on its capping agent, which can alter the solubility and reactivity of these particles (Tejamaya *et al*, 2012). We found no evidence that there were differences in toxicity to natural bacterial communities between PVP and citrate capped AgNPs. One difference we observed was an increase in bacterial production in one week in communities exposed to PVP-capped AgNPs but no

such increase with citrate-capped AgNPs (Figure 3). This could be merely a transient effect, but previous studies have shown that PVP-capped AgNPs are more persistent/stable compared to citrate capped AgNPs (Huynh & Chen, 2011; Tejamaya *et al.*, 2012). The stability of PVP AgNPs could allow them to persist in the water column longer and exert greater effects on natural microbial communities. However, we did not see this pattern in either TAg concentrations or bacterioplankton Ag fractions, as concentrations in treatments with both citrate and PVP capped concentrations were not significantly different from one another (Figure 2). It is possible that PVP capped AgNPs were bound less strongly by aquatic ligands and this increased their toxicity. While PVP capped AgNPs appear to remain bioavailable even after partial sulfidation in wetland ecosystems (Lowry *et al*. 2012), whether this differentiates them from citrate-capped AgNP is not clear. Nonetheless, our results demonstrate that little toxicity difference between the two capping agents studied here.

2.4.4. Comparison between chronic and pulse AgNP additions

We also found little difference in bacterioplankton responses between a single large pulse and the highest dose of chronic additions of AgNP. While the total Ag concentrations, at their highest, was similar between these two different exposure regimes, the method of addition was very different with one increasing over seven weeks and one over a few minutes. Despite this rapid increase, we did not observe substantially different results (e.g., acute or severe toxicity) in the pulse enclosures. The pulse addition of PVP AgNP was followed by increased bacterial production, similar to that observed in the highest dose of chronic PVP AgNP, which is further evidence that AgNP altered some aspect of the bacterial community dynamics, or possibly by influencing higher trophic levels leading to reductions in grazing pressure. Consequently, the toxicity of

AgNP appears generally limited to bacterioplankton even under conditions (e.g., single large dose) that are most likely to produce the most severe effects.

2.4.5 AgNPs effects on bacterioplankton

AgNPs are known for their antimicrobial properties and have been demonstrated to negatively impact biofilm development and bacterial species composition in marine waters (Fabrega *et al.*, 2009). It is thus perhaps surprising that we found no large or sustained changes in the bacterial communities over the course of our seven week experiment. Unlike studies with single strain lab cultures, on which much of the AgNP literature is based upon, natural bacterial assemblages can harbor high diversity, which has been shown to make a community after more resistant to perturbations (Girvan *et al.*, 2005; Baho, Peter & Tranvik, 2012; Bouvier *et al.*, 2012). While we did not assess microbial diversity in this study, bacterial communities are generally composed of taxa that respond to AgNPs as a stressor in four ways: intolerant, affected but recover, tolerant and stimulated (Das *et al.*, 2012b). A shift in the taxonomic composition of the bacterial community from sensitive to tolerant taxa may have reduced or eliminated the negative effects of AgNP. In addition, high quantities of DOM may have altered AgNP chemistry and led to its removal from the water column or reduced bioavailabilty (Chapell *et al.*, 2011; Guo *et al.*, 2013; Furtado *et al.*, in press). Finally, our weekly sampling regime may also partly account for the absence of observed treatment responses to bacterial abundance, bacterial stoichiometry, and bacterial chlorophyll a. By sampling once per week, the bacteria could have fully recovered in number, stoichiometry and chlorophyll a to initial acute AgNP exposures.

2.5. Conclusion

We assessed the responses of natural pelagic lake bacterioplankton communities under realistic environmental conditions and exposure scenarios to two differently capped AgNPs. We found that AgNPs did not negatively affect natural bacterial communities over six weeks, this does not rule out a possible loss of community function, which could be occurring as the result of a community shift. Exposure to PVP capped AgNPs created conditions for increased bacterial production at TAg concentrations at or greater than 30 µg/L. These responses are likely due to selection of tolerant taxa or changes in the biomass of other resource consumers (e.g., algal), or changes in grazing pressure due to toxic effects on higher trophic levels (e.g., protists). Introductions of AgNPs at realistic environmental concentrations (<5 µg/L) may not necessarily have adverse effects on natural bacterial communities but may still affect ecosystem services. Future work should assess changes in bacterioplankton diversity and functions as this will help better determine the causes of limited AgNPs toxicity in nature and its implications for lake ecosystems.

Chapter 3: Interactive influence of silver nanoparticles and phosphorus supply on lake bacterioplankton

3.1. Introduction

Silver nanoparticles (AgNPs) are currently the most widely used nanoparticle in commercial and industrial enterprises (www.nanotechproject.org). Due to their antimicrobial properties (Langenheder *et al*. 2010), AgNPs are now found in a variety of medical textile and household items (Salata 2004; Buzea *et al*. 2008; Sharma *et al*. 2010; Sotiriou & Pratsinis 2010). Recent increases in the use of AgNPs mean that there is a greater likelihood that they will reach the aquatic environment both through waste water discharge and from other indirect sources (Benn & Westerhoff 2007; Blaser *et al*. 2008; Mueller & Nowack 2008; Gottschalk *et al*. 2009). Once released into the aquatic ecosystem, AgNPs could negatively affect microbial communities, and this could compromise ecosystem function and the ecosystems services which they provide, such as decomposition and nutrient cycling (Throback *et al*. 2007; Choi & Hu, 2008; Choi *et al*. 2008).

In the laboratory, AgNPs have been demonstrated to reduce and inhibit growth of cultured strains of *E. coli* (Morones *et al*. 2005), alter bacterial peptide profiles (Shrivastava *et al*. 2007), destabilize membranes resulting in the disruption of ATP production (Lok *et al*. 2006), and cause the formation of pits and gaps in the membrane (Li *et al*. 2010). One mechanism responsible for the negative effects of AgNPs on microbes has been, in part, attributed to the release of Ag^+ ions, although the precise mechanisms for toxicity remain in contention (Fabrega *et al*. 2011). Recently, AgNPs have been shown to negatively affect natural bacterial communities by reducing their abundance, production, diversity and extracellular enzyme activity in the water column

and sediments of various aquatic ecosystems (Wigginton *et al*. 2010; Das *et al*. 2012a, b; Doiron et al, 2012).

Investigations of AgNP toxicity on aquatic microbes have not typically considered the importance of natural environmental factors. The applicability of laboratory studies to natural environments is unclear, given the differences between laboratory conditions and natural aquatic ecosystems. In particular, matrix ionic strength (high in cultures) can affect the toxicity of AgNP by influencing the precipitation of Ag^+ (Stebounova *et al*. 2011; Huynh & Chen, 2011). Dissolved organic matter (high in nature) may reduce AgNP disassociation or bind free Ag^+, both of which would reduce AgNP toxicity (Fabrega *et al*. 2009; Liu & Hurt, 2010). Lab cultures are typically selected for rapid growth, in nutrient balanced and rich media; whereas lake communities are typically nutrient limited and unbalanced, with communities composed of a vast range of taxa. The abundance, activity and stoichiometry of bacterioplankton communities will vary with environmental conditions and this could affect AgNPs toxicity to bacterioplankton (Van der Gucht *et al*, 2005; Bian *et al*, 2013). Viral lysis of bacterial cells is important in the natural bacterial loop which is not considered as a factor when assessing bacterial communities' responses in the laboratory. AgNP toxicity may also be mediated by free cations (e.g., phosphorus) in the water that can precipitate out mineral forms of silver (Xiu, Ma & Alvarez, 2011).

