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The influence of humic acid and water hardness on the partitioning of silver ions and nanoparticles between fresh water and freshwater algae

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THE INFLUENCE OF HUMIC ACID AND WATER HARDNESS ON THE PARTITIONING OF SILVER IONS AND NANOPARTICLES BETWEEN FRESH WATER AND FRESHWATER ALGAE

By

Matthew D. Ferguson

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

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MASTER'S THESIS

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THE INFLUENCE OF HUMIC ACID AND WATER HARDNESS ON THE PARTITIONING OF SILVER IONS AND NANOPARTICLES BETWEEN FRESHWATER AND FRESHWATER ALGAE

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment of the
Requirement for the Degree of
Master of Science

by
Matthew D. Ferguson
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ABSTRACT

Silver nanoparticle (AgNP) containing products are abundant in consumer goods. If trends continue, AgNP levels will continue to rise as innovative applications continue to be realized. These nanoparticles (NPs) can enter the environment as their uses can transport them to natural waters (e.g., washing socks containing AgNPs).

Research on the behavior of AgNPs and Ag^+ in artificial fresh water is presented in this thesis. Specifically, their sorptive properties between fresh water and freshwater algae, *Pseudokirchneriella subcapitata* (Korshikov) Hindák, as a function of hardness, humic acid (HA) content, and silver type were investigated. The experimental design was modeled after a 2^3 factorial analysis in which each factor is varied at two levels: (1) no added HA and with HA, (2), Ag type [AgNP versus Ag^+], and (3) low hardness and high hardness.

The Freundlich Isotherm method was used to determine the K_F partitioning constants at the varying conditions. The effects of each factor on partitioning constants were evaluated. A normal probability plot was used to determine which factors had the greatest effect. Results were that the greatest effects were caused by hardness and the interaction between hardness and HA.

An increase in hardness caused a decrease in Ag sorption by an average log K_F of 0.46475, whereas the interaction between hardness and HA caused an increase in Ag sorption by an average log K_F of 0.40375. The other two main effects (Ag type and HA content) also had an effect on sorption. However, these main effects were not as great as that observed with hardness.

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ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
AgNP	Silver Nanoparticles
BL	Biotic Ligand
BLM	Biotic Ligand Model
DLS	Dynamic Light Scattering
DL	Detection Limit
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EDTA	Ethylenediaminetetraacetic acid
ENP	Engineered Nanoparticles
H	Hardness
HA	Humic Acid
HS	Humic Substances
IC_{50}	Inhibitory Concentration; 50%
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IHSS	International Humic Substances Society
ISA	Ion Strength Adjuster
ISE	Ion Selective Electrode
K_F	Freundlich Isotherm coefficient
M_{algae}	Mass Ag associated with algae
$M_{container}$	Mass Ag sorbed to container
$M_{dissolved}$	Mass Ag dissolved in media
M_{total}	Total Ag mass
NP	Nanoparticles
PEN	Project for Emerging Nanotechnologies

PVA	Polyvinylalcohol
QL	Quantitation Limit
SWHA	Suwannee River Humic Acid
T_0	Time zero
T_{24}	Time 24-hours
USEPA	United States Environmental Protection Agency
UTEX	University of Texas
WET	Whole Effluent Toxicity

GLOSSARY

Ag^{+}_{NP}	Silver ions released from AgNPs.
Agglomeration	The phenomenon of nanoparticles coalescing into larger size particles by means of weak attractive forces such as van der Walls interactions (ISO, 2008).
Allotropic silver	Term used to describe AgNP suspensions prior to the discovery that the suspensions were indeed nanoparticles.
Bioaccumulation	The accumulation of a toxicant in an organism from all sources of exposure (e.g., food, water, etc.).
Bioavailability	Describes a toxicant as being in a form that is readily available for uptake into an organism.
Biotic ligand model	A model used to predict toxicity that uses environmental data to determine the levels of a toxicant on a binding site in or on an organism and predict the resultant toxicity.
Colloid	Materials that are 1 nm – 1 μm in diameter.
Dissolved organic carbon	Carbon products from decayed organic matter that is less than 0.45 μm.
Dissolved organic matter	Naturally occurring organic matter in solution that typically contains 50-55% dissolved organic carbon.
Dynamic light scattering	Uses Rayleigh scattering effects to measure the size distribution profile of a solution containing particles.
Engineered nanoparticles	Nanoparticle material that is man made.
Free silver ions	Silver in the form of Ag^{+} and Ag^{+}_{NP}.
Humic acid	A large component of dissolved organic carbon.
IC_{50}	Concentration that inhibits 50% of the sample measurement (such as growth).
Interaction effects	The combined effects of the experimental factors, either comparing the effects of two factors or all three.
Ion selective electrode	Instrument used to determine free Ag ion levels.

Main effects	The effects of the individual experimental factors, i.e., humic acid, Ag type, and water hardness as independent variables.
Matrix Matched	Using experimental conditions in calibration standards, e.g., added PVA at concentrations that would be expected in experimental samples.
Nanoparticles	Materials that are 1 - 100 nm in any one dimension.
Percent recovery	Levels of analyte observed or measured as compared to actual amounts.
Surface ligands	Associated surface molecules on the nanoparticles.
Total silver	All silver, including elemental silver from nanoparticles and silver released as ions from the nanoparticles.

1. INTRODUCTION

In human history, most tools have been improved through time as an enhanced understanding of scientific fields, such as chemistry, leads to technological advances (e.g., the advancement from stone hammers to iron hammers). With time, more and more products have taken advantage of available technology. This is just as true today, as our growing understanding of the biochemical and physical realms open new avenues of discovery. Innovation creates new and improved products, and today, these products are increasingly likely to contain engineered nanoparticles (ENPs).

Engineered nanoparticle products have great potential to enhance our daily lives; the toxicological studies, however, investigating current and emerging particles are lagging behind the development and application of these materials. A distinction is made between nanoparticles (NPs), which can occur naturally, and ENPs, which are manmade.

The influences of ENPs on environmental systems are not well known. One area of uncertainty is the behavior of ENPs when they reach an aquatic environment. Simple fate and transport algorithms require an understanding of the chemical properties of the media in which the contamination occurs in addition to knowledge of the chemical behavior once the contamination is in the media. A key aspect to consider in the modeling of fate and transport is the partitioning behavior of a chemical species in aquatic media. My research presented here was aimed at determining ENP partitioning coefficients in aquatic media at different environmental conditions. Partitioning coefficients for the ionic form of Ag were also determined and compared.

1.1. Nanoparticle Terminology Used in this Thesis

Nanoparticles are defined as materials that are 1 - 100 nm in any one dimension. Silver in this thesis will be referred to as Ag when it is not necessary to distinguish the form (ionic or NP). Ionic Ag from Ag_2SO_4, and ionic Ag in general (when source of the ion is not relevant) will be referred to as Ag^+, and Ag that is part of a NP (Ag^0) will be referred to as AgNP. Silver ions released from the AgNPs, are referred to as, Ag^+_{NP}. Given that Ag^+_{NP} and Ag^+ are both ionic forms of Ag, it will be of value to identify the differences between the two (i.e., the source) when reporting experimental results. Both Ag^+ and - Ag^+_{NP} are also referred to as free Ag ions. Here, the term colloid is used interchangeably with AgNPs as the prepared Ag colloids used in this thesis qualify as AgNPs (Table 1).

Table 1. Terms used to describe the referred forms of silver.

Silver form	Term used
Ag^+	Silver (Ag) from Ag_2SO_4 and ionic Ag when source (i.e. AgNP or Ag_2SO_4) is not relevant
Ag^+_{NP}	Silver released from AgNP
AgNP	Silver nanoparticles

1.2. Nanoparticles

According to the United States Environmental Protection Agency (USEPA), the convergence of nanotechnology, biotechnology, and information technology is expected to hasten over the coming decades (USEPA, 2007). There are four major types of ENPs that have permeated into these markets: 1) Carbon-based materials (e.g., fullerenes, nanotubes); 2) metal-based materials (e.g., zinc oxide); 3) dendrimers (e.g., nano-sized branched polymers); and 4) composites (e.g., combined NPs; USEPA, 2007). A review of the current state of NPs in consumer products is presented in the *Project on Emerging Nanotechnologies* (PEN), available at www.nanotechproject.org. The group responsible for this resource was built as a partnership between the Woodrow Wilson International Center for Scholars and the Pew Charitable Trusts to help "ensure that as nanotechnologies advance, possible risks are minimized, public and consumer engagement remains strong, and the potential benefits of these new technologies are realized".

The Project on Emerging Nanotechnologies (PEN, 2011) provides the first on-line open resource "inventory of nanotechnology-based consumer products" providing easily accessible data on recent trends. A graph of their total products listed are plotted by year in Figure 1. Also on their inventory webpage are figures providing information on the number of products within each of the major types of NPs available. Products are searchable in the on-line inventory, which includes information based on three criteria: (1) the products must be accessible and available for purchase, (2) the manufacturer or another source acknowledges the existence of "nano-based" attributes in the products,

and (3) that the claim that the product contains nano-based materials is rational. Also, all product information PEN obtained is listed in their on-line inventory. This accessibility allows all users of the PEN on-line inventory to source their findings. Currently, their product inventory evaluates consumer data from 30 different countries, including markets such as China, Mexico, Italy, and Israel. The data provided in their on-line inventory and summarized here are current as of March 10, 2011.

Figure 1 illustrates the linear growth in consumer products that contain NPs. These data have been categorized based on the most common NP type in these products. The results indicate that AgNPs are the most abundant (55.4%), followed by carbon NPs (16.1%), titanium NPs (10.4%), silica NPs (7.6%), zinc NPs (5.5%), and gold NPs (5.0%; PEN, 2011). A search in the PEN on-line inventory using the term, "silver nanoparticles" resulted in a list of many personal products that contain AgNP. For example, mineral supplemental drinks (e.g., MesoSilver®), slippers (e.g., Contour-Foam™ Silver Slippers), pillows (e.g., Contour-Foam™ Silver Neck-Support Pillow), socks (e.g., Sharper Images Antibacterial Silver Athletic Socks), and teddy bears (e.g., Pure Plushy®) are all products that contain AgNPs.

Hansen et al. (2008) also support these findings. In their research, they further state that for any risk assessment, understanding *where* the particles reside in the product is important. The authors divide the potential nanomaterial locations into three main categories: (1) in the bulk (e.g., one-phase materials like ceramic zeolites), (2) surface products (e.g., nanoscale thick structured film, or film with nanoscale attributes), and (3) as particulates (e.g., particles suspended in liquid or bound to a surface). The researchers

assigned 435 products (their data was obtained from the *Project of Emerging Nanotechnologies*) to the appropriate location category. Their results suggest that of all NPs that could be classified into a location category, 95% of those are as particulates, with the remaining 5% is located on the surface (4.4%) and the in the bulk category (0.6%). The particulate category can be further categorized into one of 4 sub-categories, which included suspended in liquid (50%), bound to surfaces (29%), suspended in solids (20%), and airborne (1%). Finally, of all the listed 28 NP types, AgNPs were again the most abundant (~ 40% of all classifiable NPs evaluated), followed by ZnO at ~ 10% of total classifiable NPs.

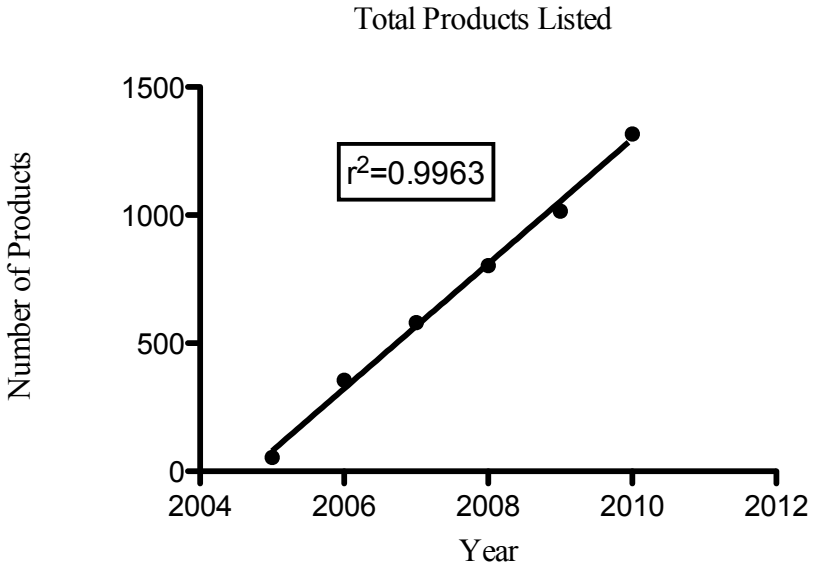

Figure 1. Number of total consumer products containing NP per year, with regression analysis (data from PEN, 2011).

1.3. Brief History of Silver Nanoparticles

Silver NPs were first added to consumer products more than 100 years ago, particularly in photography products and in biocides (Nowack et al., 2011). The first notable synthesis of AgNPs was reported by Lea (1889) in preparing an "allotropic" form of silver. Lea did not know the exact form of his silver, however, Frens and Overbeek (1969) synthesized silver colloids after the methods outlined in Lea's manuscript. They determined that the size of their AgNPs was 37 – 45 Å (3.7 – 4.5 nm) and that they were spherical.

