FATE OF SILVER NANOPARTICLES DURING SIMULATED COMMERCIAL PROCESSING OF FRESH-CUT LETTUCE

By

Gayathri Upeksha Gunathilaka

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science - Doctor of Philosophy

2022

ABSTRACT

FATE OF SILVER NANOPARTICLES DURING SIMULATED COMMERCIAL PROCESSING OF FRESH-CUT LETTUCE

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The use of silver nanoparticles (Ag NPs) in pesticides could lead to residual levels in food crops, thus raising both food safety and environmental concerns. The effectiveness of typical produce processing practices to remove Ag NPs from fresh produce is poorly understood. Further, little is known about the behavior of Ag NPs in wash water during commercial production of fresh-cut produce, which limits our ability to design effective mitigation strategies.

The first study evaluated the behavior of Ag NPs over time when exposed to commercially applicable chlorine concentrations (2–100 mg chlorine/L) in simulated lettuce wash water. Aggregation and dissolution of Ag NPs (5 mg/L) were evaluated in the presence and absence of dissolved lettuce extract (DLE, 0.1%). Aggregate size of Ag NPs increased faster in the presence of chlorine (49 to 431 nm) compared to the control ($P < 0.05$). Lower dissolved Ag concentrations and more negative zeta potentials were found in the presence of chlorine (0.01 to 0.03 mg/L and -39 to -95 mV) and DLE (0.01 to 0.14 mg/L and -28 to -32 mV), as compared to the control (0.54 to 0.8 mg/L and -10 to -20 mV) ($P < 0.05$). Transmission electron microscopy with energy dispersive spectroscopy confirmed the formation of composite AgCl-Ag NPs particles in the presence of chlorine. The increased aggregate size over time likely resulted from nucleation and crystal growth of AgCl. In the presence of DLE, Ag NPs and AgCl precipitates were embedded in and bound to the DLE matrix. These observations suggest that chlorine and plant-released organic matter could substantially change the fate of Ag NPs in wash water and subsequently their environmental impact.

In the second study the removal of Ag NPs from Ag NP-contaminated lettuce leaves was investigated during batch and small-scale pilot processing. First, a batch-type system (4-L carboy jar) was used to evaluate the impact of commercial produce sanitizers and simulated leafy green processing water on the removal of Ag NPs from contaminated lettuce. Peroxyacetic acid (PAA; 80 mg/L) and chlorine (100 mg/L and pH 6.5) were used with/without 2.5% (w/v) organic load as washing treatments with deionized water serving as the control treatment. Treating lettuce with the organic load alone, organic load with chlorine, chlorine alone, 0% organic load and peroxyacetic acid removed about 2.7%–6.6% of Ag from the lettuce after 5 min of washing. Thereafter, the removal of Ag NPs from contaminated lettuce was assessed using a small-scale pilot processing line during 90 seconds of flume-washing followed by centrifugal drying (a typical washing practice in commercial produce processing). The Ag removal efficiency ranged between 0.3% to 3%, probably resulting from strong binding of Ag with plant organic materials. Significantly greater Ag concentrations were found in the centrifugal water than in flume water, suggesting that centrifugation removed additional Ag from lettuce.

The low Ag removal observed in both the simulated batch-type and pilot-scale produce washing systems demonstrate that typical produce processing conditions may not be effective in removing Ag NPs from contaminated produce. Thus, it is necessary to further investigate and develop effective methods for the Ag NP removal from contaminated produce.

This dissertation is dedicated to everyone who has supported my education.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. Elliot Ryser, for his continuous guidance, valuable support, and patience throughout my Ph.D. study. He always had time to guide me whenever I stepped into his office regardless of how busy he was, and I am very grateful for all of his support. I would also like to express my sincere gratitude to Dr. Wei Zhang for all the guidance and valuable assistance provided to me throughout my Ph.D. This research would not have been possible without the continuous guidance and unlimited support of Dr. Ryser and Dr. Zhang. I would also like to thank Dr. Jeffrey Swada for his advice and directions for my research, especially the pilot-scale processing study. I am also grateful to the other members of my dissertation committee, Dr. James Pestka and Dr. Hui Li, who provided me with guidance, assistance, and insightful feedback.

I would like to convey my thanks to Dr. Xudong Fan at the Center for Advanced Microscopy for assisting me in microscopic analysis, the CANR Statistical Consulting Center (Junjie Han, Sukhdeep Singh, and Dr. Robert Tempelman) for helping me with statistical analysis, and Michigan Elemental Analysis Lab at the University of Michigan for sample analysis. I am grateful to Dr. Scott Burnett, David McSherry, and Dr. Junzhong Li for their valuable insight regarding sanitizer interactions. Dr. Gordon Davidson also provided important feedback which furthered the development of my research. My sincere thanks also go to Dr. Bradley Marks, the two faculty instructors for the courses I worked as a TA (Dr. Ustunol and Dr. Strasburg) as well as all my MSU course professors, Ms. Marcia Hardaker (graduate coordinator) and my masters advisor Dr. Yifan Zhang for all their support.

I would like to acknowledge financial support for my Ph.D. research provided through the USDA-National Institute of Food and Agriculture, Agriculture and Food Research Initiative (Grant No: 2017-67017-26168).

I would also like to thank my previous lab members and friends at Michigan State University – Jianzhou He, Yike Shen, Nurul Hawa Ahmad, Natasha Sloniker, Quincy Suehr, Sihan Bu, Hamoud Alnughaymishi and many others for all the valuable support and friendship given to me.

Lastly, I would like to thank my family for their love and support. I cannot continue without thanking my husband and my best friend, Dhananjaya, for all his support and love and, my parents for their support and encouragement.

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KEY TO SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of Variance
d	days(s)
FDA	Food and Drug Administration
FSMA	Food Safety Modernization Act
g	grams(s)
h	hour(s)
min	minute(s)
ml	milliliter(s)
ppm	Parts per million
s	second(s)
SAS	Statistical Analysis Systems
US	United States of America
USDA	United States Department of Agriculture
µl	microliter(s)
µm	micron(s)

INTRODUCTION

Worldwide production of minimally processed fresh produce has continued to increase. For several years, an ever-greater awareness of healthy diets has been driving both increased production and consumption of fresh produce. The consumption of fresh produce is estimated to increase 10-20% annually (64, 151). Alongside the heightened consumer demand for minimally processed fresh produce, the quality and safety of these products is also receiving increased attention, typically including food safety risks from microbial pathogens, conventional pesticide residues, and heavy metals (5, 135, 154). Despite considerable attention to food safety risks of fresh produce, limited concerns have been raised regarding engineered nanoparticles (ENPs).

Among the most important nanoparticles in agriculture are silver nanoparticles (Ag NPs), which exhibit antimicrobial properties and are used as a broad-spectrum treatment against plant pathogens (69, 142). Each year an average of 500 tons of Ag NPs are produced worldwide (111), and the rate of production will likely increase as Ag NP applications broaden. Agricultural soils and water may be overwhelmed from the potential increased use of Ag NPs in nano-pesticides and the subsequent release after use. The amount of Ag NPs released from nanopesticides is estimated at 54 times that of all other sources combined (such as sewage sludge, wastewater irrigation, and atmospheric deposition) (46). The U.S. Environmental Protection Agency (EPA) has registered hundreds of nano-pesticide products containing Ag NPs, with many new patents on fungicides containing Ag NPs now pending (165). As such, there is a high probability that significant amounts of Ag NPs will be released into the environment based on current and future applications. Ag NP exposure results in their uptake and bioaccumulation in plants, which has been demonstrated experimentally for Ag NPs up to 40 nm in size using Arabidopsis thaliana plants as a model (102). Similarly, Ag NPs may also accumulate in fresh fruit and vegetable products destined for human consumption.

Humans are at risk of Ag NP exposure from food crops due to their movement through the food chain which may adversely affect human health. In vivo toxicity studies have shown liver and DNA damage in rodents after Ag NP exposure to > 125 mg/kg and > 20 mg/kg, respectively (76, 145), as well as accumulation of silver in the liver and kidneys of rodents exposed to > 20 mg/kg (145). In vitro toxicity tests have also shown damage in human lung fibroblast cells, glioblastoma cells, and skin keratinocytes at Ag NP exposure levels of 20-100 μg/ml (8, 93). Therefore, chronic human exposure to trace-levels of Ag NPs should be prevented as much as possible to minimize health risks.

Because fresh produce is minimally processed before consumption, effective processing strategies need to be developed to decrease the level of Ag NPs in fresh-cut produce. However, the effectiveness of present produce-processing practices in removing Ag NPs is currently unknown. In commercial processing, leafy greens are washed in a flume tank with sanitizers and then drained on a shaker table before being centrifugally dried to further remove excess water. Sanitizer addition to processing water is a standard practice in the produce industry to reduce microbial populations and minimize cross-contamination during washing (31, 98). Chlorine-based sanitizers are favored and widely used within the produce industry due to their cost-effectiveness and minimal adverse impact on end product quality (21, 30, 97, 117). Maintaining the processing water at 4 to 7 °C helps to minimize microbial growth and maintain product quality (64). During washing and recirculation, the produce wash water will accumulate increasing amounts of organic matter from the cut produce, tissue exudates, and other debris (e.g., soil, insects, and microbes). The efficacy of chlorinated sanitizers decreases during the washing due to reaction with other organic matter (30), thereby reducing its ability to minimize microbial cross-contamination during washing. Furthermore, as the organic matter accumulates in the wash water, the chlorine

concentration decreases from its starting value in the range of 50 to 100 mg/L (30, 127). Sodium hypochlorite has a long history of use as a chlorinated sanitizer in the produce industry (70). Because of concerns regarding the efficacy of chlorine being negatively impacted by organic load, there is increasing interest in alternative sanitizers such as peroxyacetic acid (32). Unlike chlorine-based sanitizers, the efficacy of peroxyacetic acid is unaffected by organic load (32). The impact of current processing practices on the removal of Ag NPs from fresh produce is currently unknown. Therefore, the impact of chemical sanitizers on Ag NP behavior in produce processing water needs to be better understood in order to develop effective strategies for the removal of Ag NPs from fresh produce during the washing.

Therefore, this dissertation research aims to: 1) review of the pertinent literature 2) evaluate the behavior of Ag NPs over time when exposed to commercially applicable chlorine concentrations in simulated lettuce wash water (Chapter 2); and 3) determine the impact of commercial sanitizers and simulated leafy green processing water on the removal of Ag NPs from contaminated romaine lettuce using both a cost-effective model bench-top carboy system and a pilot-scale processing line (Chapter 3).

Objective 1 is covered in Chapter 2 and Objective 2 in Chapter 3. The dissertation concludes with a summary of the findings and recommendations for future research.

CHAPTER 1:

LITERATURE REVIEW

1.1 USE OF ENGINEERED NANOPARTICLES (ENPS) IN AGRICULTURE

Particles with at least one dimension between 1 to 100 nm in diameter are typically referred to as nanoparticles *(46)*. Production and application of engineered nanoparticles (ENPs) have dramatically increased worldwide during the past several decades due to their unique physical and chemical properties *(46)*. At present, ENPs are increasingly incorporated into many industrial and consumer products, including textiles, paint, clothing, sunscreen, cosmetics, antimicrobial agents, medicine, food additives, and pesticides. Nano formulations are already used widely in pharmaceutical and personal care products. Nanotechnology is the next groundbreaking technology in agriculture and has many applications including improvement of agricultural productivity and sustainability. Through nanotechnology, nanopesticides and nanofertilizers for sustainable farming are also possible *(46, 148)*. The development of pesticides using nanotechnology has drawn immense scientific and industry attention. Applications within the agrochemical sector are still in their infancy, although some nanopesticides are already on the market *(46)*. Over 3000 patents for nanopesticides were registered within the last decade *(71)*, with expectation of rapid growth in the coming years.