Another potentially important environmental controller of AgNP toxicity is nutrient supply. The toxicity of chemicals to aquatic organisms may vary with their nutritional state (Frost *et al*. 2005; Hansen *et al*. 2008; Lessard and Frost 2012). The nutritional status of bacterial communities are often inferred from their C:N, C:P, and

N:P ratios (Sterner and Elser 2002, Wagner *et al*. 2013). It has been shown that AgNP toxicity to algae depends not only on dose but on algal P content, suggesting that P deficient cells are more susceptible to AgNP toxicity (Das *et al*, 2014).

Changes to bacterioplankton community stoichiometric ratios can provide information on cellular responses to chemical stress. AgNPs could result in altered bacterial C:N:P ratios in a number of ways. For instance, AgNPs could increase the cellular demand for N and P. Exposed cells may increase the production of stress-response proteins, which requires more N- and P- for ribosomes and proteins (Elser *et al*, 1996). Such responses, should increase the uptake of N and P by bacterial communities and lower C:N and C:P ratios. AgNPs could also impair uptake and transport processes of N and P, or compromise the cell membrane integrity, which could lead to increased C:N and C:P ratios. Such changes in bacterial stoichiometry could alter the balance of elements cycling within and among trophic levels (Sterner & Elser, 2002). Altogether, there is a need to better understand how AgNPs affect the metabolic processes of natural bacterial communities, including their demand for and uptake of dissolved nutrients.

We examined the responses of natural bacteria communities in two boreal lakes at the Experimental Lakes Area, ON, Canada, to exposure to polyvinylpyrrolidone (PVP) capped AgNP in bags suspended *in situ* in the water column. Specifically, we quantified changes in the abundance and elemental composition of bacterial communities exposed to three concentrations of AgNP and two concentrations of dissolved phosphorus (P). We predicted bacterial abundance would decrease with increasing concentration of AgNP but that these effects would change with P supply, such that P-limited communities would be more susceptible to AgNP toxicity. It was also expected that differences would emerge

between responses in the two lakes, with bacteria in the humic-rich lake (i.e. L222) experiencing less toxicity compared to the bacteria in a more algal-rich lake (i.e. L114) due to particle stabilization and reduced dissolution of Ag^+ through complexation with humic substances (Fabrega *et al*, 2009).

3.2. Methods

3.2.1. Study Locations

We conducted our experiments at the Experimental Lakes Area (ELA) in northwestern Ontario using microbial communities from two different experimental lakes. L114 is a mesotrophic lake that has relatively high ambient phosphorus concentrations and elevated algal biomass during the summer (Table 3).

Table 3: Lake Characteristics for Experimental Lakes 114 and 222.

Lake	DOC (mg C/L)	Seston Chlorophyll α (µg/L)	Total dissolved nitrogen (µg/L)	Total Phosphorus (µg P/L)
114	8.6	13.7	353.5	10.5
222	10.8	3.3	367.2	7.5

Average values for each variable were taken over four weekly sampling dates spanning the end of July until early August 2012.

Compared to L114, L222 is phosphorus-poor, with a higher dissolved organic carbon concentration (DOC). The DOC and total dissolved nitrogen in both lakes are typical of boreal forest systems. L222 has significantly less phytoplankton biomass compared to L114 as evidenced by its lower chlorophyll a (chl a) concentrations (Table 3).

3.2.2. Experimental Design

The experiments were conducted *in situ* in the lakes by suspending bags of water containing their natural bacterial communities. To begin each experiment, we collected

water one meter from the surface, screened it through a 35 µm mesh and then filtered it in the laboratory through 0.2 µm polycarbonate 142 mm (PCTE) membrane filters (Millipore, Toronto, Canada). The filtrate was refrigerated at 4ºC until transported back to each respective lake, where it was mixed with whole lake water (also 35 µm screened) at 60% filtrate to 40% whole lake water ratio, (to reduce competition and allow room for growth) and placed into 1 L whirlpak bags (Nasco, WI, USA). We manipulated AgNP and total dissolved P (TDP) to produce 6 treatment combinations with each AgNP-P level replicated three times in each lake. Each bag then received one of three AgNP (0, 10, or 80 µg Ag/L) and one of two TDP (0 or 20 µg P/L) additions. We used PVP capped AgNPs as described below, and phosphorus was added in the form of sodium phosphate monobasic ($NaPO_4$-P) (Sigma Aldrich, MO, USA). Each bag received a spike of 500 µg/L NO_3-N as $NaNO_3$, in order to reduce the likelihood of acute N limitation during our experiment. The bags were sealed, gently inverted (to ensure adequate mixing) and secured by tethering them to a floating pole attached to a buoy at the deepest part of the lake, where they were left floating at the surface for 72 hours. Incubations of 72 hours were chosen to ensure sufficient time for exposures of AgNPs and P enrichment effects to be observed, while avoiding situations of nutrient limitation over longer time periods. Water samples were collected in triplicate upon the start of our incubation, screened through 35µm mesh and processed in the exact manner which is described below for our experimental treatments. These samples were called “time zero” and served as a baseline for each assessed variables.

3.2.3 AgNP Characterization

A PVP capped BioPure nanosilver solution was purchased from nanoComposix, Inc (San Diego, CA). The nanoparticles were received as purified monodispersent

spheres at a concentration of 1.0 AgNP mg/mL in milliQ water. The stock concentration was confirmed by acid digestion and inductively coupled plasma mass spectrometry (ICP-MS) using analytical methods as described by Das *et al*. (2012a). Information from the manufacture indicated that the particles had a diameter of 48.3 nm, as determined using TEM and a median hydrodynamic diameter of 56.3 nm and surface charge of -33.9 mV, as determined by Dynamic Light Scattering (DLS).

3.2.4. Sample Processing

Following the 72 hour *in situ* incubation, we collected the bags and returned them to the laboratory for processing. Samples of 10 mL and 4 mL were removed from each bag and stored as a 4% solution with HNO_3 for analysis total Ag (TAg), and preserved in a 10% formaldehyde solution for total bacteria abundance (TBACT). The remaining water (986 mL) was passed through a GF-C filter (nominal pore size 1.2 µm, Whatman, NJ, USA). Subsequently, 50 mLs of the GF-C filtrate was passed through a 0.8 µm polycarbonate filter (Isopore Cork, Ireland), which was preserved in 5% nitric acid. This filter was saved in the fridge at 4ºC for analysis of total Ag and total Ag bound or accumulated by the bacterioplankton.

Two aliquots of the remaining GFC filtrate were filtered through ashed GF-F filters (nominal pore size 0.7 µm, Whatman, NJ, USA). These filters were dried at 60ºC and used for analysis of particulate carbon (C), nitrogen (N), and P. The remaining GF-F filtrate was filtered through a 0.2 µm membrane filter and frozen (-20ºC) until analysis for TDP.

3.2.5. Total Bacteria (TBACT)

TBACT was enumerated on a flow cytometer after staining with SYBR Green Stain I (Invitrogen-Thermo Fisher Scientific, MA, USA). A Cytonomics FC 500 flow

cytometer with CXP data processing software (Beckman Coulter, CA, USA) was used for all measurements (Marie *et al*. 1997). Cell counts were determined by adjusting for dilution with formalin and SYBR Green I, then multiplying the adjusted particle count by the flow rate and dividing by the time (1 minute) that the flow cytometer was running for.