The antimicrobial activity of these products fueled its popularity in the early 20th century. Brand names like Collargol, Argyrol, and Protargol were accepted as a medicinal Ag and popularized for more than 50 years. During that time, doctors would commonly administer AgNPs for the treatment of bacterial infections, such as syphilis (Fung and Bowen, 1996). They are still currently being used as biocidal agents and health supplements (e.g., MesoSilver®). If these trends of AgNP usage remain, and if the increased usage in the recent past is any indication, they will continue to be used in great quantities.

1.4 Silver Nanoparticles and the Environment

Silver NPs are small pieces of elemental silver (Ag^0) that are 1 - 100 nm in at least one dimension. These NPs are different than dissolved silver ions (Ag^+) in many ways. For example, associated surface ligands and AgNP size are some of the major identified attributes contributing to the NP chemical behavior in the environment and are properties absent with Ag^+.

1.4.1. Associated Surface Ligands

The surface ligands associated with AgNPs are commonly formed during their production. For example, Lee and Meisel (1982) used three different methods for making AgNPs via wet chemistry. The first two types of AgNPs were made with Ag_2SO_4 or $AgNO_3$ and polyvinyl alcohol (PVA) to make AgNPs with PVA surface ligands. The authors also heated up a solution containing $AgNO_3$ and a 1% sodium citrate solution to make AgNPs with sodium citrate surface ligands. These surface ligands were chosen by the authors because they would adsorb particular dyes for Raman scattering analyses.

Other researchers vary the AgNP surface ligand types depending on the application. For example, Zhang et al. (2005) used tiopronin monolayer surface ligands on their AgNPs for use in fluorometry, as these ligands can be partially exchanged by fluorescein-labeled thiolate oligonucleotides. There are many potential ligands that exist for AgNPs and these can be varied to elicit a desired effect, such as to control the toxicity of a particle (Lu et al., 2010).

1.4.2. Evidence that Silver Nanoparticles Enter the Environment

The antimicrobial properties of AgNPs (Kim et al., 2007) are a major contributor to their popularity in many consumer products. A study by Benn and Westerhoff (2008) evaluated the potential of AgNP used as an antimicrobial in socks to be released into water. They found the highest measured concentration of Ag in the socks was 1360 μg-Ag/g-sock. To evaluate the propensity of AgNP to be released into the water during washing, they placed socks directly into 500 mL of ultrapure water and shook the bottles for 1 hour or 24 hours. The researchers determined that after 4 washes of a

particular brand of socks, a cumulative amount of 1845 μg of Ag was released into the wash water. In some brands, after the first or second wash the release of Ag from the socks was much less or negligible, suggesting that newer socks are more likely to release more Ag. Interestingly, one brand of socks that had the highest levels of Ag before the leaching experiments leached less than 165 μg of Ag. These results suggest that the manufacturer might have some control over the release potential of AgNPs from the socks.

Other products have been shown to release AgNPs. Benn et al. (2010) studied the potential release of AgNPs from common consumer products often found in the home. They evaluated the release of Ag in toothpaste, shampoo, detergent, and cloth products containing AgNPs (advertised as colloidal). With the cloth products, AgNP loading was determined by washing these products in tap water followed by digestion and Ag analysis via ICP-MS; the other products followed the same protocol except for washing. The researchers determined that the toothpaste contained 18 μg Ag g^{-1}, shampoo contained 0.9 μg Ag g^{-1}, detergent contained 1.8 μg Ag g^{-1}, and cloth products contained 0.5 μg Ag g^{-1}. It can be concluded that the potential for these AgNPs to enter the environment exists as consumer products containing the ENPs are constantly being used and washed down the drain. Depending on the municipal wastewater treatment, it is possible that AgNPs will ultimately enter into the environment.

1.5. Silver Nanoparticles

Research that evaluates AgNP chemical behavior will improve upon the ability to predict the causal effects of AgNPs in the environment, including their bioavailability and possible bioaccumulation in the food web.

1.5.1. Toxicity and Bioavailability of Ag^+ versus Silver Nanoparticle

Tests comparing the Ag^+ and AgNP toxicity can provide valuable information regarding the toxic contribution of Ag^+ released from the NP versus the toxicity of the NP itself. According to Navarro et al. (2008), toxicity of carbonate coated AgNPs to the freshwater algae, *Chlamydomonas reinhardtii*, was partly due to Ag^+ (which was 1% of the total Ag in solution). They deduced that the toxicity of AgNPs appears to be more than that of just Ag^+ alone. Comparing the EC_{50} values of $AgNO_3$ with AgNP, both as a function of Ag^+, support this, where the $AgNO_3$ EC_{50} was 20.3 μg/L and AgNP EC_{50} was 3.6 μg/L. The exact mechanism of AgNP toxicity is not well understood, however, in studies using bacteria, protein and enzyme interactions with Ag^+ and oxidative stress were determined to be potential mechanisms of toxicity (Yamanaka et al., 2005; Kim et al., 2007, respectively).

In aquatic animals such as fish, crayfish, and daphnids, silver is an ion that disturbs the ionoregulatory system by binding and inhibiting Na^+ channels (Bury et al., 2002; Grossel et al., 2002; Bianchini et al., 2003). Levels of dissolved organic carbon (DOC) in the water may affect the ability of Ag^+ to bind to those Na^+ channels, effectively influencing the effect of Ag on the organism. In the biotic ligand model

(BLM), Bury et al. (1999A) showed that by increasing DOC, the amount of Ag^+ on the gills of rainbow trout was significantly decreased.

In other research, Karen et al. (1999) showed that humic acids (HA) significantly reduce the acute toxicity (reported as the LC_{50}) of Ag^+ in rainbow trout (*Oncorhynchus mykiss)*, fathead minnows (*Pimephales promelas*), and water fleas (*Daphnia magna*). Increasing hardness (measured as a function of $CaCO_3$), on the other hand, afforded little protection against Ag^+.

1.5.2. Silver Nanoparticle Fate and Transport

The USEPA has made strong recommendations regarding the research of nanoparticle contaminants. To assist in the field of risk assessment research, they propose a "better understanding and [application of] information regarding (nanoparticles)" for environmental fate, detection and analysis, human exposure probabilities, and human and ecological health effects (USEPA, 2007). This agency supports the need to more fully understand the behavior of AgNPs in the environment.

There are many aspects of a NP to consider when modeling its behavior. For example, a decrease in aerodynamic diameter (a theoretical diameter of an irregularly shaped particle) would normally result in an increase in dispersion. Though NP size is important to consider when determining the chemical behavior in aquatic media, the effect of the surface chemistry of NPs, being largely a function of the associated surface ligands, may be of greater importance.

The surface chemistry, which relates to the ligand coatings, of ENPs will affect their distribution (i.e., fate) as it will have a direct effect on their behavior in the

environment. For example, Chinnapongse et al. (2011) evaluated the potential for citrate stabilized AgNPs to maintain dispersion in aqueous media. The authors determined that, depending on the chemistry of the water (field collected natural fresh water or synthetic sea water), the concentrations of AgNP stabilized with citrate surface ligands (AgNP-cit) decreased as a function of the water type. Concentrations decreased most rapidly in sea water samples where less than 20% of the AgNPs remained in solution after 1 hour. At least half of the AgNP-cit, however, remained suspended in solution (dispersed) after 24 hours and remained moderately constant for another 24 hours in natural fresh water samples. The difference in fresh water chemistry, which depended on the source of the sample, revealed that this had an influence on the potential for agglomeration. This data suggests that ENPs have the ability to remain in the water column for extended periods of time. This is an important feature in fate and transport as it is evident that the chemistry of the water has a significant effect on ENP dispersion. Knowledge of these effects will, for example, aid in determining the distance an ENP might travel down a stream or in determining the rate of dispersion in a large body of water. The result of evaluating aquatic chemistry and ENP behavior enables an increased predictability in fate and transport modeling.

1.6. Environmental Chemistry

The partitioning of ENPs into or out of the aqueous phase will depend on the chemistry of the ENPs, the water, and natural ligands (e.g., HA) present in the water column. Understanding the chemistry of the water column aids in determining how these factors directly affect the toxicity, bioavailability, and fate of ENPs (e.g., AgNPs) in a system.

Humic substances and water hardness are two major factors that are known to affect the behavior of many metals as they enter the environment (Karen et al., 1999; Smith et al., 2002; Steinberg et al., 2006).

1.6.1. Humic Substances

Naturally occurring organic matter in solution can be described as dissolved organic carbon (DOC) or dissolved organic matter (DOM). Dissolved organic material typically contains 50-55% DOC (Harding et al., 2006). In freshwater ecosystems, humic substances, which include humic acids and fulvic acids, can make up 50-80% of the DOM; the naturally occurring concentrations of HAs in oligotrophic water bodies range from 0.5 to 80 mg/L (Steinberg et al., 2006). In other work by Thomas (1997), the reported mean concentrations of HSs in Lewes Brooks (a protected freshwater wetland in East Sussex, England) in October, February, May, and August were 0.54, 0.30, 0.25, and 0.20 mg HS/L, respectively. The size of humic acids suspended in the water are normally in the nanometer range and thus can be classified as naturally occurring NPs.

The humic components of DOC can play an important role in the behavior and effect of a metal toxicant in an aquatic system. In a study by Bury et al. (1999B), increases in DOC had a significant influence on decreasing Ag^+ toxicity to fathead minnows (*P. promelas*). Under normal environmental conditions, Ag^+ (a soft metal) will strongly bind to soft ligands because the soft metals are highly polarizable due to their atomic deformation potential (Smith et al., 2002). Of all soft ligands, Ag^+ has the greatest affinity for reduced oxidation states of sulfur. Research by Xia et al. (1998) showed that

50% of sulfur in HA exists in reduced oxidization states, supporting the theory that HA will have an affinity for Ag^{+}.

Impacts of HAs on algae can be advantageous or detrimental, depending on the scenario. Lee et al. (2009) studied the effects of DOM on the growth of *Pseudokirchneriella subcapitata* (Korshikov) Hindák. In their experimental design, each independent sample contained static HA concentrations ranging from 0.1 mg/L to 11 mg/L and at pH's 7.0, 7.5, and 8.0. It was demonstrated that HA concentrations higher than 10 mg/L did not stimulate significant growth; at pH's 7.5 and 8.0, there was a slight decrease. Whereas, exponential growth was observed when HA concentrations ranged between 1 and 5 mg/L, and a slight increase between 0.1 and 1 mg/L. The decrease in cell concentrations (measured as a function of cell count and chlorophyll analysis via UV_{686}) above 10 mg/L may be due to a decrease in photosynthesis, which can be explained by the capacity for HSs to capture photons (Thomas, 1997). The addition of HA might also inhibit the precipitation of essential nutrients (e.g., iron as $Fe(OH)_3$) keeping the bioavailable form of that micronutrient present for longer periods of time (Lee et al., 2009).

Predictions of how ENPs and HAs will interact when in a mixture is challenging given the individual complexity of each. This complexity will likely be magnified when in a mixture and further so when other environmental factors, like hardness are considered.

1.6.2. Water Hardness

Hardness is a measure of divalent cation levels, such as Ca^{2+} and Mg^{2+}. It is an essential component for predicting the species of chemicals in an environment. For example, in chemical equilibrium modeling software, such as Visual MINTEQ (Gustafsson 2010), hardness values are utilized along with other factors such as pH, to determine the speciation of metals. Hardness will affect the extent to which Ag^{+} associates to a surface and which anions to complex with. In essence, an increase in hardness results in an increase in competition between cations (including Ag^{+}) and their potential ligands. There is conflicting evidence as to whether or not hardness may play a significant role in ameliorating toxic effects of Ag^{+} to fish and daphnia. Karen et al. (1999) did not observe significant changes in Ag toxicity to *D. magna* and *P. promelas* in response to increases in hardness, however, they did see a significant effect to *O. mykiss* when hardness was doubled from 30 mg $CaCO_3$/L to 60 mg $CaCO_3$/L. The researchers assert, therefore, that hardness has little protective influence on Ag toxicity to *D. magna, P. promelas,* and *O. mykiss*. They further state that DOC components are "more important than hardness" when determining the toxic effects of Ag.

Bianchini and Wood (2008) assessed the effect of water hardness on silver toxicity to *D. magna*. To elucidate the effectiveness of using the BLM in *D. magna* toxicity, they exposed the organisms to Ag^{+} in moderately hard water (~ 111 mg/L $CaCO_3$) and high hard water (~1250 mg/L $CaCO_3$; calculated as a function of Ca^{2+} and Mg^{2+}). The effect of increasing hardness on Ag mortality induced a 2.5 fold decrease in the LC_{20} suggesting that water hardness plays a role in Ag exposure predictions in the

BLM. They propose that the protective aspect of increasing water hardness can be partly explained by the competing ions, i.e. Ca^{2+} (log K = 3.3)[1] and Mg^{2+} (log K = 3.0), at the biotic ligand (BL) binding sites where toxicity is observed.

In another study using *P. subcapitata* a similar log K for Ca^{2+} complexation to the algal cell was found, where the BL for the algal cell is "a hypothetical ligand consisting of all internal and/or external metal binding sites involved in metal toxicity" (Heijerick et al., 2002). These researchers determined that with the algae, Ca^{2+} had a log K of 3.2, however Mg^{2+} had a higher affinity to *P. subcapitata* (log K = 3.9) than *D. magna* based on a comparison to the results of Bianchini and Wood (2008). This increase in Mg^{2+} affinity supports that that there could be increased competition between Ag^{+} and Mg^{2+} at the BL for algae compared to *D. magna* and so hardness may be a more important consideration when working with algae.