Nanoformulation of conventional pesticides from polymers and metallic nanoparticles has become an area of high demand in the pesticide industry *(71)*. Nanopesticide formulations aim to increase solubility of poorly soluble active ingredients, protect the active ingredients from premature degradation, and slowly release the active ingredient in a controlled manner *(20, 40, 71, 136)*. Nanopesticides can be produced as micro- and nano-emulsions or through dispersion of ENPs as either direct active ingredients or as pesticide carriers. In nanoencapsulation of pesticides, the outer shell of the nanocapsules is manipulated and the active ingredient is slowly released at low doses over time to prevent excess pesticide run-off *(24)*. Active ingredients in nanocarriers are

highly stable and attach to specific target sites. In addition, nanoemulsions of pesticides with oil or water enhance their solubility and efficiency against pests *(25, 113, 168)*. Nano-clay and silica nanoparticles have been used as pesticide carriers for controlled release*(12, 45, 91)* and titanium oxide, silver, and copper nanoparticles are being tested as active ingredients in fungicides for crop protection *(16, 28, 74, 125)*. However, more extensive research is needed to develop effective mitigation and regulation strategies to minimize their harmful impact on the environment.

1.2 AG NPS AND THEIR ENVIRONMENTAL IMPACT

Ag NPs predominate in the nano-family and have the highest degree of commercialization *(106, 162)*. To date, Ag NPs are most commonly used in consumer products compared to other ENPs because of their inherent antimicrobial properties *(124)*. These consumer products include deodorants, air filters, textiles, toothpastes, cosmetics, bandages, food packaging, household appliances, and baby products *(124)*. Ag NPs have been used in medical imaging and electronics as well as in the food industry, due to their unique physicochemical properties such as high thermal and electrical conductivity *(110, 115, 141)*.

Ag NPs have been used in making broad-spectrum antimicrobials for use against plant pathogens such as *Fusarium culmorum, Botrytis cinerea, Phoma* spp.*, Scalerotinia sclerotiorum, Rhizoctonia solani, Sphaerotheca pannosa, Pythium ultimum, Bipolaris sorokiniana, Colletotrichum gloeosporioides, Trichoderma* spp. and *Magnaporthe grisea (24)*. Furthermore, silica nanoparticles containing Ag are effective in treating powdery mildew disease on plants in the gourd family (Cucurbitaceae) including melon, pumpkin, squash, and cucumber *(24)*.

Capping agents are used in the production of Ag NPs to prevent aggregation by steric and electrostatic repulsion *(10)*. They affect Ag NP stability in addition to solution chemistry such as electrolytes, NOM, pH, and ionic strength *(169)*. ENPs naturally tend to aggregate and settle easily because of their high surface to volume ratio *(122, 147)*. Capping agents protect ENPs and provide stability *(10)*. Polyvinylpyrrolidone (PVP), citrate, and sodium borohydride ($NaBH_4$) are the most prevalent capping agents used in the production of Ag NPs *(10)*.

Average worldwide production of Ag NPs is approximately 500 tons/year *(85)*, with rapid increases expected in the future due to the continually expanding applications for Ag NPs. For example, out of a total of 1827 products with ENPs, there are currently 442 Ag NP-containing

products in Europe *(106)*. Additionally, in Denmark alone, 340 out of 2329 ENP-containing products contained Ag NPs *(106)*. Because of their current widespread and increasing use, increasing levels of Ag NPs will likely be released into the environment in the future.

The release of Ag NPs into the environment can happen though multiple pathways, such as atmospheric deposition, irrigation with wastewater effluent, land application of sewage sludge, and stormwater runoff. The increasing use of Ag NPs in agriculture (e.g., nanopesticides) will potentially become a significant source of release of Ag NPs to agricultural soil and water. Hundreds of Ag NP-containing products are currently registered in the U.S. by the EPA *(165)*. Further filings for many patent applications for Ag NP-containing fungicides have already been made. The release of Ag NPs into the environment from nanopesticide use is estimated to be 54 times higher than the combined release from all other sources *(43, 46, 48, 78, 152, 153)*. From 1983 to 2002, the concentration of Ag in Asian surface water changed from 0.03 ng/L to 1.3 ng/L *(100)*. Ag concentrations were as high as 27-36 ng/L in San Diego Bay and San Francisco Bay by the late 1980s *(100)*. In 2018 , Ag NPs were found in the range of 0.3 to 2.5 ng/L in Dutch surface water *(162)*. In addition, wastewater effluent in Germany reportedly had a Ag NP concentration of 12 ng/L*(162)*. Nonetheless, information on current environmental concentrations of Ag NPs is generally lacking. However, a few studies modeling predicted environmental concentrations provide valuable insight in lieu of actual measurements. The total Ag concentration is estimated to be in the ng/L range for water, and in the mg/kg range for soil and sediment *(14, 106)*. The predicted Ag NP concentrations were 0.016–0.127 μg/L in wastewater effluents and 1.29–6.24 mg/kg in sewage sludge *(48)*. In another study, the predicted concentrations of Ag NPs in surface waters were between 0.09 and 0.43 ng/L in the U.S. and between 0.59 and 2.16 ng/L in Europe *(160)*. However, the concentration of Ag NPs was measured in the effluent from one U.S.

wastewater treatment plant was as high as 0.1 μg/L *(109)*. Environmental concentrations of Ag NPs are expected to increase rapidly, thus greatly elevating the exposure of humans and ecosystems to Ag NPs. Due to their very dynamic behavior in aqueous environments, Ag NPs may undergo various morphological and chemical transformations after release *(157)*. Thus, environmental factors play a critical role in the transport, fate, and toxicity of Ag NPs *(157)*.

1.3 SYNTHESIS OF AG NPS

Ag NPs are synthesized using physical, chemical, and biological methods. "Top-down" and "bottom-up" are the primary two main methods for synthesizing Ag NPs *(34)*. Bulk metals are mechanically ground and then stabilized using protective agents in the "top-down" method *(6, 104)*. Chemical reduction, sono-decomposition, and electrochemical techniques are used in the "bottom-up" method *(140, 161)*. Evaporation-condensation is used to physically synthesize Ag NP in a tube furnace *(55, 82, 163)*. The advantages of physical methods include lack of hazardous chemicals and rapidity of the process. However, poor uniform distribution, high-level energy consumption, poor yield, and solvent contamination are the pitfalls of this method *(1, 129, 130)*. Water or organic solvents are used to synthesize Ag NPs in the chemical method. Disadvantages of using chemical methods are generation of hazardous byproducts during the process, low purity of Ag NPs, extremely high cost of the process, and poor quality of the synthesized Ag NPs (variations in size, tendency for aggregation) *(59, 103)*. However, compared to physical methods, chemical methods are far more economical and produce a higher yield.

Additionally, biological methods have been developed to overcome the limitations associated with the chemical synthesis of Ag NPs. Extracts from various plants (e.g., *Allophylus cobbe (57)*, *Artemisia princeps (61)*, and *Typha angustifolia (56)*), as well as different bacteria (e.g., *Pseudomonas stutzeri (79)*, *Lactobacillus* strains *(114)*, *Bacillus licheniformis (72)*, *Escherichia coli (62)*, *Brevibacterium casei (73)*), fungi (*Fusarium oxysporum (132)* and *Ganoderma neo-japonicum Imazeki (58)*), small biomolecules (e.g., biopolymers *(87)*, starch *(83)*, fibrinolytic enzymes *(34)*, and amino acids *(131)*) have been used to synthesize Ag NPs biologically. The three major components in biological synthesis include a solvent, reducing agent and non-toxic material. The advantages of biological synthesis include a high yield, rapid

production, , minimal impact on the environment, ready availability of biological materials, cost effectiveness, and simplicity *(143)*. Further, extra steps are not needed to prevent NP aggregation, unlike for chemical methods. The quality and stability of Ag NP is also higher since both the shape and size of can be controlled during production *(60, 143)*.

1.4 CHARACTERIZATION OF AG NPS

Ag NPs are characterized based on various properties including shape, surface area, aggregation behavior, morphology, particle size distribution, dispersivity in solution, and crystallinity. Dynamic light scattering (DLS), zetasizer, transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), scanning electron microscopy (SEM), UV-Visible spectroscopy, X-ray diffraction (XRD) and Fourier Transform Infra-Red (FTIR) spectroscopy are the most widely used techniques for characterization.

UV-Visible spectroscopy provides the absorbance spectra of the NP with both shape and size determined from the wavelength corresponding to the peak absorbance (λmax, 400 – 450 nm for Ag NPs) *(2, 112)*. Changes in the shape of Ag NPs also can be determined from the absorbance spectra. In addition, UV-Visible spectroscopy is also used to study nanoparticle stability and aggregation over time *(35)*. When the colloidal dispersion is unstable resulting in aggregation, the wavelengths are longer and broad due to the increased particle diameter.

Electron microscopy techniques such as TEM and SEM can also be used to measure and visualize the shape and size distribution of Ag NPs (morphology). Energy dispersive X-ray Spectroscopy (EDS) is used for elemental mapping of Ag and other elements on the surface of the specimen. Therefore, EDS can be used to confirm the presence of Ag and other elements in the specimen by revealing their distinctive peaks. Electron microscopy can be combined with EDS to reveal the elemental composition of the NP containing sample.

X-ray diffractometry (XRD) is used to determine Ag NP crystal structure, its chemical bonds, disorder, and other related information. When an X-ray beam passed through crystalline NPs, the X-ray beam will deflect in different directions with the angle, pattern and the intensity of the diverted x-ray beam helping to determine both the crystalline phase and size of the NP *(112)*.

FTIR is used to identify unknown compounds and the number of components in NPs (e.g., aliphatic and aromatic amines, carboxylic acid groups, alkane etc.) *(3, 112)*. After passing an IR beam through the samples, the resulting absorption and transmission spectra produce a molecular fingerprint of the sample.

A zetasizer can be used to determine both particle size (hydrodynamic size distribution) and the zeta potential of Ag NPs by dynamic light scattering (DLS). The zeta potential indicates the charge (+/-) on the NP surface which relates to the stability of NPs. A lower (i.e., more negative) zeta potential indicates that the particles are more dispersed due to electrostatic repulsion. In contrast, particles with the charge near neutrality are less dispersed due to weaker electrostatic repulsion. Therefore, higher stability particles exhibit less aggregation over time due to greater electrostatic repulsion between particles. The zeta potential is also impacted by the pH and ionic strength of the suspension (water chemistry) in addition to surface charge of the particle. When ionic strength of the solution increases, zeta potentials become more positive while surface charge of the particle remains unchanged *(22, 112)*.Any suspended material in a sample (e.g., soil) will impact the light scattering intensity and therefore particle size measurement which is a limitation of DLS *(106, 144)*. However, DLS has been successfully used to determine the size of Ag NPs in suspension *(106)*, aggregation behavior *(68)*, stability under different conditions *(26)* and behavior in environmental water *(89, 156)*.

1.5 QUANTIFICATION OF AG NPS

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is most commonly used to quantify total Ag levels due to its high speed, sensitivity and accuracy *(92, 106)*. After passage through a plasma flame, the Ag in the Ag NP-containing sample is ionized with the Ag ions generated passing through the mass spectrophotometer for quantification. The total Ag concentration in the sample relates to the total number of released ions and the emitted radiation intensity *(9, 106)*.

ICP-MS alone provides only the total Ag content and is unable to differentiate between Ag NPs from ionic or complexed Ag. However, ICP-MS combined with chromatographic techniques can be used to separately quantify ionic and complexed Ag. Using this methods, the particles are separated based on size (smaller particles elute slower than larger particles) after which the element distribution is measured within the size range *(92, 106)*. Unlike these ICP-MS techniques (standard ICP-MS and ICP-MS combined with chromatography), single particle ICP-MS can chemically characterize individual Ag NPs and differentiate Ag NPs from Ag ions monitoring the signals at a higher time resolution.