3.2.6. Particulate Carbon, Nitrogen & Phosphorus

Particulate C and N were analyzed in duplicate using a Vario EL III CN analyzer (Elementar, Hanau, Germany). Phosphorus analysis for both TDP and Particulate P was determined after persulfate digestion with the molydbate-blue reaction (APHA, 1992). For bacterio-chlorophyll, filters were stored frozen (-20ºC) until extraction. A 24 h dark ethanol extraction (EPA method 446.0) was performed and absorbance was measured at 885 nm on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, CA, USA).

3.2.7. Total and Bacterioplankton Silver

Silver concentrations were determined by ICP-MS, essentially as described in Das *et al*. (2012a). All samples were digested using a 4% HNO_3 digestion in a heat block at 70ºC. For cell-associated bacterial Ag (bact Ag), Ag filters were removed after the digestion and the remaining solution was filtered through at 0.7 µm Whatman GF-F filter. Indium (In) was added to the samples as an internal standard and samples were run on inductively coupled plasma-mass spectrometry using a Varian 820 instrument (Varian Canada Inc., QC, Canada). Standard solutions were prepared from 1000 mg/L Ag in 2% HNO_3 in milliQ water. The ICP-MS was operated in peak hopping scan mode with a dwell time of 10 ms for monitoring ^{107}Ag and ^{115}In. The method parameters (ion optics, nebulizer and plasma parameters) were auto-optimized by Varian expert Software

(Varian Canada Inc., QC, Canada) using a 5 mg/L tuning solution of Be (beryllium), In, and Th (thorium).

3.2.8. Statistical Analysis

Statistical analysis was run using SAS 9.2. (SAS Institute Inc., NC, USA) Differences from time zero (baseline measurement) were taken and a 2-way analysis of variance (ANOVA) test using AgNP and TDP levels as independent variables with a Type III sum of squares was employed to test for treatment and interaction effects. A Tukey test (corrected to control experiment-wide error) was employed to discern any significant differences between treatment combinations.

3.3. Results

3.3.1. Recovery of Added AgNPs and P

TAg analysis showed that there was generally a greater recovery of the added Ag in treatments with L222 water than in L114. The highest percent recovery was observed in treatments with addition of 80 µg/L AgNPs in both lakes (Table 4).

Table 4: Recovered average TAg and percent recovery of Ag in each AgNP and P exposure (all units µg/L).

P	AgNP	Lake 114 TAg	Lake 114 % Ag recovery	Lake 222 TAg	Lake 222 % Ag recovery
0	10	7.3	73.1	9.4	94.3
0	80	61.1	76.4	78.7	98.4
20	10	7.4	73.8	7.7	77.3
20	80	73.2	91.5	79.6	99.5

Calculated: % recovery= (TAg fraction recovered)/ (AgNP exposure level-10 or 80 µg/L))*100%

The highest percent P recovered was observed in L114 treatments with nominal 80 µg/L AgNPs addition. Conversely, the lowest percent P recovered was observed in the same treatment (e.g. 80 µg/L) in L222 (Table 5). Unaccounted Ag and P was likely due to the Ag being incorporated into algae or other biomass not measured by our techniques

(e.g., algal uptake > 1.2 µm, Das *et al*, 2012 a) or as a result of adsorption to the plastic bags. However, our data on Ag and P indicate that we did manipulate Ag and P as planned during these experiments.

Table 5: Percentage recovery phosphorus, based upon P and AgNP treatment additions (all units µg/L).

P	AgNP	Lake 114 TDP + BPP	Lake 114 % P recovery	Lake 222 TDP + BPP	Lake 222 % P recovery
20	0	12.8	63.9	5.0	24.8
20	10	11.1	55.3	6.3	31.5
20	80	14.5	72.7	1.8	9.1

Calculated: %recovery= (Bacterioplankton particulate P (BPP) + TDP)/ (20 µg/L P)* 100%.

3.3.2. Response of Total Bacterial Abundance (TBACT) and Carbon Biomass

TBACT was significantly influenced by the P supply and AgNP addition in both lakes. We used two-way ANOVAs to examine for interactive effects of AgNPs and P in each lake. In L114, AgNP and P supply both significantly affected TBACT, whereas they had an interactive effect on carbon biomass. These effects reflected the significant increases in TBACT and carbon at concentrations of 20 µg/L P but only in bags in L114, where we added 10 and 80 µg/L AgNPs in L114 (Figure 5). In contrast, a decrease in bacterial abundance was observed in L222 in response to the AgNP (80 µg/L) treatment. In that lake, we observed neither effects of P supply nor any interaction between P supply and AgNP addition on TBACT. In L222, we found no change in carbon biomass resulting from exposure to AgNP or P (Figure 5).

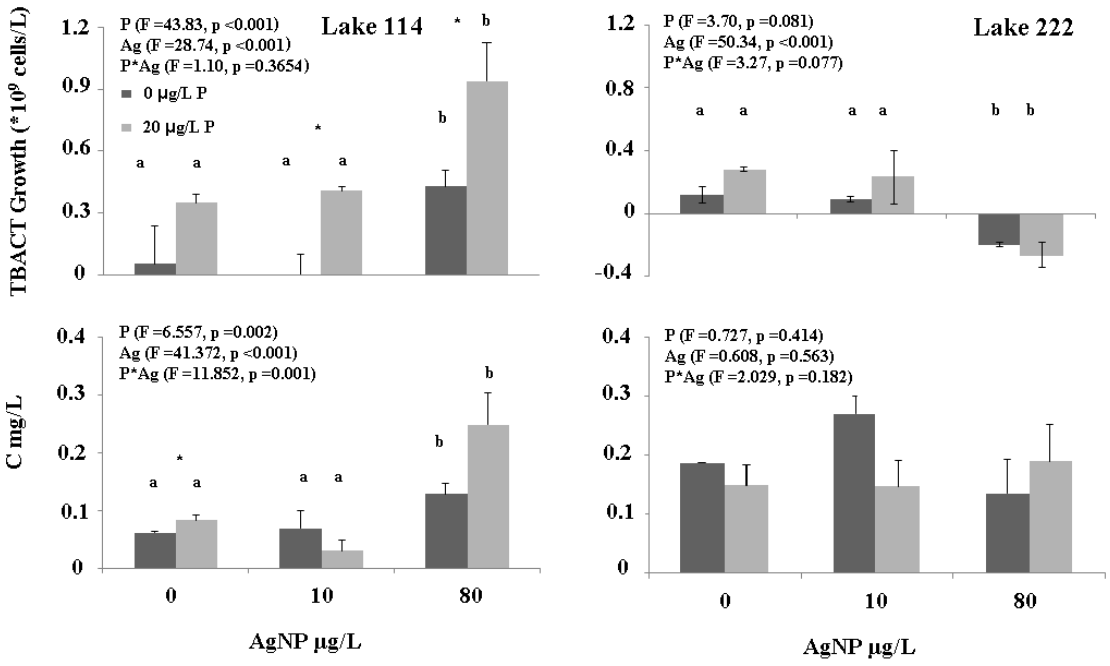

Figure 5: Net Bacterioplankton Abundance and Carbon Biomass in response to three AgNP and two P exposure levels.

Values given following the 72 hour *in situ* incubation in response to two phosphate levels and a concentration gradient of PVP capped AgNPs**.** Error bars represent ± 1 standard deviation of the replicate (n=3). Shown are the main effects and interaction statistics (F ratios and p value) determined using a 2-way ANOVA (Type III SS) run in SAS. Pairwise comparisons (corrected to control experiment wide error -Tukey tests) were employed to determine differences between the treatments. Differences in letters indicate significant differences between Ag treatments at $p<0.05$. * indicates differences between P treatments (0-20 µg/L) at $p<0.05$.