1.6.3. Effects of Humic Acid Combined with Hardness

Humic substances contain high levels of carboxyl and amino moieties that have a strong affinity to group A metals, such as Ca^{2+} and Mg^{2+} (Smith et al., 2002). This characteristic can partly explain why levels of Ag sorbed to humic substances can be affected as a function of hardness; the group A metals will compete with Ag^{+} for binding sites, resulting in desorption of the Ag^{+}.

Though DOC components at high levels might decrease Ag^{+} toxicity, there are a myriad of conditions to consider when predicting potential changes in bioavailability. For example, in an oligotrophic lake with very low DOC content (e.g., 0.1 mg/L HA), but

[1] Where log K values are used to describe the affinity of an ion to a specific ligand (e.g., fish gills); the higher the log K of a particular ion, the higher the affinity of that particular ion to the ligand.

with high hardness, hardness *may* play a more significant role than DOC in the bioavailability of particular metal contaminants. There has been little done on this type of combined effects analysis. Research looking at water quality effects on NPs in general is lacking but is expected to increase as the NP industry continues to grow.

Studies evaluating the influence of water hardness and/or HA on Ag^+ chemical behavior and bioavailability are readily available (Smith et al., 2002; Bury et al., 1999B; Karen et al., 1999). There has not been much research, however, investigating the effects of water hardness and/or HA on AgNPs. A few studies evaluated the influence of AgNPs on bacterial growth using DOM as an experimental factor and are discussed later (Section 4.). This lack of publications investigating the effects of both hardness and HA on AgNP chemical behavior, and more specifically with respect to interactions with freshwater algae, supports that the work presented here is a novel contribution to the scientific literature.

1.7. Algae as a Test Model

Algae are used often in the field of aquatic toxicology as they are important organisms to consider in aquatic environments. They are primary consumers and provide nutrients for higher trophic organisms; disturbances that cause a decline in algae populations, therefore, may have considerable effects on the ecosystem. A common algae utilized in the laboratory is *P. subcapitata*. Several publications exist that evaluate the effects of Ag^+ toxicity on this alga and illustrate that it is particularly sensitive to Ag (e.g., Lee et al. 2004; Lee et al. 2005; Hiriart-Baer et al. 2006;).

Another important feature of algae, specifically single-celled algae, is that they often reside in the water column and are subject to currents that can transport them. This characteristic plays a large role in the fate of a toxicant. As toxicant loading (i.e., sorbed chemicals) onto algae increases, the fate of that toxicant is largely a function of where the algae are destined. Therefore, knowing the dynamic influences of each environmental condition and how it affects the levels associated to the algae, Ag fate and transport predictions can be done with more accuracy.

1.8. Partition Coefficients

The USEPA (2007) acknowledges gaps in understanding NP behavior in the environment; any well-designed study investigating the aquatic behavior of NPs would be an important scientific contribution. Furthermore, specifically understanding the partitioning of toxicants in an aquatic system in response to environmental conditions is a practical and necessary component for fate and transport models.

Gaps exist in the understanding of how NPs partition in an aquatic environment. To narrow these gaps, investigating how a metal NP (e.g., AgNP) behaves as compared to its ionic form might help to determine potential differences between the two forms. Partitioning coefficients are accepted tools in fate and transport modeling and are often used to show the portion of total toxicant sorbed to a substrate. For example, Miles et al. (2001) used *P. subcapitata* to model the relationship between different species of methylmercury (i.e., MeHgOH and MeHgCl) and their partitioning between the water column and algae. The partitioning constants were in the following units and will also be applied to the research presented in this thesis (Equation 1):

$$\frac{\left(ng \text{ of MeHg kg}^{-1}\right)_{cell}}{\left(ng \text{ of MeHg L}^{-1}\right)_{water}} \qquad \text{Equation 1}$$

1.9. This Work

This research was designed to provide a better understanding of the behavior of Ag^{+} and AgNPs in solution with the microalgae, *P. subcapitata,* also present. Humic acid, hardness, and silver type (Ag^{+} and AgNP) were the factors used in this study. These factors were varied and their potential effects on the partitioning of total Ag evaluated. The goal of this research were to: (1) obtain phytoplankton:AgNP and Ag^{+} partitioning coefficients (K_f); and (2) analyze data to understand the effect of each environmental factor on AgNP partitioning behavior. This work aids in the prediction of the fate and effects of AgNP contamination in aquatic environments.

1.9.1. Methodology

Methods for evaluating the partitioning of AgNP between water and algae were based on both standardized methods and literature. The "List of Approved Biological Methods" in the most recent guideline for the USEPA's (2002A) whole effluent toxicity (WET) tests recommends, using test method number 1003.0 with the alga *P. subcapitata*. The USEPA's method provides directions for preparing the algal culture medium, stocking algal cultures, and maintaining stock cultures (USEPA, 2002B).

This thesis illustrates how humic acids and hardness influence the partitioning of Ag between water and freshwater algae. The Freundlich Isotherm partition coefficient (K_f) was used to model the partitioning to algae, and the effect of the environmental

conditions (HA and hardness) on K_f was determined. The effects the different forms of Ag (AgNP vs. Ag^+) had on the partitioning behavior were also determined. The AgNP used in this study contained the surface ligand PVA. Previous research has shown that citrate, another commonly used surface ligand, binds the AgNP with less efficiency than that of PVA (Gómez et al., 2007).

2. METHODS

Suwannee River Humic Acid (SWHA) standard II was obtained from the International Humic Substances Society (IHSS; St. Paul, MN). Trace metal grade nitric acid (HNO_3) was obtained from Fisher Scientific. Fluka® Silver Atomic Absorption Spectroscopy (AAS) Standard (1g Ag/L) was used for ICP-MS calibrations. Ionic Ag exposures were conducted with silver sulfate (Ag_2SO_4; JT Baker; 99.5%). Silver NPs were made with silver nitrate ($AgNO_3$; Sigma-Aldrich; 99%), sodium borohydride ($NaBH_4$; ACROS organics; 98%), and PVA (ACROS Organics; 98%). Filters used were Whatman GF/C (1.2 μm) and Millipore (0.2 μm). All water was purified with a Barnstead NANOpure™ ultrapure water system and will be referred to as ultrapure water.

2.1. Algae Culturing

Culturing methods were performed by following standard procedures. The cultures were renewed and monitored for biological contamination regularly. This procedure ensured healthy and abundant *P. subcapitata* were always available.

2.1.1. Methodology

The culture of the test species, *P. subcapitata*, was maintained by following the USEPA method 1003.0 (USEPA, 2002B). For the congruency of previous editions, method 1003.0 maintains the outdated name, *Selenastrum capricornutum.* This thesis will use *P. subcapitata*, as it is the currently accepted scientific name for this species (Vigneault and Campbell, 2005).

2.1.2. Culture Media

Culture medium was prepared as described by USEPA (2002B). Briefly, five different stock solutions were prepared ahead of time and combined as needed to make the culture media.

2.1.3. Method Adjustments

The USEPA recommends adding Ethylenediaminetetraacetic acid (EDTA) at a final concentration of 300 µg/L as part of a toxicity test exposure, as it lowers the rate of type I errors (USEPA, 2002B). When conducting metal toxicity tests, however, they advise that EDTA has the potential to "underestimate the toxicity of metals". Specifically, EDTA will complex some of the Ag^{+} (log K = 8.05; Smith et al., 2001), which results in less of the form (Ag^{+}) expected to cause toxicity. This suggests that EDTA will also affect partitioning results with Ag. Since the goal of the current work was to determine the interaction of metals (specifically Ag) with algae, EDTA was not used in any of this research.

2.1.4. Algae

The *P. subcapitata* strain was obtained from UTEX (University of Austin, Texas): The Culture Collection of Algae (UTEX number: 1648). This primary culture was shipped on Bristol medium and stored in a refrigerator at 4°C upon arrival (USEPA, 2002B). Cultures for experiments were initiated by adding a small sample of the stored algae to each of four Erlenmeyer flasks containing 100 mL of filtered (0.2 μm) culture medium. New cultures were initiated by adding 1-2 mL of a previous culture (1-2 weeks old) to new culture medium (100 mL).

Algae were grown in a Percival Scientific environmental chamber at 25°C, with 85.49 $\mu E/m^2/s$ of continuous light exposure (measured with a Li-Cor LI-210 Photometric Sensor), and on a shaker table at 100 rpm. Based on the literature, *P. subcapitata* exhibits exponential phase growth for up to 7 days prior to stationary growth-phase (Antunes et al., 2007; Moreira-Santos et al., 2004). In other research, *P. subcapitata* exhibited exponential phase growth for up to 10 -14 days prior to stationary growth-phase (Tien et al., 2002). Miles et al. (2001) harvested their cells in both exponential (about 4 days) or stationary (> 6 days) growth phases for partitioning experiments and they concluded that the partitioning of certain metal containing toxicants (e.g., monomethylmercury) was not affected by the growth phase (i.e., exponential or stationary) of their algae.

In my research, algae growth was visually apparent within 1 or 2 days of a new culture as a green mat accumulated on the bottom of the sample flasks. Growth rates were not determined. For this research, algae were harvest between days 7 and 10 after initiating new cultures. Phase of growth was assumed to be in either an exponential or

stationary phase of growth. Observations confirmed an increase in the levels of dead algae (no chlorophyll or green color in algal cell as observed with a compound microscope; cell wall only) after 14 days.

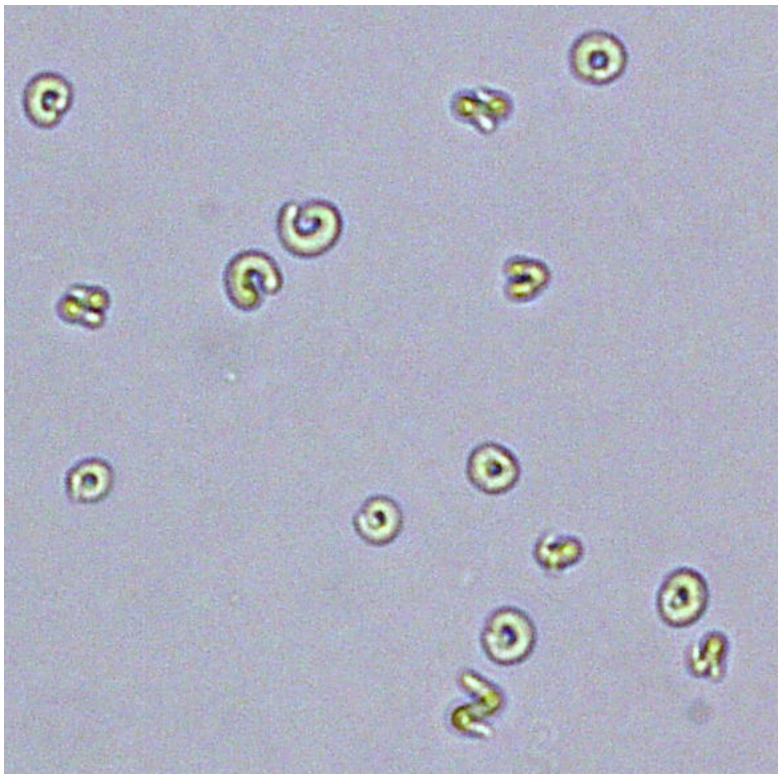

Figure 2. Image of *P. subcapitata* used in this study (magnification at 60X). Algae concentrations were measured with a Levy Ultra Plane hemocytometer.

Weekly observations were conducted to evaluate the sterility of the cultures. This was achieved by viewing a sample from each culture flask through a compound microscope (Figure 2). Each sample was thoroughly scrutinized to ensure no other organisms were present. The algae were approximately 10 μm in average diameter, measured within 10 days of new culture growth with a micrometer. This implies that the 1.2 μm filters will not allow *P. subcapitata* to pass.

Dry weight of cells was determined by evaluating the weight of known cell concentrations of desiccated algae. To accomplish this, Whatman GF/C filter papers were pre-rinsed with ultrapure, pre-dried at 80°C for 24 hours and weighed prior to filtering known concentrations of algae. Six concentrations of algae (25×10^3, 10×10^4, 25×10^4, 5×10^5, 10×10^5, and 15×10^5 cells/mL) were filtered (1.2 μm) and dried at 80°C for 24 hours (in duplicate). Each filter was then weighed and the relationship between cell counts and dry mass of cells was used for the partitioning calculations.

If necessary, the algae were concentrated for inoculation of the experimental tubes by decanting the top water surface of a settled culture. This algae culture was then placed on a stir plate at a low setting (to disperse the settled algae) and a sub-sample enumerated with the hemocytometer. An addition of 2.5 to 3.5 mL of stock culture was added to each partitioning experiment tube to achieve 5×10^5 cells/mL in a total volume of 30 mL at the beginning of the Ag exposure.

A 24 hour IC_{50} toxicity test was also performed to determine appropriate Ag concentrations to expose algae to. The test conditions here consisted of eight concentrations of Ag^+: 0, 0.1, 10, 20, 40, 75, 200, and 500 μg/L (nominal concentrations).