Surface enhanced Raman spectroscopy (SERS) is also used to analyze Ag NPs in different environments. In SERS, the Ag NPs are first conjugated with and firmly bound to an indicator *(53, 106)*. Thereafter, the Ag NP conjugates are usually concentrated by ultracentrifugation and ultrafiltration before SERS analysis for quantification *(106, 146)*.

1.6 HEALTH RISKS ASSOCIATED WITH AG NPS

The presence of Ag NPs in the environment causes potential adverse risks to ecosystems and the food chain. With their increased production and widespread use, public concern regarding the safety and potential risks of Ag NPs to humans has also increased. Ag NPs are toxic to plants, bacteria, and both vertebrate and invertebrate animals, including human cells *(160)*. They induce the formation of reactive oxygen species that increase cell membrane permeability and DNA damage after entering cells *(160)*. Ag NPs are also toxic to freshwater algae including *Chlamydomonas reinhardtii* and to aquatic life *(116)* and can bioaccumulate in some aquatic organisms like clams *(99)*. Furthermore, morphological, physiological, and genetic alterations in different unwanted plants that inhibit crop growth and yield have been associated with prolonged exposure to Ag NPs *(123)*. Ag NPs retained in food crops can then transfer through the food chain and adversely affect human health.

The toxicity of Ag NPs is presumably associated with the release of Ag ions into the environment *(95)*. However, Ag NPs themselves are also toxic, not only Ag ions. In studies conducted with algae *(95, 116)*, photosynthesis was inhibited by Ag ions about 18 times greater than Ag NPs during the first hour of exposure. However, Ag NPs became even more toxic than Ag ions after 2 hours of exposure *(95)*. It is believed that Ag NPs attach to the cell surface and then disrupt cell behavior *(95)*. Ag NPs might also bypass the cell barriers unlike "normal" larger sized Ag, releasing Ag ions that inhibit cellular function *(95)*.

Animal model studies suggest that Ag NPs pose a substantial human health risk. For example, *in vivo* exposure of rodents to Ag NPs at 20 to 125 mg/kg resulted in liver and DNA damage, neurotoxicity, and Ag accumulation in the kidneys *(75, 76, 145, 164)*. Several studies have also demonstrated toxic effects on human cells, including glioblastoma cells, skin

keratinocytes and lung fibroblast cells following Ag NP exposure to 20-100 µg/ml *(8, 93)*. Because of these toxicity studies, prevention of chronic human exposure to trace-level Ag NPs through dietary intake is important.

1.7 EXPOSURE OF FOOD CROPS TO AG NPS

Food crops will inevitably experience elevated exposure to Ag NPs due to increasing concentrations of Ag NPs in the environment. Sewage sludge application to agricultural land and use of nano-pesticides for crop protection are two major pathways where Ag NPs can get into food crops *(148)*. The other pathways include, but are not limited to, irrigation with reclaimed water, atmospheric deposition, and wastewater treatment plant effluents, all of which can lead to the uptake and bioaccumulation of NPs in plants. Consumption of these food crops, including fresh produce, then becomes an important route of human exposure. The roots of both *Arabidopsis thaliana* and zucchini plants can take up and transport Ag NPs up to 40 nm in size to the shoots *(102)*.

Fresh produce can also be contaminated by Ag NPs through routes other than root uptake *(85)*. Since nanopesticides can both penetrate and stay on the surface of produce, nanopesticide use represents a significant contamination pathway. Fruit, vegetable, and leaf surfaces can absorb Ag NPs through rainfall splash, irrigation water, and different practices. Fresh produce can be further cross-contaminated with Ag NPs through post-harvesting processes such as shredding, conveying, flume washing, and centrifugal drying.

Various solutes, including ENPs, can be absorbed by leaves through both the cuticle and stomata *(85, 101)*. Lipophilic substances can be absorbed into the cuticle by diffusion, whereas ionic and polar solutes can pass through pores of the cuticle having a diameter of 0.6–4.8 nm *(101, 108)*. Hydrophilic substances can penetrate the stomatal pores of leaves *(38)*. The diameter of the stomatal aperture can increase to 30 times its original width *(38, 108)*. Using *Arabidopsis thaliana*, nano-TiO_2 particles were found in stomatal guard cells, epidermal tissues, pavement cells and palisade cells of leaves *(84)*.

The extent of ENP interaction with produce depends on the degree and persistence of contamination. To date, there have been limited studies on the interactions between ENPs, produce, and contamination through above-ground pathways like foliar exposure. Studies involving foliar or above-ground exposure include nano-CeO_2 with maize leaves *(13)* and cucumber *(67)*, nano-Al_2O_3 with tomato skin *(119)*, nano-polystyrene with leek and broad bean *(39, 86)*, nano-SiO_2 with tomato skin *(119)*, nano-TiO_2 with tomato skin *(119)*, lettuce *(86)*, and spinach leaves *(137)* and Ag NPs with lettuce *(85)* and pear *(167)*. These studies have shown that ENPs can attach to the plant surface and then internalize, making their removal during washing difficult *(85)*.

1.8 FOOD SAFETY RISKS ASSOCIATED WITH AG NPS IN FRESH PRODUCE

The production of minimally processed fresh produce is increasing worldwide, with consumption increasing 10-20% per year due to heightened awareness of healthy diets *(64, 151)*. Fresh produce provides high levels of vitamins and minerals and may help in preventing chronic diseases when combined with other healthy habits. Given the increased production and widespread use of Ag NPs, public awareness surrounding their use along with possible health issues will undoubtedly increase.

The food safety concerns associated with the uptake, translocation, and accumulation of Ag NPs in food crops are currently unknown. As fresh produce is often minimally processed before consumption, improving the safety of fresh produce requires a fundamental understanding of the interactions between Ag NPs and fresh produce, as well as the fate, dissemination, and transfer of Ag NPs in fresh produce through wash water during commercial processing.

1.9 POST-HARVEST RISKS AND CURRENT FRESH PRODUCE PROCESSING PRACTICES

Commercial fresh-cut produce is usually only minimally processed between harvesting and marketing. For example, post-harvest processing of leafy greens involves field trimming, cold storage, shredding, flume washing and rinsing, draining, drying, packaging, cold storage, and distribution. Leafy green processing involves a range of different components including shredders, conveyor belts, flume tanks, shaker tables, and dewatering centrifugal dryers.

Fresh produce contamination with Ag NPs can occur in multiple ways even after harvesting, such as possible cross-contamination during washing and rinsing, shredding, slicing, peeling, drying, and packing. Washing of produce is essential to remove soil, microbial and chemical contaminants, and other debris and improves quality and appearance, thereby enhancing shelf-life and safety of the product. Addition of chemical sanitizers to the processing water helps to minimize microbial cross-contamination during washing *(66)*. However, the ability of chemical sanitizers to decrease the level of Ag NPs on fresh produce during washing remains unknown.

1.9.1 Sanitizer addition to wash water during produce processing

In produce processing, washing is a critical step to ensure produce safety. There is a common misconception that the sanitizers are added to wash water to inactivate microbial populations attached to produce surfaces *(32)*. However, sanitizers are primarily used to minimize microbial cross-contamination from the flume water during washing *(158)*. Microbial populations on produce generally decrease 1 to 3 logs after sanitizer washing with water alone generally achieving only a 1 log reduction *(31)*. Recirculating the flume water in produce processing facilities helps to reduce operational costs *(96)*. Addition of sanitizers also helps to control the spread of microbial pathogens in recirculating processing water.

Chlorine-based sanitizers are most commonly used to minimize microbial populations in commercial flume water followed by peroxyacetic acid, with chlorine dioxide (ClO_2), ozone and electrolyzed water seen less often. Maintaining the flume water temperature at 4°C is standard practice in produce processing. In 2013, the U.S. Food and Drug Administration (FDA) Food Safety Modernization Act (FSMA) released recommendations for the growing, harvesting, packing, and holding of produce for human consumption *(41)*. It advises monitoring and adjusting the concentration of the active sanitizer ingredient and the pH of the processing water, especially when sodium hypochlorite is used in recirculating systems.

1.9.2 Chlorine use in wash water

Currently, chlorine is the most commonly used sanitizer in commercial produce processing due to its relatively low-cost and minimal negative impact on product quality *(21, 33, 97, 117)*. NaOCl is the most commonly used chlorine source by commercial processors.

When NaOCl mixes with water, the following reaction occurs:

$NaOCl + H_2O \leftrightarrow HOCl + NaOH$

$HOCl \leftrightarrow H^+ + OCl^-$

$HOCl + HCl \leftrightarrow H_2O + Cl_2$

During the reaction, hypochlorous acid (HOCl; pK_a 7.5) is formed, which is the main/active ingredient and the most effective form of chlorine for disinfection *(30)*. HOCl dissociation into the hypochlorite ion (OCl^-) is pH dependent. Between pH of 2 to 7.5, more than half of the chlorine is available as HOCl *(33)*. However, the pH of chlorine-based sanitizers is around 9.5 to 10. Commercial produce processors must maintain the pH of processing water between 6 and 7.0 by adding an acid, typically citric acid, to maintain sanitizer efficacy *(33)*. Processing conditions also influence sanitizer efficacy in wash water. Maintaining the temperature of processing water

between 4 and 7 °C helps to maintain sanitizer efficacy *(64)*. The initial total chlorine concentrations in produce processing water typically range from 50 to 100 mg/L *(21, 33, 97, 117, 127)*. During processing, the free chlorine concentration gradually decreases with time as organic debris accumulates in the recirculating wash water.

There are some concerns regarding the use of chlorine as sanitizers. Those concerns include environmental damage and worker safety with high concentrations of chlorine and potentially hazardous by-products associated with chlorine. Most importantly, the efficacy of chlorine decreases rapidly with accumulation of organic load in recirculating wash water. Therefore, because of these reasons, interest in the use of peroxyacetic acid (PAA) as a produce sanitizer has increased.

1.9.3 Peroxyacetic acid use in wash water

Peroxyacetic acid (i.e., peracetic acid, POAA or PAA) is another sanitizer approved by the FDA and EPA for fresh produce *(165)*. Eighty mg/L is the maximum allowable PAA concentration for produce processing *(105)*. The reaction of hydrogen peroxide (H_2O_2) reacts with acetic acid (CH_2CO_2H) generates peroxyacetic acid ($C_2H_4O_3$).

$$CH_3CO_2H + H_2O_2 \leftrightarrow CH_3CO_3H + H_2O$$

When added to water, PAA breaks down to form acetic acid, oxygen, and water *(7, 19, 80)*. There are several advantages of using PAA as a sanitizer over chlorine. The concentration or antimicrobial efficacy of PAA is not impacted by the accumulation of organic load or changes in pH *(150)*. Furthermore, hazardous byproducts are not generated when PAA is used *(30)*. Unlike chlorine, PAA is effective at a wide range of temperatures. Although produce processing water is maintained at 4°C, PAA retains its effectiveness at up to 13°C *(32)*. In addition, PAA can also be combined with other acids to increase its efficacy. For example, there have been studies that show

the increased effectiveness in controlling molds and yeasts in produce processing water when using a PAA-octanoic acid mixture (Tsunami 200) compared to PAA alone.

However, in the U.S. the cost of PAA sanitizer is four to five times higher compared to chlorine-based sanitizers. Also, the use of PAA increases the organic load production in processing water because of the presence of acetic acid, although this increase does not affect the efficacy of PAA. The higher organic load from PAA may also increase the cost of wastewater disposal.