3.3.3. Bacterioplankton Ag (bact Ag)

The bact Ag (0.8-1.2 µm) size fraction was affected by AgNP exposure in both lakes. While the accumulation of Ag within this size fraction was highest at 80 µg/L AgNPs in both lakes, we found more Ag accumulated in bacterial communities in L114 than L222 (Figure 6). Bacterial Ag:C molar ratios were significantly affected in both lakes by exposures to AgNPs (Figure 6). We also found an interactive effect between AgNP and P supply on the bacterial Ag:C ratio L114. In both lakes, the Ag:C molar ratio increased with the addition of AgNPs. In L114 the bacterial Ag:C molar ratio increased at

low AgNP additions but this effect was absent at the higher AgNP concentration (80 µg/L) where the ratio remained the same (Figure 6).

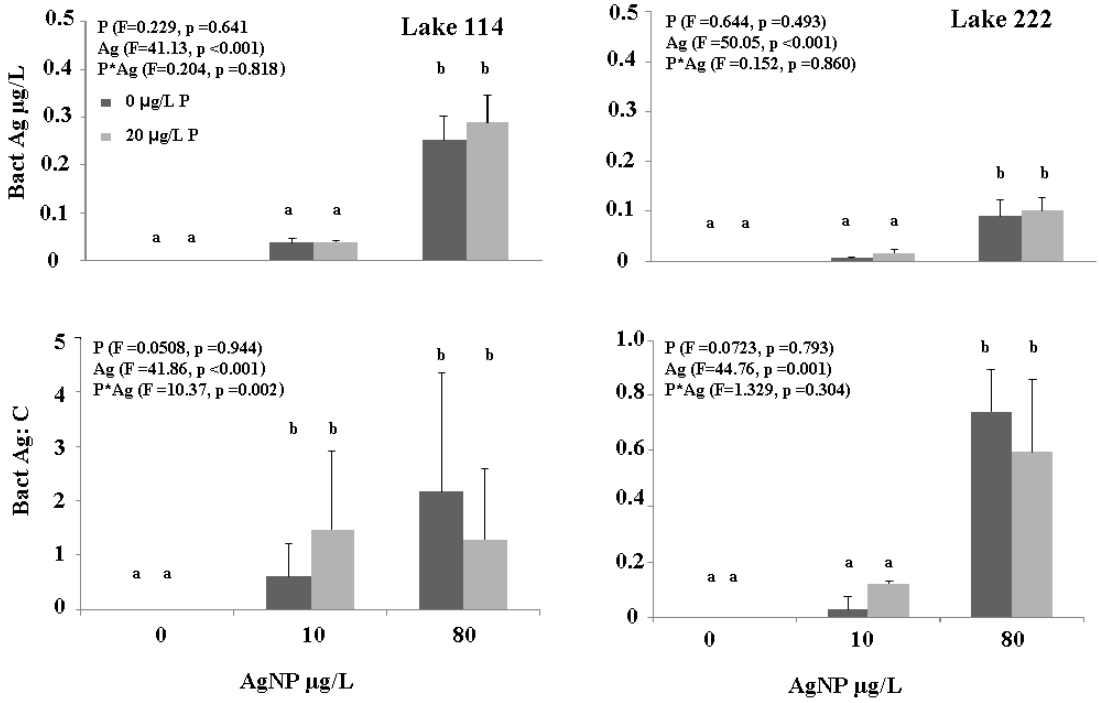

Figure 6: Bacterioplankton bound Ag and C:Bact Ag molar ratios in response to three AgNP and two P exposures.

Values given following the 72 hour *in situ* incubation in response to two phosphate levels and a concentration gradient of PVP capped. Error bars represent ± 1 standard deviation of the replicate (n=3). Shown are the main effects and interaction statistics (F ratios and p value) determined using a 2-way ANOVA (Type III SS). Pairwise comparisons (corrected to control experiment wide error-Tukey test) were employed to determine differences between the treatments. Differences in letters indicate significant differences between Ag treatments at $p<0.05$. * indicates differences between P treatments (0-20 µg/L) at $p<0.05$.

3.3.4. Response of Bacterioplankton C:N:P Stoichiometry

We found AgNP generally reduced bacterial C:N ratios but these effects interacted with P supply in both lakes. In L114, bacterial C:N ratios decreased with the increased P supply at concentrations of 0 and 10 µg/L AgNP. However, increased P

supply did not affect C:N ratios at the highest AgNP concentration (80 µg/L). In L222, increasing AgNP reduced bacterial C:N ratios at low P supply, but this effect was largely absent at the high P concentration (Figure 7). C:P ratios were significantly affected by an interaction between AgNP and P supply, albeit only marginally in L114 ($p=0.058$) as shown in Figure 7.

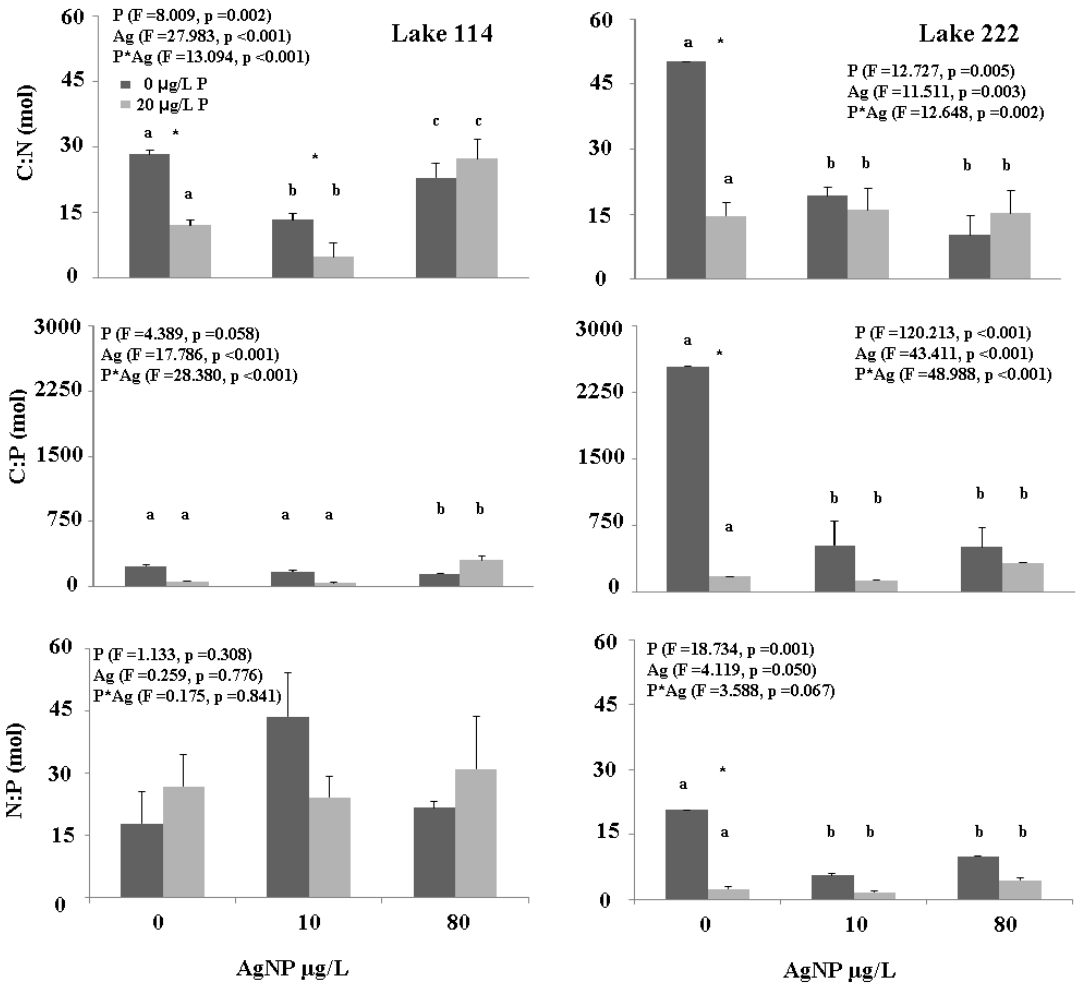

Figure 7: Bacterioplankton C:N:P stoichiometry (C:N, C:P & N:P)