All exposures were in 30 mL of media and algae were diluted to 5×10^5 cells/mL. Growth inhibition was the effect endpoint and was determined from the number of viable cells (where viable was a cell containing visually noticeable concentrations of chlorophyll, i.e., green) after 24 hours of exposure. Each algae sample was enumerated at the end of the exposure period using the hemocytometer and change in cell number over the exposure period was compared to the change in the negative control (Equation 2). The IC_{50} was calculated with a variable slope, 4 parameter non-linear regression with log transformation of the nominal concentrations. Prism® (GraphPad Software, La Jolla, CA) statistical software was used for this analysis.

$$\frac{(\text{Cell concentration at } T_{24} - \text{Cell concentration at } T_0)}{(\text{Mean of negative control cell concentration at } T_{24})} \times 100 \qquad \text{Equation 2}$$

2.2. Synthesis of Silver Nanoparticles

The production of surface ligand stabilized NPs was achieved with the methods outlined by Lee and Meisel (1982). The resulting AgNPs were stabilized with PVA surface ligands. Stabilizers like PVA aid in decreasing agglomeration, which increases the dispersion of these NPs (Porel et al., 2005).

2.2.1. PVA Stabilized Silver Nanoparticle Production

To make PVA stabilized AgNPs (AgNP-PVA), 100 mL of 0.85 g $AgNO_3$/L were added to 300 mL of cold ultrapure containing 0.0227 g of $NaBH_4$ (placed in an ice bath prior to addition of $AgNO_3$ solution). After mixing the $AgNO_3$ and $NaBH_4$ solutions, 50 mL of 1% (M/V) PVA was added to the solution and boiled for 1 hour. The final volume was adjusted to 500 mL. The resulting solution was a dark storm-cloud gray. The total Ag

concentration of the final AgNP-PVA solution was calculated to be 107.9 mg/L Ag. A 500 mL solution was also made containing PVA at the same concentration as above (1 g/L PVA), but without Ag. This was used to make up the inductively coupled plasma (ICP) standards.

2.3 Methods for Analysis/Quantification

Silver was analyzed by ICP coupled with a mass spectrometer (ICP-MS) and sample preparation was optimized to produce a higher percent recovery. Calibration of the ICP-MS was done with matrix-matched solutions. Quality control measures of detection and quantitation limits were assessed.

2.3.1. Silver Concentration Analysis

The percent recovery for the NPs was assessed for two types of sample preparation methods. In both cases, 40 mL of ultrapure was spiked with Ag as AgNP for a target total [Ag] of 250 μg/L in 50 mL polycarbonate Nalgene® Oak Ridge tubes, hereafter referred to as Oak Ridge tubes. The Oak Ridge tubes were shaken before samples (10 mL) were removed at the beginning (T_0), at 8 hours, and at 16 hours.

The first sample preparation used only HNO_3 to acidify the samples. Addition of trace metal grade HNO_3, using Whatman pH indicator test papers (Integral Comparison Strip; 1.0 – 12.0 range), brought the pH <2.0 without heat digestion (hereafter referred to as acidified samples). The second sample preparation used USEPA method 1638 (USEPA, 1996), with an overnight heat digestion instead of the recommended 2 hours. Briefly, 10 mL/L of concentrated HNO_3 and 5 mL/L of concentrated HCl were added to the sample. It was heated in an 85^{o}C oven overnight in a 15 mL polypropylene

centrifuge tube (Fisher Scientific). Both the acidified and heat digested samples were analyzed with an Agilent 7500ce ICP-MS. The percent recovery was calculated as the [Ag] measured compared to the target [Ag]. Percent recovery analysis was not conducted for Ag_2SO_4.

Total Ag concentrations for the partitioning experiments were determined at 0 and 24 hours. For the partitioning experiments, 10 mL from each sample was filtered (1.2 μm pore size) to remove algae; for uniformity, samples containing no algae were also filtered. Time 0 (T_0) samples (defined as samples collected immediately after Ag was added, but before algae or HA was added to an experimental tube) received the HNO_3 and HCl immediately following the transfer into the centrifuge tubes. The samples collected after the 24 hour partitioning experiments (T_{24} samples) had the same volumes of acids added. Both sets of samples (T_0 and T_{24}) were heat digested as described above at the same time (within 28 hours of the beginning of the partitioning experiment).

When the AgNP samples were analyzed on the ICP-MS, each calibration standard had the same concentration of PVA as the samples (1000 mg/L). A second set of calibration standards without PVA was used for the Ag_2SO_4 sorption experiments. In both cases, Fluka AAS Ag^+ standards were used to make the standards.

2.3.2. Analytical Detection and Quantitation Limits

The detection limits and quantitation limits (DL and QL, respectively) were derived from Equations 3 and 4 (FDA, 1999):

$$DL = \frac{3.3\sigma}{S} \qquad \text{(Equation 3)}$$

$$QL = \frac{10\sigma}{S} \quad \text{(Equation 4)}$$

where σ is the standard deviation of 7 digested ultrapure (blank) samples and *S* is the slope of the calibration curve. Samples that were below the QL were not used in modeling or statistical analysis.

2.3.3. Oak Ridge Tube Sorption and Filter Removal of Silver Nanoparticles

Preliminary experiments were conducted to determine the influence of filtering and the degree of sorption of Ag as Ag_2SO_4 or as AgNP-PVA onto polycarbonate Oak Ridge tubes. Ag_2SO_4 and AgNP-PVA were added to 30 mL Oak Ridge tubes at 50 and 350 µg/L, for 24 hours. The environmental conditions, HA and hardness were also included, so that tube sorption of the two Ag types at all combinations of HA and hardness were assessed. These conditions are the same as those varied in the partitioning experiments, i.e., presence or absence of HA, high or low hardness, and Ag type (Ag^+ versus AgNP-PVA). Algae were not added in this Oak Ridge tube sorption experiment. The sample was removed after 24 hours. Each Oak Ridge tube was then rinsed with ultrapure water to remove any remaining sample and the tubes were air-dried. At that time, 100 µL of concentrated HNO_3 was added to the dried Oak Ridge tube and rolled around the tube to ensure that the acid covered all surfaces. This was followed by the addition of 10 mL ultrapure and 50 µL of HCl; this sample was analyzed on the ICP-MS. Two of the Ag^+ samples (i.e., one at high hardness and no HA, and one with HA and at high hardness) had total Ag sorption to the tube of 0.33% and 0.46%, respectively. All other samples had Ag levels below detection. All the AgNP-PVA samples at 350 µg/L had sorption to the tubes ranging from 0.12 to 0.72 ng (1.2% and 8.8%, respectively, of total Ag mass) in all

samples. These results indicated that sorption of Ag^{+} from Ag_2SO_4 to the Oak Ridge tube was very small, or negligible, therefore it was not considered in the algae partitioning experiments. Since AgNP-PVA sorption to the Oak Ridge tubes was greater, sorption to each tube in the partitioning experiments with the NP was determined as in the preliminary sorption experiments and accounted for in the final mass balance (Section 2.4.1).

The influence of the filtering (1.2 μm) step on the removal of AgNPs was evaluated by determining the differences between filtered samples and unfiltered samples. This experiment was done in triplicate. Ten mL of a 24 hour solution containing culture media (no algae), AgNPs at nominal concentrations of 150 μg/L, and HA at 1 mg/L was filtered through a Whatman GF/C filter. Samples were then heat digested and analyzed on the ICP-MS. This was compared to an unfiltered sample that was also heat digested. If filtered concentrations were greater than unfiltered, they were reported as 0% loss from filtering. The difference in Ag between filtered and unfiltered samples ranged from 0% to 2.8% and so loss of AgNP from filtering was concluded to be insignificant.

2.3.4. Ion Selective Electrode

Prior to partitioning experiments, Ag ion activity was assessed using a Thermo Scientific Silver/Sulfide ISE probe. Silver measured by the ISE probe will be referred to as Ag^{+}_{NP} if the Ag ions are from AgNPs. Since low μg/L concentrations of Ag^{+}_{NP} are expected, the manufacturer's *Low-Level Measurements* procedure was followed as outlined in the manual. Briefly, low-level ionic strength adjuster (ISA) solution was added to each

sample, followed by the addition of ultrapure water. Calibration standards were matrix matched with PVA and with Ag concentrations at 0, 5, 10, 40, 100, and 300 μg/L. Two samples were made per concentration, either containing no algae, or containing algae at 500,000 cells/mL. Samples were then measured (mV) in duplicate. Each measurement was recorded 5 minutes after introducing the ISE probe to the stirred samples. Samples of AgNP-PVA were prepared at 50 and 350 μg/L total Ag (nominal concentrations). The activity of Ag^{+}_{NP} was measured every 8 hours for 24 hours, starting at 0 hours. A total of four conditions were evaluated (i.e. the two concentrations, and with or without algae; N=1). Detection and quantification limits were also determined to be 0.23 μg/L and 2.5 μg/L, respectively. Due to the expected low concentrations (1-10 μg/L), concentrations above the DL were used in this analysis rather than the QL.

2.3.5 Dynamic Light Scattering Measurements of Silver Nanoparticles

Dynamic light scattering (DLS) technique was used to determine the sizes of the AgNP-PVA. Samples were transported to Colorado School of Mines after all algae experiments were completed. This method uses Rayleigh scattering effects to measure the size distribution profile of a solution containing particles. The DLS results showed that the AgNP-PVA were 100-110 nm in diameter. It is unclear how transport and storage affected aggregation state, but assuming these samples were representative of the ones used in the algae experiments, these particles are right on the boundary between NPs and fine particles. For continuity, even though a fraction of the AgNP-PVA may be slightly larger than a NP, AgNP-PVA will still be referred to as NPs.

2.4. Partitioning Experiment

The partition coefficients (K_F) were obtained as outlined in the following sections. Two levels of each exposure condition (hardness, HA, and Ag type) were used. These levels were either a high or low setting of the conditions, e.g., two hardness levels were chosen at a high and low levels. Humic acid and Ag type conditions were varied as outlined below.

2.4.1. Partitioning Method

One 50 mL Oak Ridge tube was used for each sample. The initial volume in each tube was 40 mL of media, which was adjusted for hardness before adding any Ag. Two levels of hardness were used. The high hardness samples were adjusted with stock solution A and B (from USEPA method 1003.0; USEPA, 2002B). High hardness was measured in representative samples using a Hach® digital titrator and was on average 160 mg/L calcium carbonate (measured twice: 158 mg/L and 161 mg/L). Low hardness samples were unadjusted media (USEPA method 1003.0; USEPA, 2002B) and calculated to be 20 $CaCO_3$ mg/L. Then Ag^+ and AgNP-PVA were added. The low setting for this factor in the factorial analysis is Ag^+ and AgNP-PVA is the high setting.

At this point, prior to the addition of the HA and algae, 10 mL of sample were removed from each tube, heat digested, and analyzed as described in Section 2.3.3. Prior to each 10 mL removal of samples from the Oak Ridge tubes, the tubes were inverted several times to shake the sample. This measured concentration was used as the T_0 (or initial) Ag concentrations.

After removing the T_0 sample, the correct amount of algae and HA were added for a final volume of 30 mL with nominal concentrations of Ag^+ and AgNP-PVA ranging from 1 to 350 µg/L. The two HA levels were either with HA (0.5 mg/L added; high) or no added HA (low). The preliminary toxicity tests conducted with Ag^+ to assess what Ag concentrations to use indicated that up to 500 µg/L would not affect cell number during the 24 hour exposure (Section 2.1.4).

Exposure conditions (i.e. light, temperature, and culture media) were the same as culture conditions with the exception of added Ag, HA, or hardness. After 24 hours, the algae were enumerated from each Oak Ridge tube with a Levy Ultra Plane hemacytometer. Ten mL from each sample was then removed immediately after shaking the Oak Ridge tube and filtered through a 1.2 µm Whatman GF/C filter. This filtrate was heat digested and analyzed on the ICP-MS. Tube sorption was also assessed for the NP samples, as described in Section 2.3.3, and included in the mass balance as $M_{container}$ (Equation 5):

$$M_{total} = M_{container} + M_{algae} + M_{dissolved} \qquad \text{(Equation 5)}$$

The T_0 concentration was the M_{total} and the filtrate collected at 24 hours was the $M_{dissolved}$. The amount of Ag associated with the algae (M_{algae}) was determined by difference. This is similar to the method outlined in Miles et al. (2001) where they determined the sorption of monomethylmercury to *P. subcapitata* by difference. After solving for M_{algae}, the masses of Ag were normalized to the masses of *P. subcapitata* as in Equation 6:

$$\frac{\mu g\ Ag}{kg\ P.\ subcapitata} \qquad \text{Equation 6}$$

These quotients are used in determining K_F.

2.4.2. Methods for K_F Determination

Partition coefficients, K_F, were obtained from the Freundlich Sorption Isotherm model as shown in Equation 7 (Chin et al., 1988; Schiewer and Patil, 2008):

$$q_e = K_F C_e^{1/n} \qquad \text{Equation 7}$$

where q_e is the Ag concentration sorbed at equilibrium (i.e., the result from Equation 6), C_e is the Ag concentration in solution at equilibrium, and K_F and n are coefficients. The linearization of this model by log transformation (Equation 8) results in a simpler form of the isotherm (Schiewer and Patil, 2008):

$$\log q_e = \log k_F + \frac{1}{n}\log C_e \qquad \text{Equation 8}$$

In the model, the log of each quotient (i.e., log q_e; y – axis) is plotted against the resulting Ag concentration at time 1 (i.e., log C_e; x – axis). K_F is obtained from the anti-logarithm of the y-intercept (Wong et al., 2000).