1.9.4 Organic load accumulation in wash water

Produce wash water contains a large amount of organic matter from cut produce, tissue exudates and other debris (e.g., soil, insects, and microbes). As produce is washed, this organic matter accumulates in the recirculating wash water, reducing the efficacy of sanitizers (i.e., chlorine-based sanitizers), and increasing cross-contamination from the processing water *(47, 139, 159)*. Several factors affect the organic load accumulation rate in processing water, including produce type, amount being processed, produce shred size, and the produce processing rate.

1.10 BEHAVIOR OF AG NPS IN DIFFERENT ENVIRONMENTAL CONDITIONS

Ag NPs experience a variety of morphological and chemical transformations in the environment. These transformations greatly influence the environmental fate, mobility, transport and toxicity of Ag NPs *(157)*. Aggregation and dissolution are fundamental processes which influence these transformations *(90)*. Different physicochemical properties like shape, size, aggregation, distribution, concentration, state of dispersion, surface charge, porosity, behavior with other chemicals in the medium, and surface chemistry affect the toxicity of Ag NPs. The release of Ag ions from Ag NPs is known as dissolution, and the rate of dissolution depends on the solution chemistry *(90)*. Aggregation and dissolution behaviors of Ag NPs have been well-studied with electrolytes (both monovalent and divalent) and natural organic matter (NOM) with different ionic strengths and different pH levels *(90)*. In those studies, the classic Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability has been used to explain the behavior of Ag NPs *(90)*. Greater aggregation of Ag NPs has been found in the presence of electrolytes *(90)*. NOM from natural water absorbs to the surface of nanoparticles, increasing their stability by steric or electrostatic repulsion *(90)*. Reduction of Ag ions is more prevalent because of functional groups such as quinones, ketones, hydroxyls, phenolic-OH and hydroxyls in NOM *(157)*. Humic and fulvic acid, which are the main constituents in NOM, induce the formation of Ag NPs; elevated temperatures, sunlight and optimum pH (9.0) further accelerate the reaction *(157)*.

Redox reactions ubiquitous in the environment also lead to morphological changes in Ag NPs which will eventually affect their transport, mobility, environmental fate, bioavailability, and behavior. The freezing or freeze-thaw cycles in the environment increase the redox reactions and

create morphological changes in Ag NPs including fusion, coalescence, aggregation, and regeneration *(54)*.

Behavior of Ag NPs with electrolytes such as NaCl and $CaCl_2$ has been studied previously with both high dissolution of Ag NPs into Ag^+ and formation of AgCl precipitates having been identified *(90)*. However, information regarding the interactions of Ag NPs with chlorine in the produce processing environment is currently lacking.

Similarly, the fate of Ag NPs with organic matter in lettuce processing water has not been previously studied. However, the behavior of Ag NPs with NOM, which is ubiquitous in natural water *(54, 90, 120)*, has been extensively studied. NOM mainly consists of high molecular weight organic matter like humic and fulvic acid *(4)*. Ag NP behavior with NOM depends mainly on the chemical composition of NOM, with higher levels of sulfur and nitrogen in NOM significantly increasing both the reduction of Ag and colloidal stability *(51)*. However, aerobic conditions exist in the produce processing environment because of oxidizers like chlorine. Furthermore, NOM in the natural environment is totally different from plant materials/organic matter in lettuce wash water. For example, on a dry weight basis, romaine lettuce contains 14.1% cellulose, 16.3% hemicelluloses, 16.2% starch, 17.5% protein, 6.2% fat, 31.3% free sugars (fructose, glucose, sucrose) and trace amounts of organic acids, fructan and pectin *(128)*. These components can leach into the flume water as plant residues during processing, but would not be present in the natural environment.

Although the behavior of Ag NPs under natural environmental conditions has been extensively studied, behavior of Ag NPs in the food processing environment has not been studied. As stated above, there is currently a lack of information regarding the interactions of Ag NPs with

sanitizers commonly used (i.e., chlorine, peroxyacetic acid) in fresh produce processing and with organic matter produced during fresh produce processing.

PAA breaks down into hydrogen peroxide and acetic acid suggesting that Ag can then be oxidized, with the release of Ag ions along with hydroxyl radical formation. This hydroxyl radical will further oxidize Ag into Ag ions *(165)*. In terms of chlorine, sodium hypochlorite will likely oxidize Ag into Ag ions because of the strong oxidizing nature of sodium hypochlorite. It is suggested that AgCl will form from the Cl^- anions and Ag^+ ions *(165)*.

The physical and chemical characteristics of Ag NPs are greatly impacted by water chemistry, causing stabilization, aggregation and dissolution *(23)*. Ions like Cl^- are associated with increasing aggregation of Ag NPs *(23)*. In addition, some studies have reported that AgCl formation and bridging between Ag NPs are also possible. This would change the surface characteristics of Ag NPs and eventually affect the toxicity *(23)*.

1.11 LITERATURE SUMMARY AND KNOWLEDGE GAPS

Nanopesticides are being developed rapidly for crop protection with over 3000 nanopesticide patents currently registered *(71)*. Many nanopesticide products contain Ag NPs as the active ingredients because of their broad-spectrum antimicrobial activity against plant pathogens (mainly fungi) *(69, 142)*. The increasing use of Ag NPs in agriculture (e.g., as nanopesticides) will substantially increase the release of Ag NPs to agroecosystems in the future. Since Ag NPs can be taken up and accumulate in food crops, their consumption becomes a direct human exposure route for Ag NPs and poses a food safety concern. The health risks of Ag NP exposure clearly emphasize the importance of minimizing chronic human exposure to trace-level Ag NPs through dietary intake.

Mitigation of human exposure to Ag NPs via food consumption would require a multi-barrier approach that reduces Ag NP levels during cultivation, harvest, and post-harvest processing. Fresh produce is often minimally processed prior to consumption. However, once it is contaminated with Ag NPs, the only logical step is to design effective control measures for use in the processing environment. To effectively address this concern, we need to improve deficiencies in two areas. First, the behavior of Ag NPs in produce processing environments is poorly understood, thus hindering our ability to design effective mitigation measures. Addition of sanitizers such as chlorine and peroxyacetic acid is a standard practice in produce processing to minimize microbial cross-contamination during processing *(30)*. Furthermore, organic matter from produce accumulates in produce wash water during processing. Thus, understanding the fundamental interactions between Ag NPs and commonly used sanitizers in produce processing water is critically important. Secondly, the effectiveness of produce processing practices, including the impact of chemical sanitizers, in removing Ag NPs from fresh produce is currently unknown.

To date, only a few studies evaluated the removal of Ag NPs from spinach and lettuce through washing *(85, 165, 166)*. However, these studies did not mimic current commercial produce processing practices. Thus, it is imperative to assess the effectiveness of flume washing practices under conditions that most closely resemble commercial operations.

The conceptual scheme for this research is shown in Figure 1.1. Improved knowledge gained from this study will contribute to developing new control strategies for mitigating the risk of Ag NPs in produce, thus protecting human health.

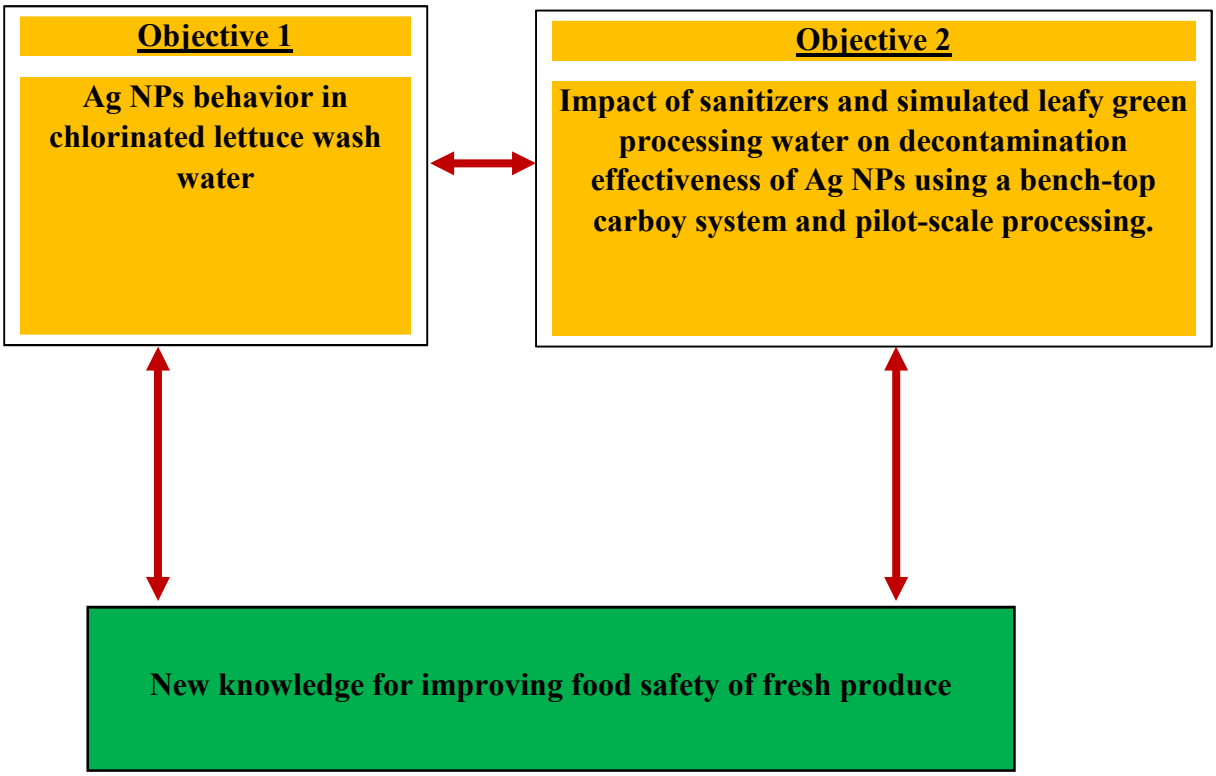


Figure 1.1 Conceptual schematic of the project.

CHAPTER 2:

BEHAVIOR OF SILVER NANOPARTICLES IN CHLORINATED LETTUCE WASH WATER

2.1 INTRODUCTION

Heightened awareness of healthy diets has increased consumer demand for fresh produce with consumption rising by 10-20% per year *(64, 151)*. At the same time, concerns over the safety of fresh produce have largely focused on microbial pathogens, heavy metals, and conventional pesticides *(5, 42, 135, 154)* with only limited attention given to emerging nanopesticides. Nonetheless, over 3000 nanopesticide patents have been granted for crop protection *(71)*. Many nanopesticide products contain silver nanoparticles (Ag NPs) that possess broad-spectrum antimicrobial activity against plant pathogens including many fungi *(69, 142)*. Given the hundreds of Ag NP-containing nanopesticides now registered with USEPA *(11, 165)*, the release of Ag NPs into agroecosystems is estimated to be 54 times greater than the combined release from all other sources including sewage sludge, wastewater irrigation, and atmospheric deposition *(43, 46, 48, 78, 152, 153)*. Since Ag NPs can be taken up by food crops *(101, 102, 138)*, consumption of fresh produce has become a direct route for human exposure. *In vivo* rodent studies have demonstrated liver and DNA damage from Ag NPs at > 125 mg/kg and 20 mg/kg, respectively *(77, 145)*. Several *in vitro* studies also reported the toxicity of Ag NPs to human lung fibroblast, skin keratinocyte, and glioblastoma cells at 20-100 μg/mL *(8, 93)*. The health risks of Ag NP exposure clearly underscore the importance of minimizing chronic human exposure to trace-levels of Ag NPs through dietary intake.