Values given following the 72 hour *in situ* incubation in response to two phosphate levels and a concentration gradient of PVP capped Error bars represent ± 1 standard deviation of the replicate (n=3). Shown are the main effects and interaction statistics (F ratios and p value) determined using a 2-way ANOVA (Type III SS). Pairwise comparisons (corrected to control experiment wide error-Tukey test) were employed to determine differences between the treatments. Differences in letters indicate significant differences between Ag treatments at $p<0.05$. * indicates differences between P treatments (0-20 µg/L) at $p<0.05$**.**

The nature of changes in C:P ratios generally mirrored those for C:N ratios with effects of AgNP differing at high and low P supply and between lakes (Figure 7). For example in L222, AgNP reduced bacterial C:P ratios at low P supply but not at high P supply. N:P ratios were not affected by AgNP or P supply in L114 but were affected by

these variables in L222. In this lake, bacterial N:P ratios decreased with increased AgNP, but this was an effect that was diminished at high P supply (Figure 7).

3.4. Discussion

In *in situ* experiments conducted in two different lakes, we found natural bacterial communities were significantly affected by exposure to AgNPs, but these effects varied depending on the lake of origin, the P supply, the concentration of AgNPs added and the response variable under consideration. For example in L114, we found AgNP addition increased bacterial growth especially at high P supply but this response was not found in L222. Given the site specific nature of responses in lake bacterioplankton to AgNP exposure, future work should carefully consider environmental relevance of results produced under highly artificial laboratory conditions.

3.4.2. Bacterial Community Response Differed Between Lakes

We found differences in the effects of AgNP on bacterioplankton abundance between L114 and L222. It is possible that bacterial communities are less affected by AgNP in L114 due to this lake's higher productivity compared to the more nutrient limited L222. Seston chlorophyll a is more abundant in L114 than L222 (Table 3). In L114, there may have been more algae (>1.2μm) in our experiment, which may have been more strongly affected by AgNP. If so, this reduced algal presence in our experiment would have reduced direct competition with bacteria for resources and increased their growth rate. In L222, there would have been a less stimulatory effect from reduced competition. Similarly, AgNP exposures could be felt differently by bacterial grazers (e.g. protists) in each site respectively; and a reduction in grazing pressure could explain increased TBACT growth observed in L114, but was not measured in this experiment. These results could also be the result of more dissociated phosphate ions in

L114, which can complex with released toxic Ag^+ ions to form silver phosphate, and reduce toxicity on exposed organisms (McTeer *et al*. 2014; Xiu, Ma & Alvarez 2011). In any case, we found a decline of TBACT at the high concentration of AgNP in L222 and an increase of TBACT in L114, effects which were clearly seen with increased P supply (20 µg/L). This result demonstrates the importance of considering ambient environmental conditions when assessing risk due to the introduction of AgNP into the environment.

3.4.3. Increased Bacterial Growth in L114

Given that AgNPs are known for their antimicrobial properties (Morones *et al*. 2005; Shrivastava *et al*. 2007; Yoon *et al*. 2007), the positive bacterial growth response we observed in L114 in AgNP-exposed communities was unexpected. The observed increased growth could be attributed to a community shift favoring silver tolerant or silver stimulated taxa within the community (Das *et al*. 2012b). Silver tolerant taxa would have a selective advantage when exposed to AgNPs and would likely further benefit from the increased nutrient availability resulting from either P enrichment or the decomposition of intolerant taxa. A hormetic effect whereby bacteria are stimulated by low doses of a toxicant and killed at a higher dosage may have also contributed to our results. Hormesis is an emerging phenomenon observed in nanotoxicology studies (Lavicoli *et al.* 2014). For example, when *E. coli* was exposed to low concentrations (<15 µg/L) of 18, 50 and 72 nm PVP capped AgNPs, the number of viable cells increased, compared to the control (Xiu *et al*. 2012). Another study showed that bacterial production, at low exposure concentrations (~10-20 ug/L) of AgNPs, exceeded control rates within 48 hours of exposure (Das *et al*. 2012a). As our experiment ran for 72 hours,

bacterial communities would have had time to be stressed and recover, which may explain the lack of any strong effect observed at 10 µg/L AgNP in both lakes. Given these differences between the effect of AgNP on bacterial abundance in L114 and L222, future work should focus on determining the mechanisms that account for lake-specific toxicity.

3.4.4. AgNP Exposure to Bacterial Communities

In both L114 and L222, bacterial Ag content increased across the AgNP exposure gradient (Figure 6), but was only significantly increased at AgNP (80 µg/L). Our results show that we altered the exposure of bacterioplankton to silver in both lakes. Interaction of AgNPs with DOC or ligands such as phosphate ions can result in precipitation or flocculation, leaving AgNP unable to interact with other particles, negating their antimicrobial effects (Navarro *et al*. 2008; Stebounova *et al* 2011). When contrasting the two lakes to one another, the lower bact Ag concentrations in L222, are likely related to its higher DOC concentrations. AgNPs and Ag^{+} ions can form complexes with sulfur-containing molecules among others in dissolved organic matter (DOM) (Bielmyer *et al*. 2002 & Choi *et al*. 2009), and these interactions with DOM often result in increased stability of the AgNP, thus less toxicity (Bone *et al*, 2012; Chappell *et al*, 2011; Guo *et al*, 2013; Unrine *et al*, 2012). Considering the relative amounts of DOC in each lake, it is interesting that a greater negative growth response is observed in bacteria from L222 than L114, and stresses the importance of site specific toxicity. We found bacterial-bound Ag to increase with AgNP exposure (increased bacterial Ag:C) in both lakes. No change in bacterial Ag:C was observed at high AgNP exposure in L114, but this can be attributed to the increase in TBACT observed in Figure 5. This is consistent with a similar nonlinear increase observed by Das (2014) between algae-silver interactions and their growth in

both the presence of AgNPs and P enrichment which should be investigated in future studies.

3.4.5. Variations in Bacterioplankton Community Stoichiometry

The effects of AgNP on bacterial stoichiometry varied between the two lakes and the two P supply concentrations. For example, bacterial C:N ratios decreased in L222 in response to increasing AgNP at the no P addition level, while AgNP did not affect C:N ratios at the higher level of P supply. The effects of AgNP on bacterial stoichiometry would be from changes in bacterial nutrient uptake and/or accumulation. The reduction in C:N ratios with AgNP exposure could either be from increased N uptake or greater C loss (perhaps due to higher respiration rates). The similarity in responses between C:N and C:P ratios suggests that AgNP exposure and P supply affect these two elements similarly. It is less clear why stoichiometric responses varied between L114 and L222. Part of this difference may be related to the nutrient deficiency observed in L222 bacterioplankton (high C:N and C:P ratios in no AgNP and no P levels) than in bacterioplankton from L114. Adding P would presumably have a stronger effect on L222 bacterioplankton, especially in the presence of greater P supply as they would be increasing their P uptake and altering their metabolism more. It is possible that this increase in available P enhances AgNP toxicity because many of these processes may be especially stress-sensitive.