The results of the partitioning experiments were plotted using Prism® software. The duplicate experiments were plotted using pooled data to reflect replication. The y-axis was the concentration of silver per mass of algae (Equation 6) and the x-axis was the log concentration of silver in solution (concentration at T_{24}).

2.4.3. Factorial Analysis

To evaluate the effects of hardness, Ag type (i.e., Ag^+ or AgNP), and HA on partitioning behavior, a full 2^3 factorial analysis was performed. The basic design of these experiments involved eight different treatments (to obtain eight partitioning coefficients). A high and low setting was selected for hardness, HA, and Ag type (Table 2). For hardness and HA, the high setting is represented by '+' and the low setting by '−'. For preparation, the '−' is Ag^+ from Ag_2SO_4 and '+' is AgNP-PVA (Table 3).

Table 2. High and low conditions of each factor used in the partitioning experiments. Hardness and HA are held constant at the concentrations listed below, while Ag concentrations are varied.

	Suwannee River Humic Acid[1]	**Ag type**	**Hardness**
High (+)	0.5 mg/L	AgNP-PVA	160 mg/L
Low (-)	0 mg/L	Ag^+	20 mg/L

[1] DOC not measured in the samples, so that HA is nominal above any background levels.

Table 3. Experimental conditions used to determine the K_F for Ag partitioned to algae. Each row is an individual test where HA, hardness, and Ag type are varied at all possible combinations. X designations are used for the factorial analysis.

	Setting		
Treatment #	**HA X_1**	**Ag type X_2**	**Hardness X_3**
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

The average effect of the each factor can be calculated along with the interactive effects. The influence of each individual (e.g., X_1) or interactive (e.g., X_{23}) effect can be easily calculated with the aid of Table 4 (Berthouex and Brown, 1994). Briefly, the '+' and '−' signs for an individual effect (i.e., X_1, X_2, or X_3) are the same as in Table 3. The '+' and '−' signs for an interactive effect is the product of the signs for the individual components; for example, in Treatment #1, the interaction for HA and Ag type (X_{12}) is '+', which is equivalent to the product of X_1, X_2, and X_3 (i.e., $^{-}1\times^{-}1\times^{-}1=^{+}1$ or just +). The log K_F values from the sorption experiments and modeling are filled into column Y and their effects are then calculated using the signs from each column to establish whether the log K_F is added or subtracted (e.g., Equation 9).

Table 4. The design model matrix used to plan and evaluate the conditions on partitioning behavior. The '+' and '−' signs for an individual effect (i.e., X_1, X_2, or X_3) are the same as Table 2. The interactive effect is the product of the signs for the individual effects, (i.e., $^{-}1\times^{-}1\times^{-}1=^{+}1$ or just +). Main effects are boxed in table.

	HA	**Ag**	**H**	**HA•Ag**	**HA•H**	**Ag•H**	**HA•Ag•H**	
Treatment # X_0	X_1	X_2	X_3	X_{12}	X_{13}	X_{23}	X_{123}	Y (log)
1	-	-	-	+	+	+	-	K_F1
2	+	-	-	-	-	+	+	K_F2
3	-	+	-	-	+	-	+	K_F3
4	+	+	-	+	-	-	-	K_F4
5	-	-	+	+	-	-	+	K_F5
6	+	-	+	-	+	-	-	K_F6
7	-	+	+	-	-	+	-	K_F7
8	+	+	+	+	+	+	+	K_F8
	0	0	0	0	0	0	0	

X_1 = HA = Suwannee River Humic Acid

X_2 = Ag = Ag form

X_3 = H = Hardness

Y = partitioning coefficient

Main effects and interaction effects of the three conditions were calculated by summing the Freundlich partitioning coefficients (K_F) and dividing by 4 (Berthouex and Brown, 1994), as in the following example:

$$\text{the effect of } X_1 = \frac{K_F2 + K_F4 + K_F6 + K_F8 - K_F1 - K_F3 - K_F5 - K_F7}{4} \qquad \text{Equation 9}$$

where X_1 is the effect of HA, and the K_Fs are all the Freundlich coefficients as log values.

Each log K_F value used in the factorial analysis uses pooled data from duplicate experiments. The main and interaction effects are interpreted as either increasing or decreasing the average value of log K_F. The magnitude of each effect was determined, as described by Berthouex and Brown (1994), by running a normal probability plot (R statistical software). The normal probability plot of the estimated main and interactive effects aided in separating random effects from effects that may be significant. Effects that may be significant do not align with random variables in the plot and are greater as they fall further from the line (in absolute value). The main effects of the duplicate experiments were plotted in such a way.

3. RESULTS

The algae dry weights, ISE measurements, toxicity, and analytical methodology are reported here. Also, the effects of each factor (HA, hardness, and Ag type) are determined and presented in this section.

3.1. Algae Dry Weight

The dry weights of cells (y – axis) are plotted as a function of cell concentration (x-axis) in Figure 3. The slope of the curve had a significant deviation from zero with a p-value of < 0.0001 (F-test). Here, the average dry weight was 5.7×10^{-14} kg/cell. The results are similar to Miles et al. (2001) in which *P. subcapitata*, in exponential growth phase, had an average dry cell mass of 1.9×10^{-14} kg/cell.

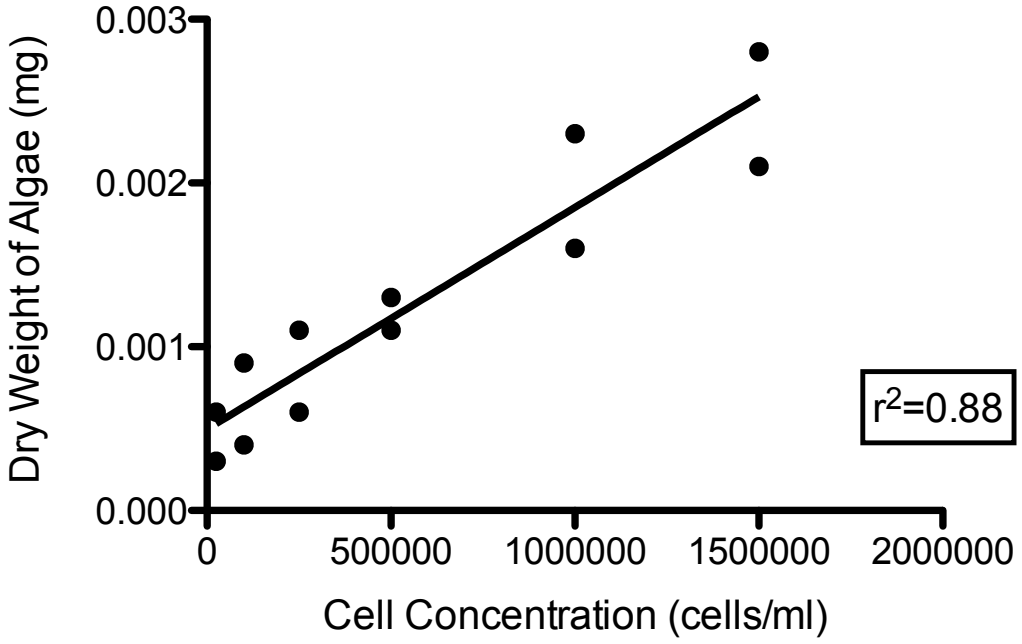

Figure 3. Linear regression of the dry algae (*P. subcapitata*) weight versus cell concentration.

3.2. Toxicity of Ag^+ to *P. subcapitata*

The 24 hour IC_{50} of Ag from Ag_2SO_4 was 4 μg/L (2.1 to 8.4; 95% CI) as calculated using Prism® statistical software. This is similar to other reports with *P. subcapitata*, in which the 24 hour IC_{50} Ag concentrations ranged from 4 μg/L and 26 μg/L (SRC, 2000; Yale, 2010, respectively). In Figure 4, change in cell number relative to the negative control (which was 100% growth) is plotted against Ag^+ exposure concentration.

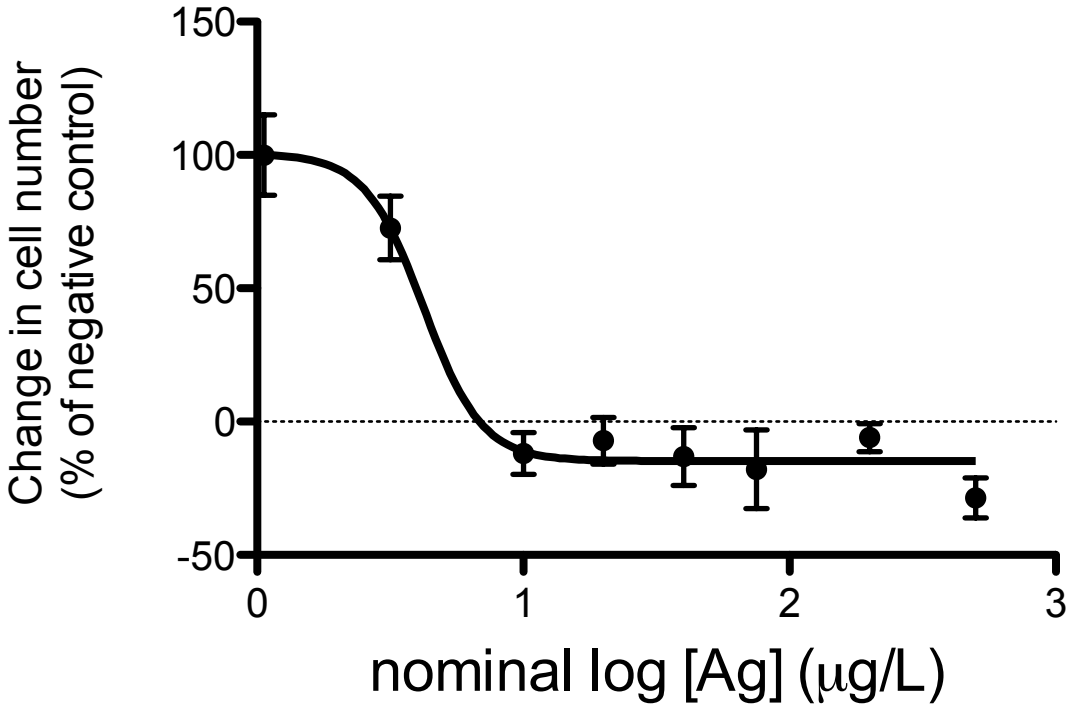

Figure 4. Silver toxicity test results showing the 24-hour concentration-response relationship of Ag from Ag_2SO_4. The error bars represent the ± standard error of the four measurements per sample. The change in cell number for the negative control during the 24 hours was 100% growth. The growth of the Ag exposed algae was then compared to the negative control. The negative growth indicates that there were less viable cells after 24 hours than there were at the beginning of the exposure.

3.3. Sample Treatment: Acidification versus Heat Digestion

Figure 5 shows the relationship between the two sample preparations (acidified and heat digested). The acidified samples had a percent recovery of 32% at T_0 compared to 83% in the heat digested sample. The other time points (8 hours and 16 hours) are not used to determine percent recovery compared to the initial spiked [Ag] since it is expected that some loss to the tube walls occurred, but a comparison between the two sample preparation methods further supports that more Ag is recovered with the heat digestion than with the acidification (67% and 7% at 8 hours and 64% and 7% at 16 hours for heat digested and acidified, respectively).

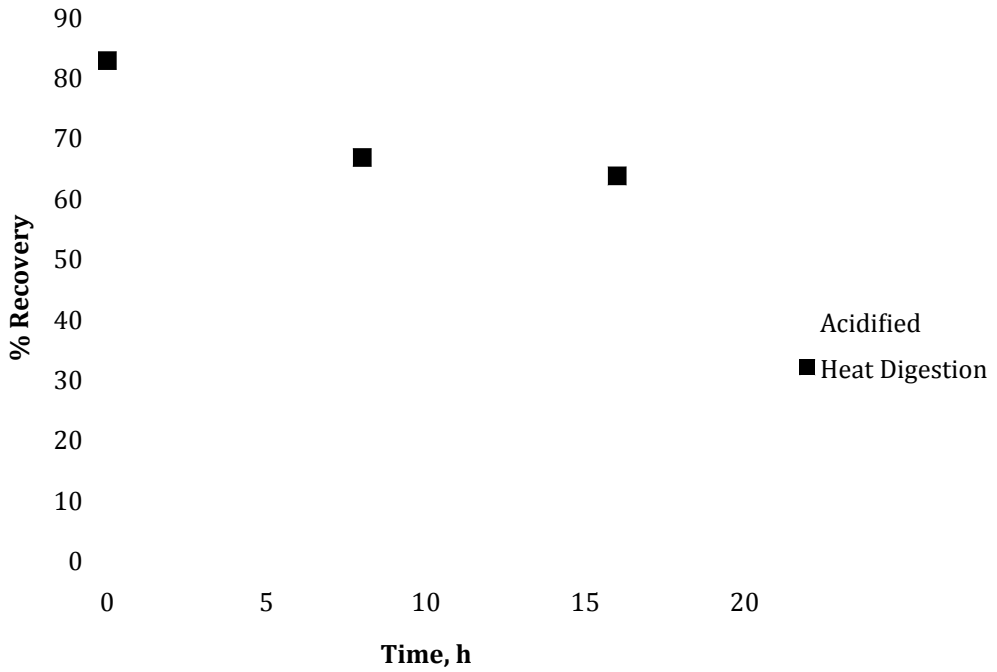

Figure 5. ICP-MS measurements of percent recovery of target concentration (250 µg/L). Acidified treatments (empty symbols) are only acidified to pH <2 with HNO_3 and Heat Digestion treatments (filled symbols) are the HNO_3 and HCl treated samples with overnight heating.