Mitigating human exposure to Ag NPs via food consumption requires the reduction of Ag NP levels during cultivation, harvest, and post-harvest processing. Fresh produce is often minimally processed before consumption. However, once fresh produce is contaminated with Ag NPs, effective decontamination methods need to be developed. During commercial flume washing of fresh produce, chemical sanitizers are routinely used to minimize microbial cross-contamination

during processing *(31, 98)*. Chlorine-based sanitizers have been preferred due to their relatively low cost and minimal negative impact on nutritional and sensorial quality *(21, 33, 97, 117, 149)*. During processing, organic matter released from fresh-cut produce accumulates in the recirculating washing water and reacts with chlorine, decreasing both the concentration and efficacy of chlorine-based sanitizers *(33, 127)*. Given the complex chemistry of flume water, a better understanding of how chlorine sanitizers and plant-released organic matter influence the behavior of Ag NPs during commercial washing of produce is needed to developing effective mitigation strategies.

Previous studies on the behavior of Ag NPs in NaCl and $CaCl_2$ solutions have shown high dissolution of Ag NPs into Ag^+ and the formation of AgCl precipitates *(90)*. Garg et al. *(44)* also also investigated the oxidative dissolution of Ag NPs at low hypochlorite (OCl^-) concentrations ($\leq$ 0.51 mg OCl^-/L), showing the rapid dissolution of Ag NPs into Ag^+. However, the interactions of Ag NPs with chlorine at typical free chlorine concentration ranges (10 – 200 mg/L *(31)*) found in commercial produce wash water remain poorly understood. Regarding interactions of Ag NPs with organic matter, previous studies largely focused on natural organic matter (NOM) including humic substances, polysaccharides, and proteins *(4, 54, 90, 120)*. However, the types and concentrations of organic matter in commercial flume water differ greatly from NOM. For example, on a mass basis, romaine lettuce contains about 14.1% cellulose, 16.3% hemicelluloses, 16.2% starch, 17.5% protein, 6.2% fat, 31.3% free sugars (fructose, glucose, sucrose) and trace amounts of organic acids, fructan, and pectin *(128)*. Since all these components will partially leach into flume water during washing, the behavior of Ag NPs in representative produce wash water warrants further investigation.

This study aimed to evaluate the aggregate size, surface charge, dissolution, and morphology of Ag NPs over time when exposed to commercially applicable chlorine

concentrations in simulated lettuce wash water. The selected free chlorine concentrations (2, 50 and 100 mg/L Cl_2) cover the range of chlorine levels typically found in processing water. Dissolved lettuce extract (DLE) was used to simulate organic matter in processing water. Finally, transmission electron microscopy (TEM) with energy dispersive spectroscopy (EDS) was used to probe the morphology and composition of Ag NP aggregates in waters containing chlorine, DLE, or both.

2.2 MATERIALS AND METHODS

2.2.1 Materials and Chemicals

A stock suspension of Ag NPs (1000 mg/L; primary particle size of 15 nm) stabilized in 0.6% polyvinylpyrrolidone (PVP) solution was purchased from US Research Nanomaterials, Inc. (Houston, TX). To remove the PVP residues in the solution, the suspension was filtered by ultrafiltration (Amicon Ultra 10K, Millipore, Burlington, MA) at 5,000 × *g* for 25 min. After washing three times (5,000 × *g* for 25 min) with ultrapure water (18 MΩ at 25 °C, Milli-Q system, Millipore, United States Filter Corporation, Palm Desert, CA), the retained Ag NPs were dispersed in ultrapure water to obtain a 240 mg/L stock suspension as determined by atomic absorption spectroscopy (AAS, AAnalyst 400, PerkinElmer, Waltham, MA). Before preparing the working suspensions, the stock suspension was sonicated for 5 min using a 90-W ultrasonic processor (Fisher Scientific, Pittsburgh, PA).

A commercial chlorine sanitizer containing 8.4% sodium hypochlorite (XY-12, Ecolab, Inc., St. Paul, MN) was used to prepare chlorine working solutions of 30, 100 and 200 mg/L free chlorine (Cl_2), which were then used to prepare the treatment solutions (i.e., 2, 50 and 100 mg/L Cl_2). The pH of each working solution was measured with a pH probe (pHTestr 30, Oakton, Vernon Hills, IL) and adjusted to pH 6.7 ± 0.1 with citric acid (Sigma-Aldrich, St. Louis, MO) to maintain the optimal sanitizer efficacy *(33)*. The final free chlorine concentration in the working solutions was measured by the N,N-diethyl-phenylenediamine (DPD) method using a DR300 chlorine meter reporting chlorine concentrations in mg/L Cl_2 (Hach, Loveland, CO). The pH of the treatment solutions at each time interval (from 0 h to 10 d) was also measured.

Dissolved lettuce extract (DLE) was used to represent the organic matter that accumulates in recirculating flume water during fresh-cut lettuce processing *(32, 133)*. DLE was prepared by

pressing cut romaine lettuce leaves in a household juicer (Hamilton Beach Juice extractor, model 67608, China). The resulting juice was then passed through two layers of cheesecloth, centrifuged at 10,000 × *g* for 30 min at 4°C to remove coarse particles, vacuum-filtered through a 0.45-μm membrane, and frozen at −20°C until further use. The pH and oxidation-reduction potential (ORP) of the DLE were determined using the pHTestr 30 and OPRTestr probes (Oakton, Vernon Hills, IL), respectively. Total solids was measured by drying a 10 mL aliquot in a pre-weighed crucible in an oven (Model 625-A, Precision Scientific Inc., Chicago, IL) at 103 ± 2 °C for 2 h, followed by weighing *(33)*. Turbidity was determined using a turbidity meter (Extech, Nashua, NH). Proximate analyses for fat, protein, and ash content were conducted by Alliance Analytical Laboratories (Coopersville, MI). Acid hydrolysis and the combustion method were used to determine fat and protein content, respectively.

2.2.2 Kinetic aggregation experiments

The aggregation kinetics of 5 mg/L Ag NP suspensions were assessed by measuring the intensity-weighted hydrodynamic diameters (Z-average) over time for the following six treatments: (1) 2 mg/L chlorine; (2) 50 mg/L chlorine; (3) 100 mg/L chlorine; (4) 0.1% DLE free of chlorine; (5) 50 mg/L chlorine and 0.1% DLE; (6) Control, ultrapure water without chlorine and DLE. Concentrations of 2, 50 and 100 mg/L free chlorine and 0.1% DLE were selected to represent those seen in commercial processing *(31, 32, 133)*.

For each trial, 10 ml aliquots of the Ag NP treatment suspension were transferred to amber-colored vials and continuously shaken at 4 °C on a rotary shaker at 50 rpm. Three vials of each treatment suspension ($n = 3$) were retrieved after 0, 2, 6, 12, and 24 h, and 4, 7, and 10 d of shaking at 4 °C and assessed for particle size, zeta potential, and dissolved Ag concentration. Solution pH was measured before and after the experiments. Triplicate measurements of particle size

(hydrodynamic diameter, nm) and zeta potential (mV) were obtained for each of the three vials using a zetasizer (Nano ZS, Malvern, UK) at 4°C. After centrifugation at 5000 × *g* for 30 min using an Amicon Ultra 10K ultrafiltration unit, the concentration of dissolved Ag in each of duplicate samples from each treatment was measured three times by AAS (Figure 2.1).

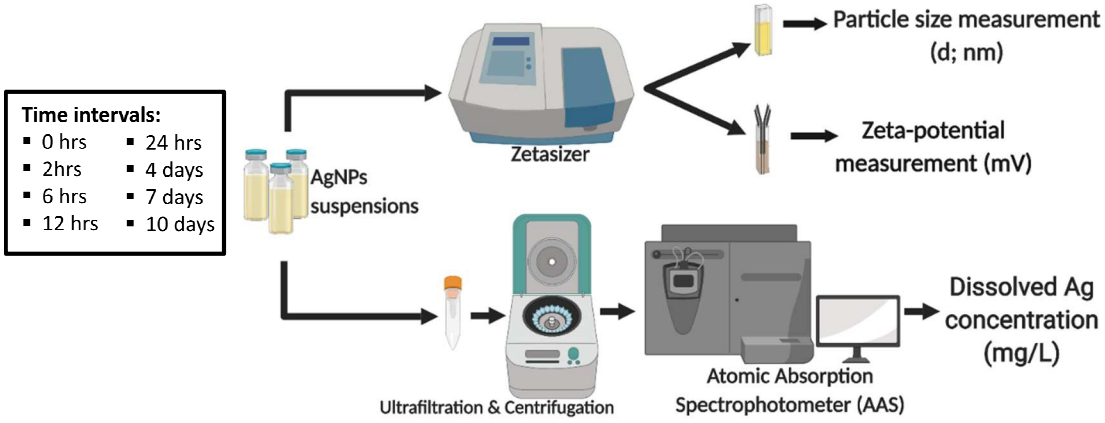


Figure 2.1 Measurement of Ag hydrodynamic diameter, (nm), zeta-potential (mV) and dissolved Ag concentration (mg/L).

2.2.3 TEM-EDS measurements

The morphological changes in Ag NP aggregates from selected samples were visualized in the presence of 2 mg/L chlorine at Day 10 and in the presence of 100 mg/L chlorine, 0.1% DLE, or 50 mg/L chlorine with 0.1% DLE at Day 0, Day 7, and Day 10 using time-resolved TEM. Before TEM analysis, the Ag NP suspensions were centrifuged at 5,000 × g for 1 h, washed twice in ultrapure water to remove free chlorine associated with the Ag aggregates, and then dispersed in 100 µL of ultrapure water. A carbon-coated copper grid was dipped in each suspension, air-dried at room temperature, and then imaged using JEM-2200FS TEM with Oxford X-Ray EDS operated at 200kV. The formation of AgCl was probed by EDS (Figure 2.2).

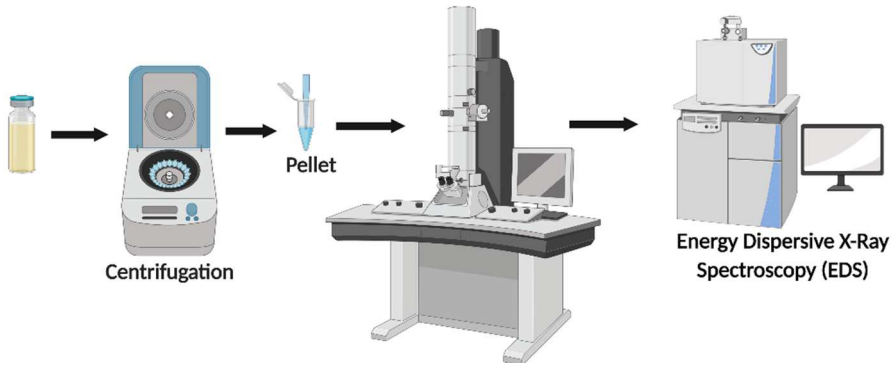


Figure 2.2 TEM imaging and Energy Dispersive X-Ray Spectroscopy (EDS) analysis.

2.2.4 Statistical analysis

One-way ANOVA repeated measures were used for multiple comparisons. Data were analyzed using proc mixed in the SAS 9.4 program (SAS Institute, Cary, NC) at a significance level of $P < 0.05$. For all particle size, zeta potential, and dissolved Ag concentration measurements, comparisons of treatments at specific time points and comparisons of the same treatment at different time points were tested by post-hoc Tukey tests.

2.3 RESULTS AND DISCUSSION

2.3.1 DLE composition

The prepared DLE contained 4 ± 0.02 % of total solids (w/v %), 6.42 g/L of ash, 10.25 g/L of protein, and 35.2 g/L of fat content. The 0.1% (w/v) DLE in the treatment solutions was used to represent organic matter in processing water and fell within the organic load range (0.05%-0.2%) used previously by Shen et al. *(133)*. The ORP, pH and turbidity of the DLE solution (0.1% w/v) were 340, 6.44 and 109.6 ntu, respectively.