3.5. Conclusion

Using *in situ* experiments, we assessed the responses of lake bacterioplankton communities from two lakes to AgNP and P supply. We found AgNP effects that were site specific, as evidenced by the differences in responses measured in bacteria from L114 and L222, and between treatments with different P supply. Exposure to AgNP will

not necessarily reduce bacterial community numbers or affect their ecosystem services. Shifts in taxa could occur which would lead to changes in microbial metabolism, nutrient acquisition, or stoichiometry, which could affect energy flows, food-web dynamics, and nutrient cycling. Our results indicate that determining the nature of AgNP effects on these processes will be difficult, given the variable responses we saw in different lakes and with different P supply.

Site specific nutrient conditions should be considered during risk assessments in order to properly manage the impacts of AgNPs and mitigate the damage done to natural freshwater bacterial communities. Altogether our results indicate that natural bacterial communities can show responses to AgNP exposure, these impacts depend, in part, on the environmental conditions within the lake of origin.

Chapter 4: Summary and Conclusions

In this research, the effects of AgNPs on natural bacterial communities were studied at the Experimental Lakes Area (ELA) near Kenora, Ontario. In a mesocosm study we examined dosing regimens (chronic versus pulse) and types of capping agents (citrate and PVP) on the responses of bacteria to AgNP exposure. The interaction between phosphorus nutrient loading and AgNP toxicity to natural bacterial communities was also explored, using *in situ* experiments in two lakes at ELA. Chapter 2 presents the results from the mesocosm study conducted on experimental Lake 239 which examined the influence of AgNP loading and surface coating. Bacterial production was measured using a tritium-labeled leucine assay, bacterial communities were enumerated using flow cytometry, chlorophyll and stoichiometry was also monitored. Related studies conducted on these mesocosms examined the fate of silver nanoparticles (Furtado, MSc Thesis, 2014), and community structure of phytoplankton and zooplankton (Vincent, MSc Thesis, In Progress). Chapter 3 presents results from an *in situ* bioassay which investigated the bacterial community nutritional stoichiometry and the interaction between nutrient loading (P supply) and AgNP toxicity, in L114 & L222. The following presents a summary of each chapter followed by suggestions for future work and a general conclusion of the entire study.

4.1. AgNP Mesocosm Summary

The objective of the mesocosm study was to examine responses of natural bacterial communities in response to two different dosing regimens and two differently capped AgNPs under natural environmentally relevant conditions. Bacterial assemblage responses monitored were bacterial production, abundance stoichiometry and chlorophyll

a. We also monitored the total silver in the water column and the sub fraction of the total which associated with the bacterioplankton size fraction (bact Ag 1.2-0.7 µm).

It was hypothesized that AgNPs added chronically would inflict dose dependent reductions to both bacterial abundance and production, and that over time silver tolerant or resistant taxa would become dominant members of the bacterial community reducing the magnitude of AgNPs negative effects. In the pulse experiment, we expected to see sharp declines in abundance and production, followed by a slow recovery over time as the AgNP leave the water column. Finally, we expected to see differences in bacterial responses to PVP and citrate capped AgNPs, with bacteria exposed to the less stable citrate-capped material showing less severe toxic responses than those communities exposed to the more stable PVP capped AgNPs.

In the mesocosm environment of L239, we observed accumulations to the bacterial Ag fraction at high loading rates (pulse and drip), but despite this increase, we observed significant increases in bacterial production in response to PVP capped AgNPs. The increase in production can likely be attributed to a community shift to tolerant taxa that utilize the newly available resources left behind by decomposing bacteria and increase their growth or cellular repair rates to fill the community's carrying capacity, or by reducing grazing pressure from higher trophic levels (e.g., protists). We did not observe this increase in response to citrate capped AgNPs. After what might be interpreted as a short period of inhibition, we did not observe long term changes in community C:N:P stoichiometry, abundance or chlorophyll a. While surprising, this observation points out the buffering capacity of AgNP toxicity to bacterioplankton assemblages as a result of interactions with DOC (Furtado *et al*, 2014). These data also

illustrate the resilience of natural communities to stressors, gained by their diversity (Baho, Peter & Tranvik, 2012; Bouvier *et al*, 2012; Girvan *et al*, 2005).

4.2. Interactive effects of AgNPs and phosphorus supply on natural lake bacterioplankton summary

The objective of the *in situ* bioassay study was to examine natural bacterial communities in two unique boreal lakes (L114 & L222), in response to phosphorus nutrient enrichment in conjunction to PVP-capped AgNP exposures. We measured bacterial abundance and community C:N:P elemental composition. We also monitored the total silver in the water column and the sub fraction associated with the bacterioplankton size fraction (bact Ag 1.2-0.7 µm).

We predicted bacterial abundance would decrease in a concentration dependent manner but that the reduction would vary in magnitude with phosphate supply, where by P limited communities would be more susceptible to AgNP toxicity. We also expected that bacteria from the humic rich high L222 would experience less toxicity than bacteria from the algal rich L114, due to reduced dissolution to Ag^{+} (Fabrega *et al*, 2009). We observed bacterial communities to vary greatly in response to AgNP exposure and P addition between the two lakes. Bacteria from L222 underwent decreases in abundance, while L114 increased in number, and these changes were enhanced by increasing P supply. The response observed in L114 was likely due to decreased algal competition or decreased predation. We changed the bact Ag fraction in both lakes but found that bact Ag was lower in L222 relative to L114. When considering the higher DOM and lower bact Ag present in L222 relative to L114, the reduction of bacterial abundance in L222 reinforces the importance of studying toxic effects in a site specific context. We also found evidence that AgNPs affect bacterial community stoichiometry, likely by altering

nutrient uptake or accumulation. However, it is less evident why stoichiometric response varied between the two lakes.

4.3. Future Work

This work was a preliminary study for the Lake Ecosystem NanoSilver (LENS) project. Future work will include a whole lake addition of AgNPs to Experimental Lake 222 at the ELA. The objective will be to determine fate and biological responses in lower trophic levels from a continuous point source addition of PVP capped AgNPs, attempting to simulate discharges of wastewater treatment plant effluent. In order to better assess bacterial community responses, sampling of the whole lake experiment should focus intensively on the following:

1) Small scale experimentation should also continue with efforts to model AgNP toxicity on a lake to lake basis.
2) Bacterioplankton community production should be examined with the intentions of determining how widespread changes to productivity are within the lake.
3) Shifts in bacterioplankton community composition and function should be assessed. This could be accomplished using 16s RNA sequencing. It would be prudent to examine functional genes pertaining to nitrogen cycling, as AgNPs have been demonstrated to inhibit nitrifiers (Choi &Hu, 2008).
4) A different index of bacterial metabolism could be employed such as a 5-cyano 2,3-ditolyl tetrazolium chloride (CTC) respiration assay or extracellular enzyme assays in order to give a broader understanding of how AgNPs affect microbial function in the environment.
5) Shifts in bacterioplankton stoichiometry while also observing algal stoichiometry

should be studied in order to investigate any interactions between the trophic levels in response to AgNP toxicity. This could also be studied using P_{32} uptake rates by each size class.