3.4. Analytical Detection and Quantitation Limits

The DL and QLs derived from Equations 1 and 2 (FDA, 1999) are listed in Table 5. Of all measurements, there was one case in which the Ag concentration was reported (via ICP-MS) below the QL and 4 cases where the concentration was reported as 0.0 µg/L. These were not included in further data analysis.

Table 5. Detection and quantitation limits for Ag^+ as Ag_2SO_4 and as AgNP.

	Detection Limit	**Quantitation Limit**
Ag^+	0.7µg/L	6.3 µg/L
AgNP-PVA	2.3 µg/L	7.0 µg/L

3.5. Ion Selective Electrode

Figure 8 shows the free Ag^{+}_{NP} ion activity from AgNP-PVA which was prepared at 50 and 350 µg/L total Ag. The *y*-axis is the ion activity as measured every 8 hours for 24 hours, starting at 0 hours. A total of four conditions were evaluated (i.e. the two Ag concentrations, and with or without algae). For each condition, the number of samples was one. Results were similar to previous research in which free Ag^{+} ions represented less than 2% of total Ag in the system (Navarro et al., 2008; Fabrega et al., 2009). At hour 16, the 350 µg/L AgNP-PVA treatments were at ~ 3% Ag^{+}_{NP}, however, all other samples at 350 µg/L remained below 2%. The DL and QL for Ag^{+}_{NP} using the ISE was calculated to be 0.59 µg/L and 1.77 µg/L, respectively (see equations 3 and 4). There were three values between the QL and the DL, each from the 50 µg/L samples, with two at 0 hour and one at 8 hours in the sample containing algae. That is, all data was above the DL and was reported (Figure 6).

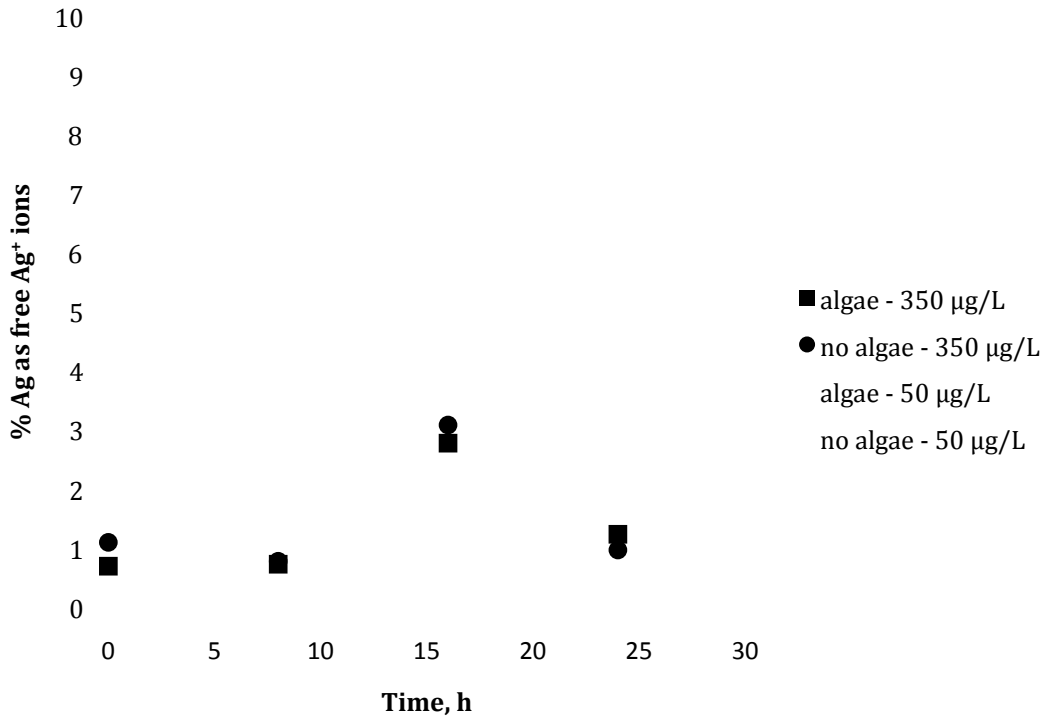

Figure 6. The free Ag^{+} ion concentrations at hours 0, 8, 16, and 24 at four different conditions: 350 µg/L AgNP-PVA with algae (filled boxes), 350 µg/L AgNP-PVA no algae (filled circles), 50 µg/L AgNP-PVA with algae (empty boxes), and 50 µg/L AgNP-PVA (empty circles).

In the 350 µg/L treatment, the Ag^{+}_{NP} percents were the same whether algae were present or not. The same was true for the 50 µg/L treatment at 0 hours. There was, however, a greater percent of Ag^{+}_{NP} at 8, 16 and 24 hours for the 50 µg/L treatments that contained no algae compared to the treatments with algae. At all measurement times, the 50 µg/L had a greater percent of Ag^{+}_{NP} than did the 350 µg/L. When these values are converted into the concentration of Ag^{+}_{NP}, however, the 350 µg/L samples had greater Ag^{+}_{NP} concentrations than the 50 µg/L, with the exception of the 24 hour samples with no algae (Table 6).

Table 6. Levels of Ag^{+}_{NP} (µg/L) at nominal AgNP-PVA concentrations of 350 µg/L and 50 µg/L after 0, 8, 16, and 24 hours as measured by ISE.

		Hours			
		0	8	16	24
350 µg/L	algae	2.43	2.54	9.71	4.32
	no algae	3.84	2.72	10.78	3.39
50 µg/L	algae	1.66	0.75	2.77	2.89
	no algae	1.61	2.19	4.50	3.95

3.6. Partitioning Experiments

Figures 7A thru 7D contain the partitioning isotherms as modeled by Prism®. The *y*-axes are the log of the Ag levels on the algae (µg/kg) as dry weight and the *x*-axes are the log concentrations of Ag remaining in solution after 24 hours (T_{24} concentrations). Each of the eight different treatments (i.e., varying hardness, HA content, and Ag type) are plotted on their own graphs. The replicate experiments are pooled for K_F determination to reflect data replication.

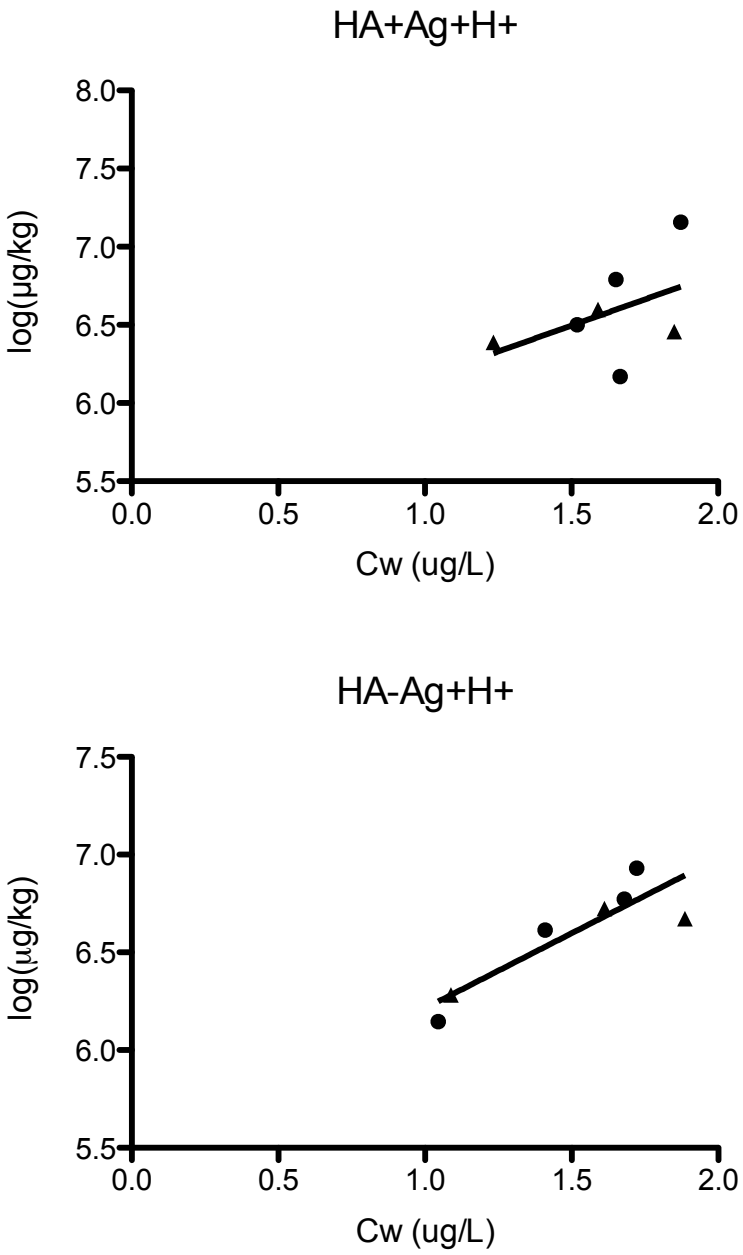

Figure 7A: The results of the 24 hour partitioning experiments showing the concentration of Ag associated with the algae at different environmental conditions. These conditions are noted by three signs, '+' or '−', with their associated factor abbreviated to the left of each (i.e., HA = HA, Ag = Ag type where "–" is Ag^+ from Ag_2SO_4 and "+" is AgNP, and H = hardness). The plotted line is from duplicate experiments to reflect replication, with circles and triangles representing the data from the two experiments. Replicate data was pooled for K_F determination.

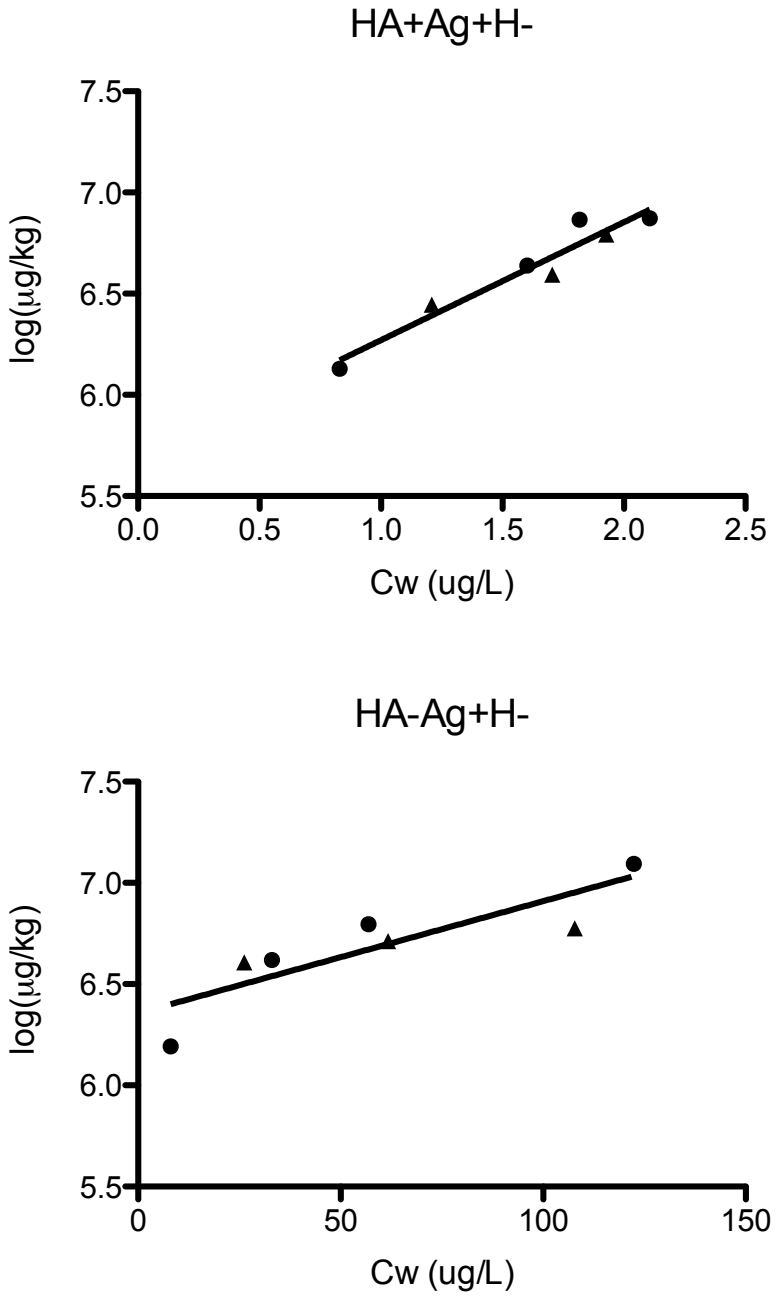

Figure 7B: The results of the 24 hour partitioning experiments showing the concentration of Ag associated with the algae at different environmental conditions. These conditions are noted by three signs, '+' or '−', with their associated factor abbreviated to the left of each (i.e., HA = HA, Ag = Ag type where "–" is Ag^+ from Ag_2SO_4 and "+" is AgNP, and H = hardness). The plotted line is from duplicate experiments to reflect replication, with circles and triangles representing the data from the two experiments. Replicate data was pooled for K_F determination.