2.3.2 Changes in hydrodynamic diameter of Ag NP aggregates

In the chlorine-only treatments, aggregate size increased with time at all chlorine levels (49–431 nm). Aggregate size was also significantly greater for all chlorine-only treatments (64 to 431 nm) ($P < 0.05$) as compared to the control (58–60 nm), except for the 100 mg/L chlorine samples analyzed at 0 and 2 h (Figure 2.3A). Therefore, the presence of chlorine facilitated the formation of larger aggregates. It is known that Ag NPs gradually dissolve into Ag^+ ions and that chlorine may enhance the dissolution of Ag NPs because of its oxidizing ability *(44, 90, 165)*. Once dissolved, Ag^+ ions can then combine with Cl^- to form AgCl crystals that may potentially co-coagulate with Ag NPs to form larger aggregates. Indeed, previous studies reported the formation of AgCl particles when Ag^+ ions were exposed to chlorine *(10, 54, 165)*. Since Ag NPs, Ag^+ and AgCl differ in their antimicrobial properties *(27, 63, 126)* and toxicities, this finding is important to assessing environmental risks of Ag species in wash water.

In the presence of DLE, aggregate size (84 to 273 nm) increased with time with or without 50 mg/L free chlorine (Figure 2.3B) and was greater than that of the control (58 to 60 nm) ($P < 0.05$). Greater variability in particle size was seen after 4, 7 and 10 days (Figure 2.3B). Aggregate size for the Ag NPs- and chlorine-free DLE treatment was indistinguishable from the other two

DLE treatments from Day 1 to Day 10, suggesting that aggregate size is predominantly representative of DLE rather than Ag NPs. Finally, aggregate size for the chlorine-only treatments were greater than that for the DLE treatments on Day 7 and Day 10 (Figure 2.3A and 2.3B), probably due to the stabilization effect of DLE on Ag NPs because of electrostatic and steric repulsion.

2.3.3 Changes in Zeta potentials

The chlorine-only treatments yielded lower zeta potentials (-39 to -95 mV) than the control treatment (-10 to -20 mV) (Figure 2.4A). Further, the zeta potentials for the 50 and 100 mg/L chlorine treatments were lower than those for the 2 mg/L chlorine treatment. This means that the charge on the particle surface became more negative in the presence of chlorine. In previous studies by Li et al. *(90)* and Badawy et al. *(10)*, AgCl particles had a zeta potential of -45.8 to -57 mV, which is much lower than that of Ag NPs (-10 to -20 mV) used in this study. Thus, AgCl particles may contribute to the more negative zeta potential of aggregates in the chlorine-only treatments. Furthermore, AgCl crystals might increase in size and coalesce with Ag NPs to form larger aggregates.

Lower zeta potentials were also found in the presence of DLE (-27 to -32 mV) with or without chlorine compared to the control treatment (-10 to -20 mV) (Figure 2.4B). Since negative zeta potentials (-20 to -33 mV) were also observed for 0.1% DLE alone, the organic matter in DLE may increase the negative charge of aggregates. In fact, DLE binding to Ag NPs stabilized Ag NP aggregates due to greater electrostatic and steric repulsion, which collectively decreased the aggregate size over time (Figure 2.3).

2.3.4 Comparison of Dissolved Ag concentrations

Dissolved Ag concentrations in the control samples ranged from 0.54 to 0.80 mg/L, whereas the chlorine-alone treatments had dissolved Ag concentrations values below the limit of detection limit of the AAS at 0.25 mg/L *(65)*. This is probably because ultrafiltration during sample preparation removed the AgCl particles. Dissolved Ag concentrations in DLE-containing samples were also below the detect limit, most likely due to the complete removal of Ag sorbed to DLE during ultrafiltration.

2.3.5 TEM-EDS analysis

In the presence of 2 mg/L chlorine, the Ag NP aggregates were non-regular as opposed to spherical in the control treatment (Figure 2.5A and 2.5B) with the AgCl crystals appearing to bind the Ag NPs together (Figure 2.5B). Based on EDS analyses (Figure 2.5II), the atomic percentages of Ag (78%) and Cl (17.8%) in the aggregates was close to that of AgCl (75.1% Ag and 24.9% Cl). Before the TEM-EDS analyses, the sample was centrifuged and washed several times to completely remove free chlorine. Therefore, it might be possible that at the relatively low chlorine concentration of 2 mg/L AgCl formed coatings on Ag NPs without largely dissolving Ag NPs into Ag^{+}, potentially resulting in an Ag-core/AgCl shell structure. As a result, the nucleation and growth of AgCl crystals between Ag NPs facilitated the formation of large aggregates (Figures 2.3A and 2.5B).

However, in the presence of 100 mg/L chlorine, cubic-like aggregates were visible in the TEM images (Figure 2.5-C, F, I), which appeared to be packed with smaller near-spherical particles (i.e., cauliflower-like structure), particularly after 7 days. (Figure 2.5-F, I). Based on EDS analysis, the atomic percentages of Ag and Cl were close to that of AgCl, suggesting the formation of AgCl. AgCl micro/nanocrystals can exhibit appear as nanocubes, octahedrals, hexapods, or

near-spherical morphologies depending on solution conditions with cubic structures predominating *(29, 37, 52, 94)*. Therefore, the Ag NPs most likely disassociated into Ag^+ in the presence of 100 mg/L chlorine to form the AgCl crystals which were larger in size compared to the Ag NPs (15 nm) (Figure 2.5C). These AgCl crystals further grew over time to form large aggregates (Day 7 and Day 10).

In the presence of DLE, Ag NPs were dispersed and embedded in the DLE matrix (Figure 2.5-D, G, J) as seen in the TEM images. Large aggregates of Ag NPs and AgCl also appeared but were more sparsely distributed than in the chlorine-only treatments. Thus, embedding of Ag NPs in the DLE matrix may provide some degree of stabilization by electrostatic and steric repulsion as well as protection against chlorine. The fate of Ag NPs with organic matter in lettuce water has not been previously studied. However, many studies have assessed the interactions between Ag NPs and NOM which is ubiquitous in natural water *(54, 90, 120)*. In the presence of fulvic acid (a major class of NOM in surface water), Li et al. *(90)* found no noticeable effect on aggregation of Ag NPs. Further, in another study, the different Ag NP morphologies remained relatively stable in the presence of dissolved organic matter (DOM), with amphiphilic surface-active DOM coating the surface and increase the stability of Ag NPs by electrostatic and steric repulsion *(54, 90)*. Reduction of Ag ions is also possible from functional groups such as quinones, ketones, hydroxyls, phenolic-OH and hydroxyls in NOM *(157)*. Similarly, in our study, Ag NPs became embedded in DLE and likely bound with different thiol groups in this plant materials *(165)*, thus increasing the stability of Ag NPs. Since the free chlorine concentration decreases rapidly in the presence of organic matter lowering its oxidizing ability, decreased dissolution of Ag NPs into Ag ions along with decreased formation of AgCl would be expected. Nonetheless, EDS was unable to detect AgCl since Cl^- was also likely sorbed by the DLE matrix.

2.3.6 Mechanism

The probable mechanism responsible for the behavior of Ag NPs in chlorinated lettuce wash water is summarized below.

In the presence of chlorine, the oxidizing power of OCl^- quickly converts metallic silver to Ag^+*(44)*. While Garg et al. *(44)* did not find the presence of Cl^- at low OCl^- concentrations (≤ 0.51 mg/L), we propose that at sufficiently high OCl^- concentrations, Cl^- would become available and react with Ag^+ to form AgCl crystals, as widely reported in the previous studies with NaCl and $CaCl_2$ solutions *(10, 54, 90)*. According to Li et al. *(90)*, the dissolution and aggregation of Ag NPs appear to occur simultaneously with the formation of AgCl particles in the presence of NaCl. Badawy et al. *(10)* and Guo et al. *(54)* also described the formation of stable, negatively charged AgCl colloids in the presence of Cl^- (at 10 mM and 100 mg/L respectively). Thus, we speculate that at high chlorine concentration (50 and 100 mg/L) dissolved Ag NPs form AgCl crystals (Figure 2.5-C) that gradually increase in size to form large AgCl aggregates as observed in Figures 2.5F and 2.5I. This scenario is supported by the increased aggregate size and decreased zeta potential observed over time for the 50 and 100 mg/L chlorine concentrations (Figure 2.3 and 2.4), indicating the continuous growth of AgCl crystals. Conversely, at the lower chlorine concentration of 2 mg/L, dissolution may be confined to the surface of Ag NPs with only AgCl coating being formed (an Ag-core/AgCl shell structure). The AgCl crystals between Ag NPs could then facilitate the aggregation of AgCl-Ag NP composite particles. This scenario is also supported the fact that aggregate size and zeta potential remained roughly unchanged over time (Figure 2.3 and 2.4). In the presence of DLE, AgCl formation, and aggregation of AgCl and Ag NPs were less pronounced. When dispersed and embedded in the DLE matrix, Ag NPs would be stabilized by

electrostatic and steric repulsion from binding with plant materials via various thiol groups. Therefore, Ag NP stability in the DLE matrix is greater than that in the chlorine-only treatments.

2.3.7 Environmental Implications

The findings from this study have several important implications. Firstly, during commercial processing of leafy greens, Ag may exist as AgCl or AgCl-Ag NP composite aggregates in wash water, depending on the chlorine level. Because Ag^+, AgCl, and Ag NPs have varying toxicities to organisms *(27, 63, 126)*, the public health impact of Ag species in wash water will vary with their forms, concentrations, and particle sizes. Additionally, due to the extended duration of this study in contrast to the typical of 2-3 min period for commercial produce washing, these results may also be relevant to the long-term behavior of Ag NPs in fresh produce wash water. Once Ag NPs and AgCl become embedded in dissolved plant organic matter, Ag is likely more stable when released into the environment. Since the fate of Ag NPs is greatly affected by both chlorine and the organic matter in lettuce wash water, future studies should evaluate the mobility and toxicity of the different forms of Ag during commercial washing of produce.