6) Monitor higher trophic levels especially grazers who use bacterioplankton as a food source (i.e. protists, zooplankton)

4.4. Conclusions

The industrial and commercial demand for AgNPs is unlikely to see a decline, due to their widespread applications. The release of AgNP into the environment is inevitable and the implications for biota exposed to these particles in environmental media are a field largely unexplored. The results presented in these two studies offer a new perspective on AgNPs effects on natural bacterial assemblages under environmentally realistic conditions, and call into question the validity of extrapolating from laboratory results to predictions of environmental responses. They also offer a realistic perspective which can be used for policy development and risk assessment. Due to the crucial role that bacterioplankton communities play in ecosystem services, such as geochemical cycling and decomposition, it is imperative that responses to AgNPs be further examined within a whole lake environment at relevant concentrations. We demonstrated that AgNPs accumulate at the size fraction of 1.2-0.7 μm, but that increased exposure of bacterioplankton assemblages to AgNPs does not always lead to an increase in toxicity. We demonstrate that bacterial communities alter their production, growth and community stoichiometry in response to low concentrations of AgNPs. Overall this work shows that variables such as the physicochemical parameters of the aquatic system in question (DOC, environmental ligands, etc.), the nutrient supply to that system, the nutritional state of the biota, the type of AgNP, and the method of exposure (chronic versus pulse)

must be taken into consideration when attempting to predict environmental outcomes. Finally, future work should examine changes in community composition and potential losses of function to bacterial communities, which would provide essential data for risk assessment.

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Appendices

Chapter 2 Supporting Information

Bacterioplankton C:P stoichiometry

The graph below shows the most "significant" observations with respect to changes in nutritional stoichiometry displayed in Table 1. Note the decrease in C:P ratios taking place during week 5, this coincides with an observed crash in bacterial abundance, and is likely a temporal change, not one influence by treatment.

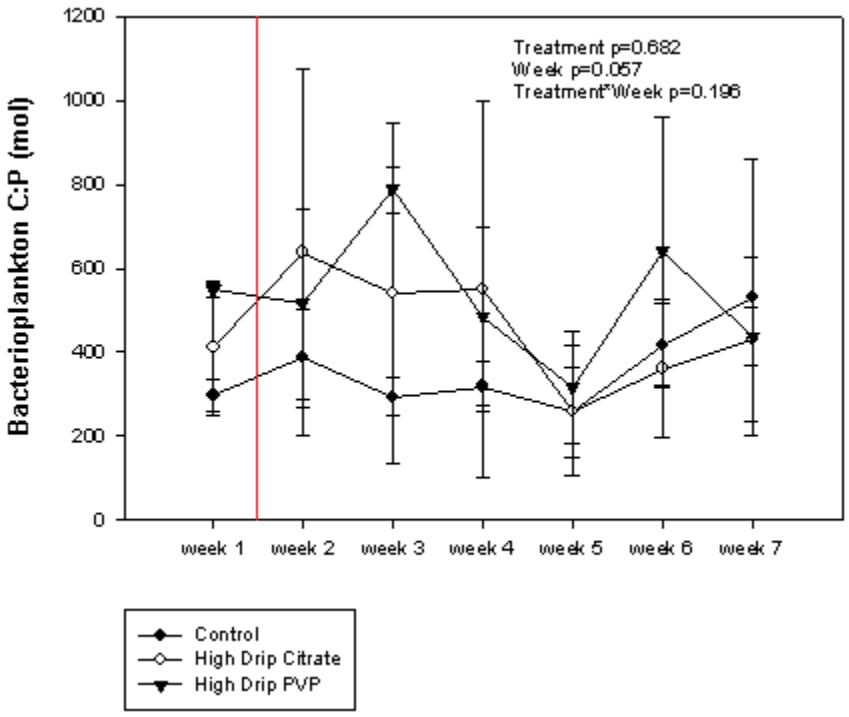

Figure 1: Drip PVP and Citrate capped exposure average silver concentrations for bacterioplankton C:P molar ratios.

Vertical red lines denote the start of the PVP capped nanosilver dosing regimen (Chronic-June 23rd, 2012). Shown are means and standard deviations of the 2 replicate enclosures. Also included on each panel are the p-values for the main and interaction effects of AgNP concentrations with time. * indicates significant differences between exposed and the zero enclosure on a given experimental week ($p < 0.05$). Note no production measurements were taken week 1.

Primary Production and Nutrient Concentrations of Experimental Lake 239

Table 1: Lake Characteristics for Experimental Lake 239.

Average values for each variable were taken over four monthly sampling dates spanning June 20th, 2012 until August 10th, 2012.

Lake	DOC (µmol /L)	Bacterioplankton chlorophyll a (ug/L)	Total Dissolved Nitrogen(TDN) (µg/L)	Total Dissolved Phosphorus (TDP) (ug /L)
239	512.9	0.026	252.9	1.550

Mesocosm silver exposures based on nominal target concentrations

Table 2: Highest achieved % TAg recovered, and the % Bact Ag

Experiment	Treatment	Target Concentration (µg/L)	Highest % TAg recovered	% Bact Ag of TAg
PVP Drip Gradient	Low PVP	5	54.8	0.97
PVP Drip Gradient	Medium PVP	20	40.2	0.40
PVP Drip Gradient	High PVP	80	43.5	1.49
Capping Agent Drip	High Citrate	80	38.8	1.19
Pulse PVP	Pulse PVP	45	88.3	4.34

Mesocosm bacterioplankton bioaccumulation factors

Table 3: Mesocosm bacterioplankton bioaccumulation factors (BCF)

	Control	Low drip PVP	Medium drip PVP	High drip PVP	High drip citrate	Pulse PVP
week 1	bd	bd	bd	bd	bd	bd
week 2	bd	bd	0.007	0.004	0.004	na
week 3	bd	0.017	0.012	0.009	0.009	na
week 4	bd	0.037	0.034	0.030	0.030	bd
week 5	0.092	0.020	0.054	0.039	0.040	0.043
week 6	0.017	0.012	0.017	0.017	0.0107	0.017
week 7	0.011	0.010	0.003	0.009	0.009	0.024
week 8	0.009	0.013	0.020	0.015	0.011	0.265

Bioaccumulation factors calculated as follows: BCF= (bact Ag µg/L)/ (TAg µg/L), bd= beyond limits of ICPMS detection, na= no sample was taken from these mesocosms on this date.

Bacterial Ag standardized to carbon biomass (Bact Ag:C)

Since we only observed transient effects on the bacterial carbon biomass, changes observed here are likely due to changes in % bact Ag of TAg shown in Appendix: Table 2 silver additions.