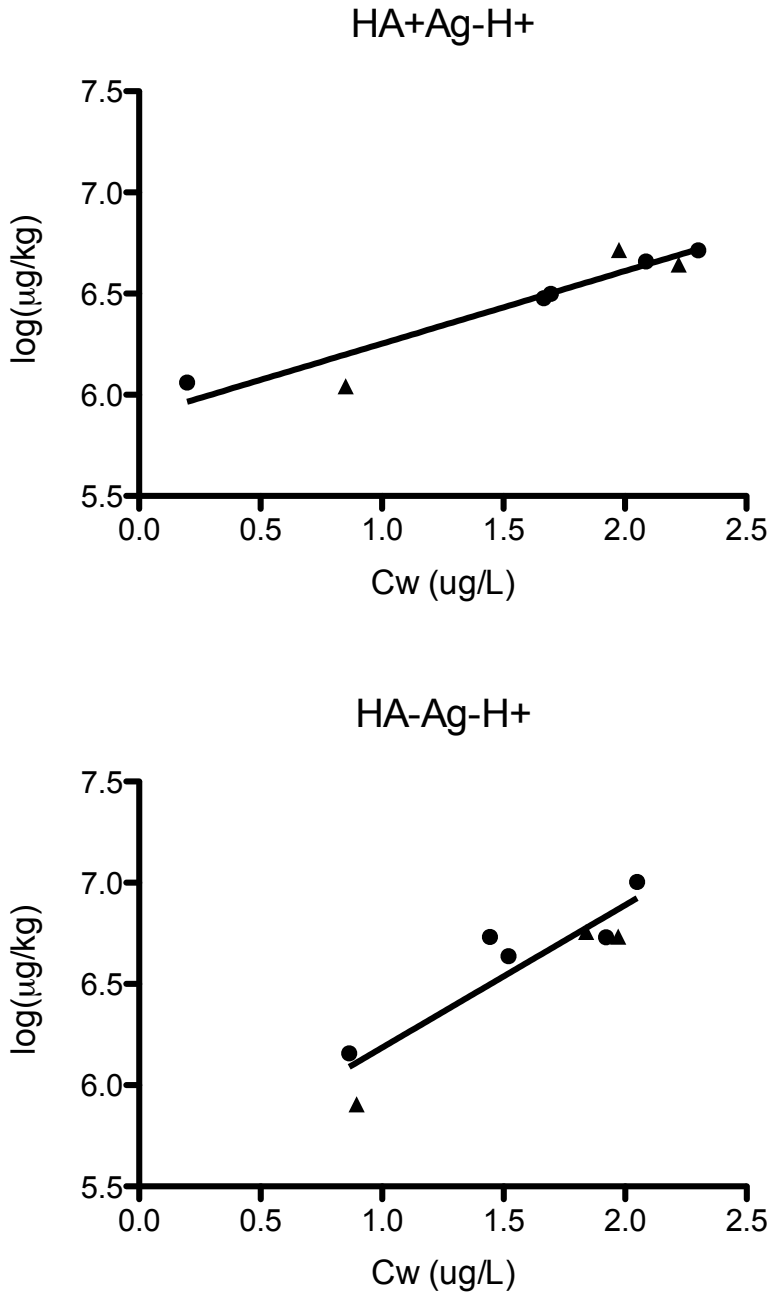

Figure 7C: The results of the 24 hour partitioning experiments showing the concentration of Ag associated with the algae at different environmental conditions. These conditions are noted by three signs, '+' or '−', with their associated factor abbreviated to the left of each (i.e., HA = HA, Ag = Ag type where "–" is Ag^+ from Ag_2SO_4 and "+" is AgNP, and H = hardness). The plotted line is from duplicate experiments to reflect replication, with circles and triangles representing the data from the two experiments. Replicate data was pooled for K_F determination.

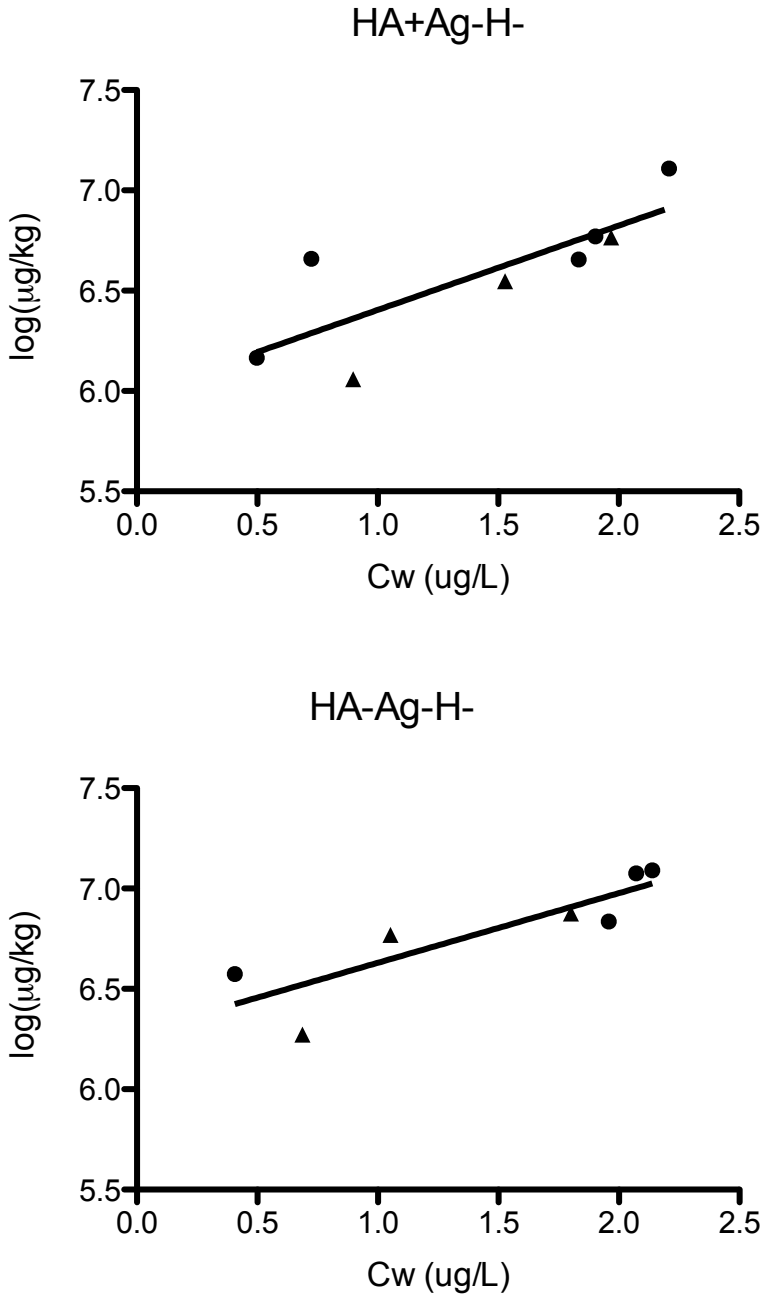

Figure 7D: The results of the 24 hour partitioning experiments showing the concentration of Ag associated with the algae at different environmental conditions. These conditions are noted by three signs, '+' or '−', with their associated factor abbreviated to the left of each (i.e., HA = HA, Ag = Ag type where "–" is Ag^+ from Ag_2SO_4 and "+" is AgNP, and H = hardness). The plotted line is from duplicate experiments to reflect replication, with circles and triangles representing the data from the two experiments. Replicate data was pooled for K_F determination.

3.7. K_F Calculations

From each plotted line, a log K_F was obtained (Figures 7A-7D). Table 7 contains the log K_F results from those isotherms. The K_F values used to determine the effect of each condition were calculated from the pooled data obtained from the duplicate experiments.

3.8. Comparing the Eight Experimental Conditions (2^3 factorial analysis)

The resulting K_F values were used to determine the relative effects of each experimental condition. Table 8 contains the relative main and interaction effect values from the 2^3 factorial designs, as calculated by Equation 9. The effect results can be interpreted as either increasing or decreasing the average value of K_F. All effects are indicative of what happens when low settings are changed to the high settings. Hardness (X_3) had the most influence on sorption as increasing hardness reduced the partitioning coefficient (log K_F) by an average of 0.465 meaning that going from 16 $CaCO_3$ mg/L to 160 $CaCO_3$ mg/L decreased the levels of Ag on the algae by an average log K_F value of 0.465. An increase in HA content (X_1) also reduced the levels of Ag associated with the algae (log K_F) by an average of 0.102. The effect of Ag type (X_2) is slightly more than HA, where replacing Ag^+ with AgNP-PVA reduces the log K_F by an average of 0.133.

Table 7. The resulting log K_F values from pooled duplicate experiments. A '+' is the high setting (i.e., AgNP-PVA, high hardness, and HA present), and a '-' is low (i.e., Ag^+, low hardness, and no HA). Boxed area borders main effects (e.g., HA, Ag, and H).

	HA	**Ag**	**H**	**HA•Ag**	**HA•H**	**Ag•H**	**HA•Ag•H**	
Treatment # X_0	X_1	X_2	X_3	X_{12}	X_{13}	X_{23}	X_{123}	Y (log)
1	-	-	-	+	+	+	-	6.287
2	+	-	-	-	-	+	+	5.942
3	-	+	-	-	+	-	+	6.353
4	+	+	-	+	-	-	-	5.686
5	-	-	+	+	-	-	+	5.485
6	+	-	+	-	+	-	-	5.890
7	-	+	+	-	-	+	-	5.418
8	+	+	+	+	+	+	+	5.616
	0	0	0	0	0	0	0	

X_1 = HA = Suwannee River Humic Acid

X_2 = Ag = Ag form

X_3 = H = Hardness

Y = partitioning coefficient

Table 8. The relative main and interactive effects of each condition. X_1 is HA content, X_2 is Ag type, and X_3 is hardness.

		Relative effects
Main	HA (X_1)	-0.10225
	Ag Type (X_2)	-0.13275
	Hardness (X_3)	-0.46475
Interaction	X_{12}	-0.13225
	X_{13}	0.40375
	X_{23}	-0.03775
	X_{123}	0.02875

One of the two largest interaction effects is that between HA and hardness (X_{13}). Their interaction increased the log K_F by an average of 0.404. Also, the interaction between HA and Ag type (X_{12}) decreased the average log K_F; this decrease is due to in the presence of HA and AgNP. The interaction between hardness and Ag type (X_{23}) slightly decreased the average log K_F and the interactions between all three conditions (X_{123}) slightly increased the average log K_F. In comparing silver types, going from the low setting (Ag^+) to the high setting (AgNP-PVA) resulted in a decrease in total silver associated with the algae.

3.9. Determining Magnitude of Effect

Potential significant effects were determined by running a normal probability plot as shown in Figure 8. Effects that may be significant fall further from the line (in absolute value). All interactive effects, except the hardness-HA interaction, fall in a straight line, and are therefore considered random (non-significant) effects. The smallest non-random effect observed was silver type, followed by the influence of HA. The greatest effects are the influence of the HA and hardness interaction (X_{13}), and hardness (X_3) alone, as it falls furthest from the line and furthest from zero. The influence of HA (X_1) and silver type (X_2) also fall off the line, but not as distant as hardness (X_3) and the interactive effect, X_{13}.