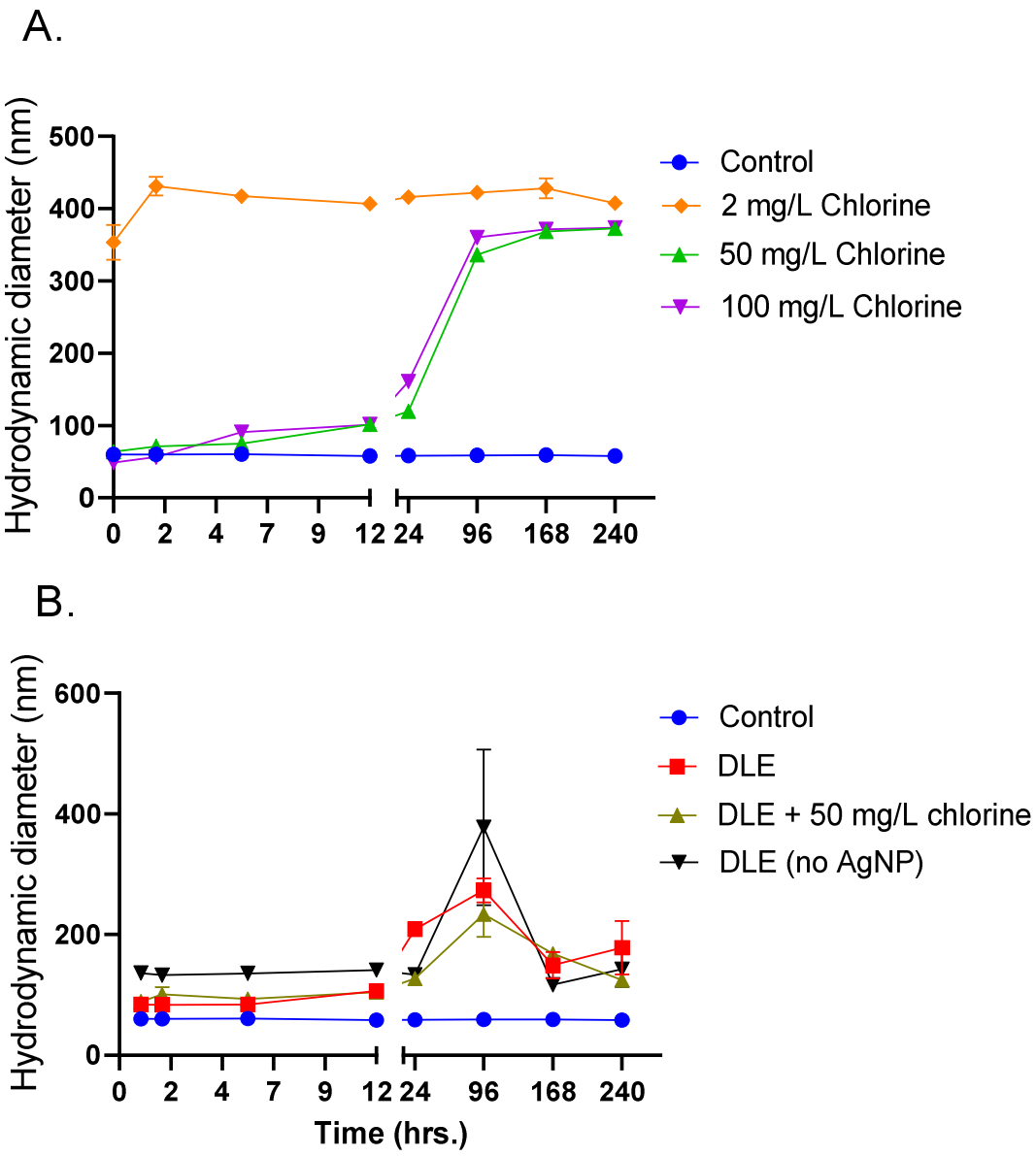


Figure 2.3 Temporal changes in hydrodynamic diameters of Ag aggregates (5 mg/L) (A) in the chlorine-alone treatments and (B) in the DLE treatments with or without chlorine (50 mg/L). Solution pH was measured before and after the experiments: Control (pH = 8.31–8.41), 2 mg/L Chlorine (pH = 6.86–7.24), 50 mg/L Chlorine (pH = 6.99–7.27), 100 mg/L Chlorine (pH = 7.03–7.30), DLE (pH = 6.53–6.80), DLE + 50 mg/L Chlorine (pH = 5.74–6.85), and DLE (no Ag NP) (pH = 6.31–6.64).

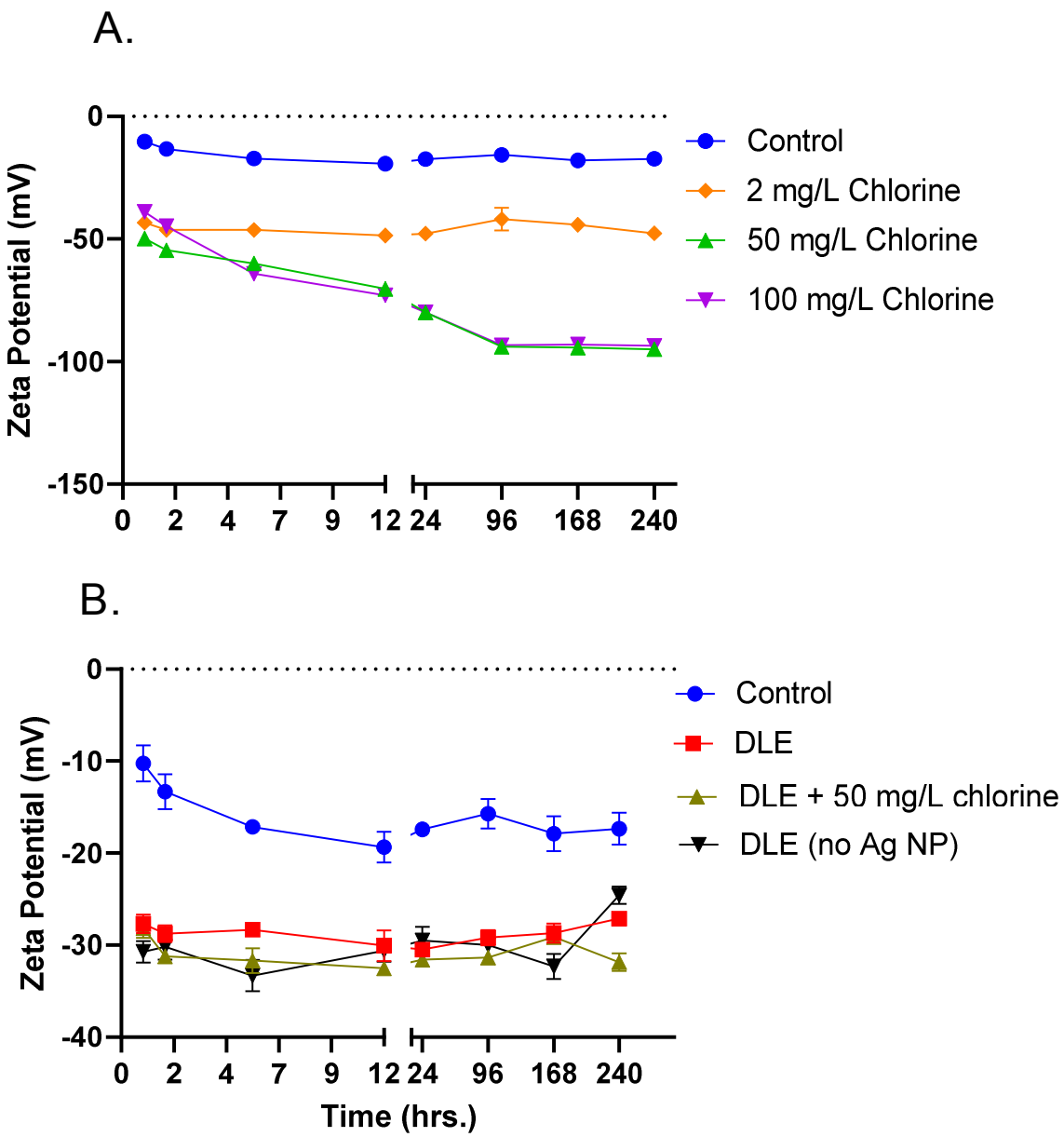
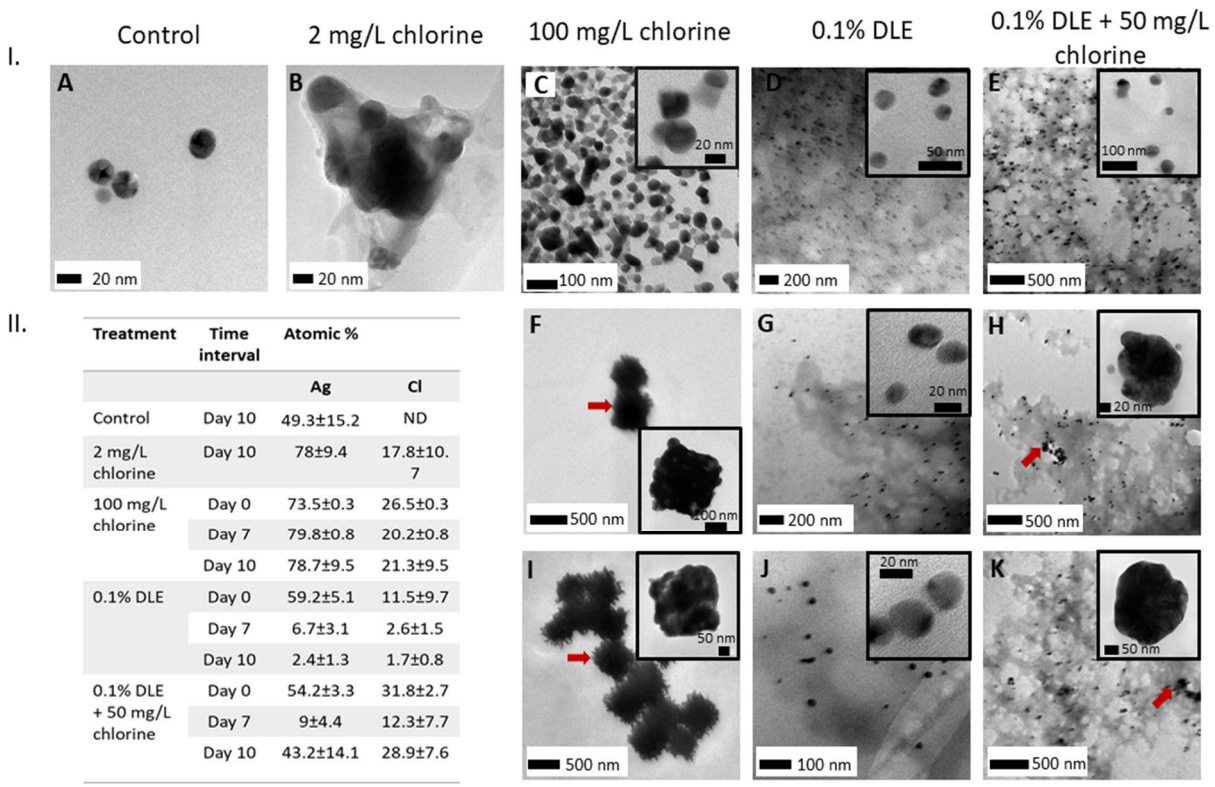


Figure 2.4 Temporal changes in zeta-potential of Ag aggregates (A) in the chlorine-alone treatments and (B) in the DLE treatments with or without chlorine (50 mg/L). Solution pH was measured before and after the experiments: Control (pH = 8.31–8.41), 2 mg/L Chlorine (pH = 6.86–7.24), 50 mg/L Chlorine (pH = 6.99–7.27), 100 mg/L Chlorine (pH = 7.03–7.30), DLE (pH = 6.53–6.80), DLE + 50 mg/L Chlorine (pH = 5.74–6.85), and DLE (no Ag NP) (pH = 6.31–6.64).



II.

Treatment	Time interval	Atomic %	
		Ag	Cl
Control	Day 10	49.3±15.2	ND
2 mg/L chlorine	Day 10	78±9.4	17.8±10.7
100 mg/L chlorine	Day 0	73.5±0.3	26.5±0.3
	Day 7	79.8±0.8	20.2±0.8
	Day 10	78.7±9.5	21.3±9.5
0.1% DLE	Day 0	59.2±5.1	11.5±9.7
	Day 7	6.7±3.1	2.6±1.5
	Day 10	2.4±1.3	1.7±0.8
0.1% DLE + 50 mg/L chlorine	Day 0	54.2±3.3	31.8±2.7
	Day 7	9±4.4	12.3±7.7
	Day 10	43.2±14.1	28.9±7.6

Figure 2.5 Transmission electron microscopy and energy dispersive spectroscopy of Ag NPs.

I: TEM images of Ag NPs in different treatments at different time points. X-axis represents the treatment and y-axis represents the time points. A: Control at Day 10; B: 2 mg/L chlorine at Day 10; C, F, I: 100 mg/L at Day 1, Day 7, and Day 10 respectively; D, G, J: 0.1% DLE at Day 1, Day 7 and Day 10 respectively and E, H, K: 0.1% DLE with 50 mg/L chlorine at Day 1, Day 7 and Day 10, respectively.

II: EDS analysis of Ag NPs in different treatments at different time points. ND: Not Detected.