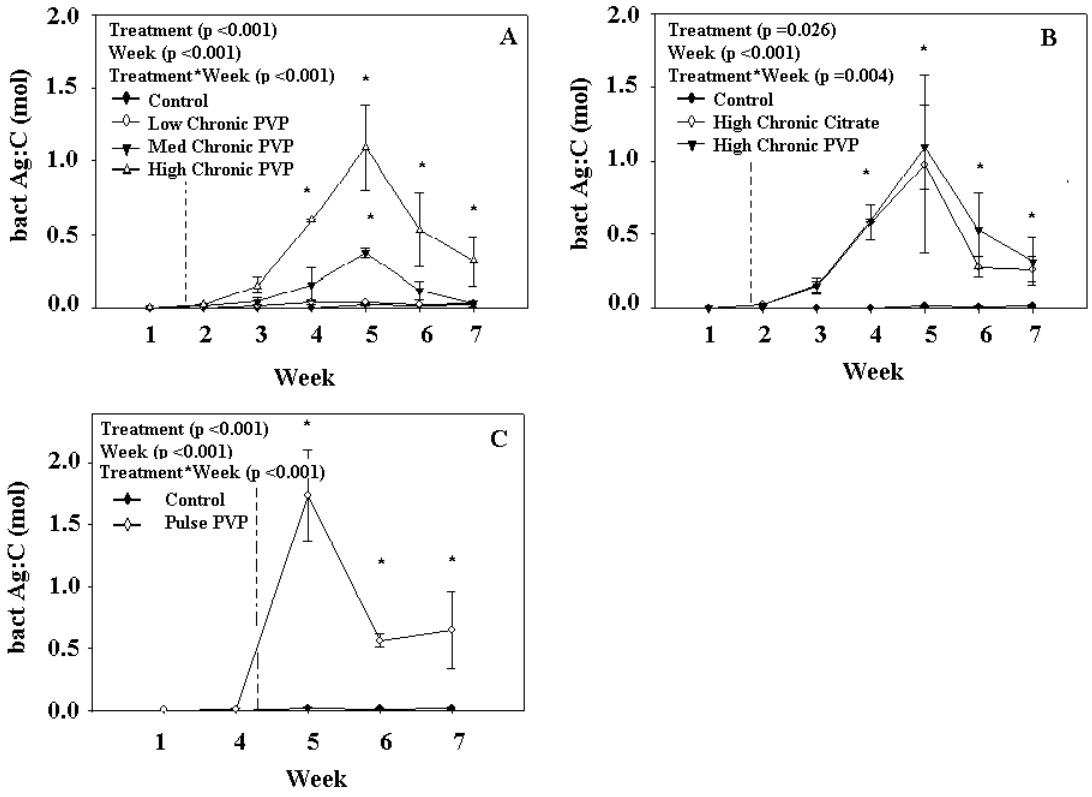

Figure 2: Bacterioplankton Ag:C molar ratios in response to A) chronic PVP, B) chronic Citrate and C) pulsed PVP capped AgNP exposures.

Vertical dotted lines denote the start of the PVP capped nanosilver dosing regimen (Chronic-June 23rd, 2012 and Pulse July 11th, 2012). Shown are means and standard deviations of the 2 replicate enclosures. Also included on each panel are the p-values for the main and interaction effects of AgNP concentrations with time. * indicates significant differences between exposed and the zero enclosure on a given experimental week ($p < 0.05$). Note no production measurements were taken week 1.

Bacterioplankton Stoichiometry Raw Data

Table 3: Bacterioplankton C:N:P molar ratios.

Ratios calculated based upon particulate C, N, P samples for the 1.2-0.7μm size fractions. sampled weekly, spanning June 21st until August 1st 2012.

Date Collected	Treatment	Bacterioplankton N:P (mol)	Bacterioplankton C:P (mol)	Bacterioplankton C:N (mol)
6/21/2012	control	29.55481	270.1786	9.1416
6/27/2012	control	130.8207	302.3527	2.3112
7/4/2012	control	18.83828	260.9769	13.8535
7/11/2012	control	20.41811	277.1328	13.5729
7/18/2012	control	11.24191	149.5169	13.2999
7/25/2012	control	24.75557	348.1199	14.0623
8/1/2012	control	16.66293	297.5635	17.8578
6/21/2012	low drip	23.59986	213.9847	9.067202
6/27/2012	low drip	34.60773	343.3635	9.921583
7/4/2012	low drip	28.84245	301.3136	10.44688
7/11/2012	low drip	18.65349	304.4544	16.32158
7/18/2012	low drip	35.37846	329.9242	9.325565
7/25/2012	low drip	89.05126	317.2674	3.562751
8/1/2012	low drip	50.55124	284.3492	5.62497
6/21/2012	Pulse	27.63431	369.1203	13.35732
7/11/2012	Pulse	88.81273	247.8805	2.791047
7/18/2012	Pulse	10.3861	145.7731	14.03541
7/25/2012	Pulse	8.712373	125.4908	14.40375
8/1/2012	Pulse	19.63648	230.8647	11.75692
6/21/2012	med drip	22.1996	283.734	12.78104
6/27/2012	med drip	25.23413	296.2365	11.73952
7/4/2012	med drip	31.72646	417.7472	13.16715
7/11/2012	med drip	18.21409	215.814	11.84874
7/18/2012	med drip	17.28336	155.3208	8.986728
7/25/2012	med drip	26.83384	322.5095	12.01876
8/1/2012	med drip	21.68423	257.5489	11.87725
6/21/2012	citrate	20.13918	296.8227	14.73857
6/27/2012	citrate	39.71945	329.3432	8.291737
7/4/2012	citrate	22.539	254.545	11.29353
7/11/2012	citrate	18.69925	232.0167	12.40781
7/18/2012	citrate	16.36318	181.937	11.11868
7/25/2012	citrate	16.23865	243.0479	14.96725
8/1/2012	citrate	52.8376	290.9079	5.505698
6/21/2012	high drip	35.48033	559.2143	15.76125
6/27/2012	high drip	56.85235	354.8282	6.241222
7/4/2012	high drip	67.88365	826.8814	12.18086
7/11/2012	high drip	29.93112	334.6426	11.18042
7/18/2012	high drip	17.79993	220.0046	12.35986
7/25/2012	high drip	40.58561	410.9975	10.12668

8/1/2012	high drip	23.7709	387.0332	16.28181
6/21/2012	control	22.60321	324.0797	14.33777
6/27/2012	control	49.63188	468.3199	9.435869
7/4/2012	control	22.05009	325.7723	14.7742
7/11/2012	control	27.69299	358.663	12.9514
7/18/2012	control	42.3105	369.1953	8.725856
7/25/2012	control	24.74012	487.4915	19.70449
8/1/2012	control	242.0071	763.6971	3.15568
6/21/2012	med drip	36.31311	530.3664	14.60537
6/27/2012	med drip	24.16905	344.3281	14.24666
7/4/2012	med drip	96.33815	570.8054	5.92502
7/11/2012	med drip	105.8855	931.8835	8.800857
7/18/2012	med drip	49.35446	467.2859	9.467957
7/25/2012	med drip	36.10505	533.1139	14.76563
8/1/2012	med drip	43.89381	437.4393	9.965854
6/21/2012	Pulse	37.20534	474.774	12.76091
7/11/2012	Pulse	54.55216	446.422	8.183398
7/18/2012	Pulse	52.67575	431.4951	8.191532
7/25/2012	Pulse	325.2364	1060.029	3.259257
8/1/2012	Pulse	45.74334	729.1928	15.94096
6/21/2012	high drip	36.06381	537.35	14.89998
6/27/2012	high drip	44.96937	675.6089	15.02376
7/4/2012	high drip	74.95479	747.2746	9.969671
7/11/2012	high drip	49.62024	634.6483	12.79011
7/18/2012	high drip	102.4599	411.1418	4.01271
7/25/2012	high drip	90.89401	867.7735	9.547092
8/1/2012	high drip	49.74648	487.3899	9.797476
6/21/2012	low drip	61.30295	823.1756	13.42799
6/27/2012	low drip	43.14088	692.6165	16.05476
7/4/2012	low drip	103.2781	978.5272	9.47468
7/11/2012	low drip	30.47044	446.8085	14.66367
7/18/2012	low drip	34.56552	447.6328	12.95027
7/25/2012	low drip	25.48753	272.4522	10.68963
8/1/2012	low drip	39.24945	529.8663	13.49997
6/21/2012	citrate	42.14553	521.8286	12.38159
6/27/2012	citrate	63.419	946.2419	14.92048
7/4/2012	citrate	71.75405	825.6672	11.50691
7/11/2012	citrate	72.71056	865.8879	11.9087
7/18/2012	citrate	45.71363	332.8731	7.281704
7/25/2012	citrate	43.06284	478.4901	11.11144
8/1/2012	citrate	31.74571	569.8029	17.94897