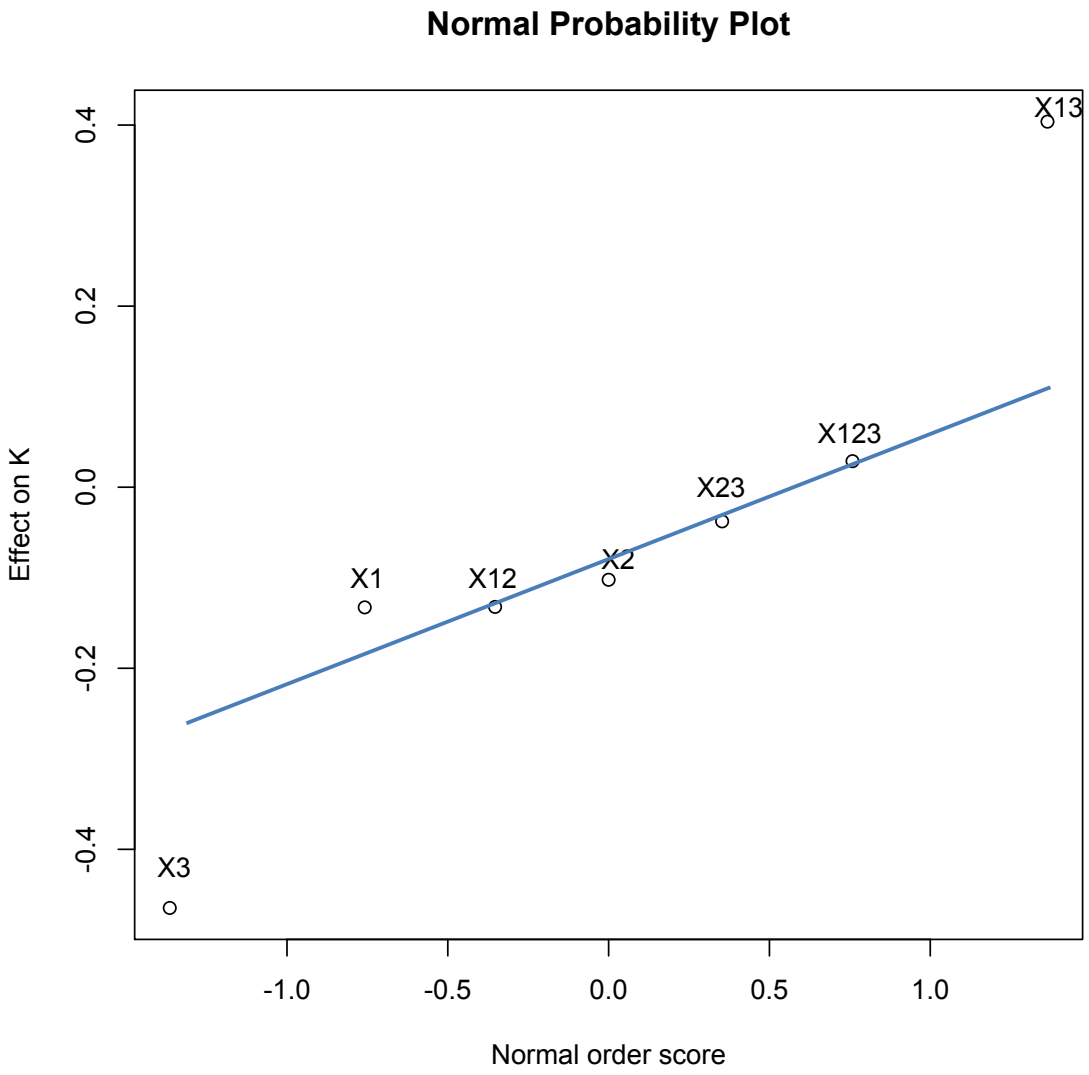

Figure 8. A normal probability plot of the effect scores over the normal order scores, comparing all main and interactive effects. The plotted line falls along the random effects. See Table 8 for explanation of data point labels.

4. DISCUSSION

The method used for algae enumeration (hemocytometer) was found to be reliable based on Figure 3, where a regression of cell concentrations and dry weight has an r^2 of 0.88. Miles et al. (2001) did not measure any differences between their hemocytometer measurements and their automatic electronic particle counter results (i.e., Coulter counter). In further partitioning experiments, however, using an electronic particle counter may increase the predictability of Ag sorption to the algae as the mode in which particles are counted does not drift (as compared to human error on a hemocytometer). Electronic counters will also decrease potential errors, such as miscalculations that may occur between two laboratory technicians counting cells using a hemocytometer (e.g., differences in analyte preparation or loading the slide with sample). Also, phase of growth and age of the exposed algae was not determined in this thesis. Further research should establish age and phase of growth in partitioning experiments as size and density of cells can vary (Allué, 2011) and this could affect sorption.

A 24 hour IC_{50} was not performed for AgNP-PVA. However the cell concentrations in the partitioning experiments exposed to AgNP-PVA did not vary significantly over the 24 hour period. For example, at high HA and high hardness and starting cell concentrations of 500,000 cells/mL, the cell concentrations at 50, 150, 250, and 350 μg/L after 24 hours were 600,000; 540,000; 600,000; and 680,000 cells/mL, respectively. Though cell concentrations did slightly increase from the nominal 500,000 cells/mL over 24 hours, there was no clear dose-response relationship between 0 μg/L and 350 μg/L AgNP-PVA.

At 50 µg/L AgNP-PVA, the percent Ag released as Ag^{+}_{NP} in the presence of algae, as measured with the ISE, was consistently less than in the absence of algae. The same was not true for the 350 µg/L AgNP-PVA; the percent total Ag as Ag^{+}_{NP} levels were similar whether algae were present or not. In the case of the 50 µg/L AgNP-PVA, it is possible that Ag^{+}_{NP} sorbed to the algal surface or to exudates in solution released from the algae, resulting in the lower Ag^{+}_{NP} when algae were present. Interestingly, the higher concentration of total AgNP –PVA had a lower % Ag released as Ag^{+}_{NP} than did the lower AgNP-PVA concentrations, whether algae were present or not. The decrease in percent Ag released as Ag^{+}_{NP} does not increase with AgNP-PVA concentration. Sotiriou and Pratsinis (2010) have demonstrated that an increase in AgNP surface area results in a "high release rate" of Ag^{+}_{NP}, and furthermore, have shown that an increase in AgNP size results in a decrease of the Ag^{+}_{NP} ion fraction. Research should be considered to investigate how AgNP concentrations affect the percent Ag released as Ag^{+}_{NP} fraction. Also, further evaluation of water quality effects (e.g., hardness, HA, pH, etc.) on ion release from AgNPs may aid in understanding their behavior, including their potential toxicities, in natural waters.

It is possible that the production of hydrogen peroxide from the algae might partly induce an increase in dissolution of silver from the NP. However, the ISE results showed that the levels of free ions did no change much at experimental conditions over the 24 hour period. This might be due to an increase in binging sites as the algae continue to secrete mucilage throughout their growth cycle. This might provide a continual sink for silver ions to bind to, as more silver ions are released from the nanoparticles. Longer

exposure periods might also show a greater effect of the H_2O_2 on the dissolution of AgNPs.

In research on the sorption of AgNPs to freshwater algae (*Chlamydomonas reinhardtii*), Allué (2011) has demonstrated that not only do ions sorb to the surface of algae, but the NPs do as well. In her research, with algae exposed to AgNPs at 108 µg/L for 1 hour about 96% of the total NP fraction that associated with the algae was sorbed to the algal surface. The other 4% had been internalized in the cell. Evaluating the amount of Ag^+ from $AgNO_3$, Allué (2011) determined that 55% of the total silver associated with the algae was sorbed to the cell wall. These results suggest that in this thesis, the AgNPs added to *P. subcapitata* are likely to sorb onto the algal surface, as hypothesized. Any agglomerated particles that would potentially sediment on the bottom of the container are likely settling along with the algae the NPs are sorbed to.

In this thesis, it was assumed that the NPs are sorbed onto the algae or physically associated with the cells in the specific case of agglomerates of NPs. In other words, it is likely that any settled NP agglomerates are associated with the algae as the NP loading onto the algal surface likely increases the propensity of the algae to settle. Further investigation would be necessary to determine the extent in which agglomerates (including the sizes of agglomerates) might sorb to or otherwise associate with algae cells.

The potential of AgNPs to sedimentate on the bottom of the Oak Ridge tubes were not determined in this thesis. Research suggests that NP interactions with organic matter can result in an increase in NP sedimentation (Arvidsson et al., 2011). This

phenomenon might be increasingly observed in samples containing HA. *Pseudokirchneriella subcapitata* secrete mucilage, which may be another cause of the lack of observed effect of changing silver type from Ag^{+} to AgNP-PVA. It is possible that this mucilage would provide an increase in area for the NPs to associate. However, whether Ag^{+} or AgNP are more likely to associate with this mucilage remains to be determined. Another source of interference might be via the filtering procedure. Filtering out algae that contain high levels of AgNP agglomerates might result in NPs coming off the algae as the algae are filtered out of solution. The physical movement of algae moving to the filter and with a high velocity of water flowing around algae caught in the filter might cause a release in NPs from the surface of the algal cells. This occurrence would underestimate the levels of Ag complexed with the algae.

The results of the partitioning experiments demonstrate that the effect of hardness on Ag sorption to algae was the most significant of all individual factors. Increasing the ions responsible for an increase in hardness (i.e., Mg^{2+} and Ca^{2+}) likely results in fewer available ligand sites on the algae that would otherwise be occupied by Ag^{+}. This is not surprising, as other research has shown (specifically in the BLM) that when hardness is increased, the levels of Ag sorbed to the BL of fish or daphnia are reduced (Karen et al., 1999; Bianchini and Wood, 2008). Likewise, more BL sites on *P. subcapitata* are likely occupied by Mg^{2+} or Ca^{2+} as hardness increases; therefore, the potential for these biotic ligands to attract and sorb Ag ions are diminished. Hence, the predicted decrease in Ag sorption due to an increase in water hardness was observed.

The influence of HA alone did not significantly affect the K_F values in this research. Other research has evaluated the influence of HA on AgNP behavior. Dasari and Hwang (2010) illustrated that HA sources (aquatic vs. terrestrial) have a potential to elicit different AgNP toxicities to bacteria. Though their research is specific to bacteria, they do point out that the sources of HA determine their chemistry (e.g., higher nitrogen content in aquatic HA and higher carbon content in terrestrial HA) and therefore elicit differences in AgNP toxicity. Significant decreases in Ag^+ toxicity in *Gammarus pulex* were observed with increasing HA as defined by the BLM, at concentrations 20 times higher (10 mg/L) than that used in this thesis (Bury et al., 2002). These researchers also mention that the levels of reduced sulfur components of DOM in most aquatic environments likely exceed that of available Ag^+. It is possible that higher concentrations of HA, or differences in sources (terrestrial HA vs. aquatic HA), may have potential significant influences on sorption in successive experiments. Future studies should evaluate many different levels and types of DOM, its components (e.g., HA and fulvic acids), and at varying degrees of hardness.

The observed interaction between hardness and HA, with an average log K_F increase of 0.404, was one of the two largest effects. Based on other research, it is possible that the HA provides sorption sites for the ions associated with higher hardness, resulting in a decrease in Mg^{2+} or Ca^{2+} available to bind to the algae. At the same time, there would be less sites on the HA to bind some of the Ag^+. This could lead to a significant increase in Ag on the algae surface, as was observed. The IHSS provides ratio data on the elemental composition of SWHA standard II. The oxygen is 53% and sulfur is .6% total mass of SWHA (IHSS, 2008). Though the form of oxygen and sulfur is not

defined, there is evidently 88 times more oxygen than sulfur. According to Smith et al. (2002), Ca^{2+} has a high affinity to particulate oxides and Ag^{+} has a high affinity to particulate sulfides. If the SWHA is proportionally higher in oxide content, the results of my work results might be explained by the nature of hardness ions preferring the SWHA to the algal surface.

Though there are many unknowns, it is clear that concomitant increases in water hardness and humic acid increase Ag sorption to the algae. Increases in water hardness alone have been shown to reduce the levels of Ag sorbed to the BL of fish or daphnia, as revealed both in the literature (Karen et al., 1999; Bianchini and Wood, 2008) and in this thesis using *P. subcapitata*. However, introducing HA to the samples containing high hardness caused a significant increase in levels of Ag sorbed to *P. subcapitata*. Winner (1985) found similar results using toxicity of Cu to *Daphnia pulex* as an endpoint. In soft and moderate water conditions, toxicity of Cu was reduced in the presence of HA. However, at high hardness levels, the effects of HA increased the toxicity of Cu. Similar results were observed using Zn as a toxicant to *D. magna* where HA reduced the toxicity of Zn in soft water more so than in hard water (Paulauskis and Winner, 1988). These results reflect a system that involves an interaction between HA and water hardness and may be due to the hypothetical mechanism described above. These effects will likely be observed in an oligotrophic lake with high hardness conditions.

Silver type effects were not observed. The levels of sorption to the algae did not change significantly between Ag^{+} and AgNP-PVA; this was the main effect was nearest to zero. Sorption levels of AgNP-PVA were the same as Ag^{+} sorption. One limitation of

this study is that the nanoparticles were not characterized after algae were added. It is possible that the AgNPs were oxidized over the 24 hour sorption experiments so that all of the AgNP existed as Ag^{+}. This would explain the lack of differences between AgNP and Ag^{+} sorption. A thorough investigation should be conducted to determine why there is no difference in Ag sorption when the two types of Ag are used. To further examine the effects of Ag type on sorption behavior, several types of AgNPs should be examined to determine their potential sorption activity based on their preparation, associated ligands (i.e., coatings), size, and/or crystallinity. These results could all be compared and used to increase the power in predicting the fate of NPs in the environment.

5. CONCLUSION

The partitioning of AgNP-PVA and Ag^{+} between the freshwater algae, *P. subcapitata*, and water is largely affected by hardness and HA. Increases in HA content alone did not influence the loading of Ag onto the algae. However, going from 0.0 to 0.5 mg/L of HA actually increased the levels of Ag associated with the algae when the water was hard (160 mg/L $CaCO_3$). Increasing hardness alone had a significant effect on the partitioning of Ag; the average logK_F decreased by about 0.465. The hardness and the HA/hardness interaction effects were the only two factors that exhibited the largest influence on Ag sorption. Changes in Ag type and all other interactions had no or less effect on the partitioning of Ag. Future studies should investigate the influence of other DOC components (e.g., fulvic acid), their levels, sources (e.g., terrestrial HA), and at higher levels of HA. Also, characterizing the AgNPs after a 24 hour or more exposure period would aid in understanding the organismal effects on the chemistry of AgNP-PVA. Probing a suite of AgNPs for their potential sorption activity should be based on their preparation, associated ligands (i.e., coatings), and/or crystallinity to further this research.

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