2.4 CONCLUSION

In the presence of chlorine, aggregate size of Ag NPs increased with time (49 to 431 nm), suggesting greater aggregation of Ag NPs than that in the control treatment ($P < 0.05$). Increasingly negative zeta potentials (-39 to -95 mV) were found in the chlorine treatments, likely due to formation of AgCl particles. Both greater aggregate size and more negative zeta potential were also observed in the presence of DLE (84–273 nm and -28 to -32 mV, respectively), as compared to the control treatment, which might be predominantly influenced by DLE than Ag NPs. Aggregate size at 4-10 days was greater in the chlorine-only treatments than in the DLE treatments, despite lower zeta potentials. These results were corroborated by transmission electron microscopy with energy dispersive spectroscopy, confirming formation of AgCl-Ag NP composite particles in the presence of chlorine and the embedding of AgCl and Ag NPs in the DLE matrix. The time-resolved TEM-EDS analysis also showed that the AgCl-Ag NP aggregates increased in size in the chlorine-only treatments over time, probably facilitated by nucleation and crystal growth of AgCl. In the DLE treatments the Ag NPs exhibited fewer changes during the experiments, likely because Ag NPs were stabilized and protected from chlorine by DLE. These results suggest that chlorine and plant-released organic matter could substantially change the behaviors of Ag NPs in wash water and subsequently their environmental fate and risks.

CHAPTER 3:

REMOVAL OF SILVER NANOPARTICLES FROM LETTUCE DURING PRODUCE PROCESSING

3.1 INTRODUCTION

Silver nanoparticles (Ag NPs) are among the most important engineered nanoparticles in agriculture, and are used as a broad-spectrum treatment against plant pathogens due to their antimicrobial properties *(69, 142)*. Annual production of Ag NPs averages 500 tons worldwide *(111)*, and is expected to increase as Ag NP applications broaden. When used in pesticides, the release of Ag NPs from agricultural soil is estimated to be 54 times greater than that from all other sources combined (e.g., sewage sludge, wastewater irrigation, and atmospheric deposition) *(46)*. The U.S. Environmental Protection Agency (EPA) has registered hundreds of nanopesticide products containing Ag NPs, with new patents for fungicides containing Ag NPs continually pending *(165)*. Therefore, increasing levels of Ag NPs will likely be found in the environment, which may bioaccumulate in fruits and vegetables for human consumption *(36, 85, 88, 102, 155)*. Exposure to Ag NP residues in food crops may adversely affect human health. *In vivo* toxicity studies have shown liver and DNA damage in rodents after exposure to > 125 and 20 mg/kg Ag NPs, respectively *(76, 145)*, and accumulation of silver in the liver and kidneys of rodents exposed to > 20 mg/kg *(145)*. *In vitro* studies using human cells have shown damage to lung fibroblast cells, glioblastoma cells, and skin keratinocytes after exposure to 20-100 Ag NPs μg/mL *(8, 93)*. Therefore, chronic human exposure to trace-levels of Ag NPs should be minimized to mitigate public health risks.

Effective washing strategies are needed to decrease not only microbial pathogens, but also chemical contaminants including Ag NPs in fresh-cut leafy greens during commercial processing. In commercial processing, leafy greens are washed in a flume tank with sanitizers and then drained on a shaker table before being centrifugally dried. Chemical sanitizers are typically added to the flume water to reduce microbial populations and minimize cross-contamination *(31, 98)* with

chlorine-based sanitizers favoured by the produce industry due to their cost-effectiveness and minimal adverse effect on product quality *(21, 30, 70, 97, 117)*. During processing, organic matter from fresh-cut produce (including tissue particulates/exudates) and soil particles will gradually accumulate in the recirculating water, decreasing both the chlorine concentration and its effectiveness *(30, 127)*. Some alternative sanitizers such as peroxyacetic acid are not affected by the organic load in the wash water *(32)*. However, the impact of chlorine- or peroxyacetic acid-based sanitizers on the removal of Ag NP from leafy greens during commercial processing remains unknown.

Only a few studies have thus far evaluated the removal of Ag NPs from spinach and lettuce during washing. When lettuce leaves were washed with 0.01 M acetic acid for 10 min, followed by final rinse with deionized (DI) water, Larue et al. *(85)* reported only a 2% reduction in total Ag from the leaves. Using DI water, peroxyacetic acid (80 mg/L) and NaOCl (200 mg/L ppm) Zhang et al. *(165)* reported total Ag reductions of 5%, 21%, and 10% for contaminated spinach leaves after washing. In subsequent work by Zhang et al. *(166)*, exposing spinach leaves to 200 mg/L NaOCl for 5 min, followed by 2.8% ammonium hydroxide for 1 min, and DI water for 1 min, removed 91-93% of the surface-attached Ag NPs. However, none of these studies mimic the commercial washing practices for fresh-cut leafy greens. In particular, the organic load of the wash may significantly impact Ag NP removal *(85)*. Therefore, additional work is needed to assess the effectiveness of washing practices under conditions that most closely resemble current commercial operations. Finding optimal wash water conditions to decrease Ag NP levels in contaminated fresh produce is another important step towards improving end-product safety.

Therefore, this study aimed to evaluate the removal of Ag NPs from contaminated lettuce. Initially, a cost-effective bench-top batch system was used to assess a range of sanitizers and

organic loads typically seen during commercial flume washing of fresh-cut leafy greens. Finally, a pilot-scale processing line was used to evaluate the removal of Ag NPs from contaminated lettuce under commercial produce processing conditions.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

First, the effectiveness of the wash-phase of produce processing in removing Ag NPs from contaminated produce was evaluated using the batch-type system (Figure 3.1). Chlorine and peroxyacetic acid solutions with and without organic load were used to simulate the wash water by washing 25 g of Ag NP-contaminated romaine lettuce in a 4-L glass carboy jars with sanitizer-free ultrapure water serving as the control treatment. Then, various water samples were collected, and water and leaf samples were quantitatively examined for Ag NPs using inductively coupled plasma mass spectrometry (ICP-MS; iCAP Q, Thermo Fisher Scientific Inc, Bremen, Germany). Ultrapure water (18 MΩ at 25 °C) produced with a Milli-Q gradient system (Millipore, United States Filter Corporation, CA) was used for the experiments. The experiments were conducted in triplicates.

Thereafter, the effectiveness of typical produce processing (wash-phase/flume washing of produce processing along with centrifugation) in removing Ag NPs from contaminated lettuce was evaluated using a pilot-scale produce processing line (Figure 3.2). The efficacy of chlorine sanitizer in removing Ag NPs from contaminated leaves was assessed in triplicates by processing 5.4 kg of romaine lettuce with tap water available on Michigan State University Campus as the control treatment. Lettuce leaves were processed by shredding, conveying, and flume washing, followed by dewatering by shaker table and centrifugal drying. At various time points during the processing, water and leaf samples were collected and quantitatively examined for Ag NPs.

3.2.2 Lettuce and Ag NPs

For the batch-type experiments, un-contaminated romaine lettuce was purchased from a local grocery store. For the pilot-scale produce processing experiments, several batches of

individually wrapped romaine lettuce were purchased from a local wholesaler, stored in a 4°C walk-in cooler and used within 3 days of delivery.

An Ag NP stock suspension (1000 mg/L; nominal particle size of 15 nm) was purchased from US Research Nanomaterials, Inc. (Houston, TX). For the batch experiments, romaine lettuce pieces (4 × 4 cm) were immersed in a container with 40 mg/L Ag NPs suspension. A wired stainless-steel mesh was used to trap the leaves inside the suspension during the immersion time. The leaves with the Ag NPs suspension were allowed to shake at 35 rpm for 1 hr in a shaker. After 1 hr, the leaves were drained out of the suspension, spun in a salad spinner (Model 32480V2, OXO, Chambersburg, PA) by hand-pumping 5 times to drain excess suspension and air-dried under the fume hood with high flow for an additional 30 min. Three aliquots of lettuce leaf samples (25 gram each) were collected for the subsequent measurement of Ag concentrations. The remainder of the Ag NPs-contaminated leaves were used for the washing experiments. Three 1-mL samples of the Ag NP suspension were collected before and after the contamination event to measure the Ag NP concentrations in the suspension using ICP-MS at a later time.

For the pilot-scale produce processing experiments, the romaine lettuce (7.5 kg) were cut into pieces (5 × 5 cm) and were used in 3 separate sets to contaminate with Ag NPs. First, 2.5 kg of the cut romaine lettuce pieces were immersed in a container with 85 mg/L Ag NPs suspension (37.5 L) for 1 hr. A wired stainless-steel mesh was used to trap the leaves in the suspension during the immersion time. After 1 hr, the leaves were drained out of the suspension, spun in a salad spinner (Model 32480V2, OXO, Chambersburg, PA) by hand-pumping 5 times to drain excess suspension, and air-dried under the fume hood in high flow for an additional 1 hr. This procedure was repeated twice to contaminate all 7.5 kg of lettuce (the same Ag NP suspension was used to contaminate 7.5 kg lettuce in three steps). Six aliquots of the leaf samples (50 gram each) were

collected to measure the Ag concentration later. The rest of the leaves were used for the washing experiments and were separated into 50-gram aliquots and placed in 28-cm-long red mesh produce bags (5-lb header bag, Pacon Inc., Baldwin Park, CA). Three 1-mL samples of the Ag NP suspensions were collected before and after each contamination event to measure Ag NP concentration in the suspensions at each set using ICP-MS later, which was used to determine the amount of Ag sorbed to the lettuce leaves.

3.2.3 Processing equipment and lettuce processing line

For the batch experiments, three 4-L Kimax glass carboy jars with 5-cm diameter openings at the top were used. Plastic stoppers were used to plug the 0.64-cm diameter discharge spouts at the bottom (Kimble Chase, Vineland, NJ). An 8-cm-long magnetic stir bar was placed into the jar before the start of each experiment. The carboy jar was placed on a stirring hotplate (Model LMS-100, Daihan Labtech Co., Ltd., Korea) at a stir speed setting of 6 without heating (Figure 3.1). Before using the three carboy jars for the next treatment, they were first washed with water, rinsed with 3% nitric acid, and then again washed with water to remove any Ag NPs.

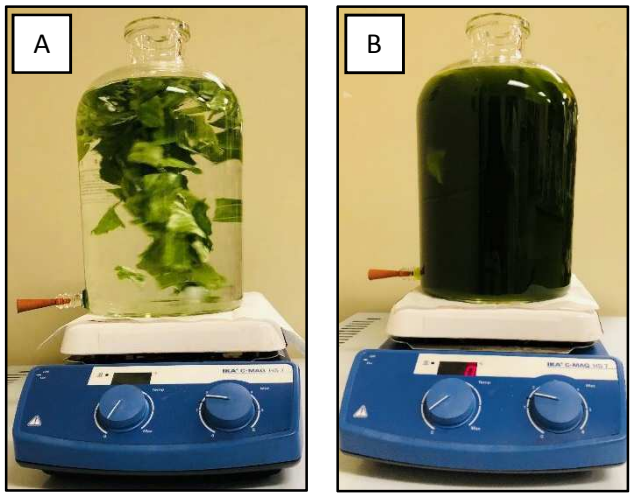


Figure 3.1 Bench-top batch system to simulate flume washing of romaine lettuce in the batch experiments. A. Wash water was used with or without sanitizers in the absence of organic load (OL); B. Wash water was used with or without sanitizers in the presence of 2.5% OL.

For the pilot-scale produce processing experiments, a small-scale pilot leafy green processing line consists of a lettuce shredder, flume tank, shaker table, and dewatering centrifuge (Figure 3.2). The lettuce shredder (Model TRS 2500 Urschel TranSlicer, Valparaiso, IN) was operated at the feed belt and slicing wheel speeds of 198 m/min and 905 RPM, respectively. It was used to shred 4.4 kg of uncontaminated lettuce (shred size of approximately 5 × 5 cm). A stainless-steel water recirculation tank (~1000 L capacity) was filled with 600 L of tap water. This water tank is connected to a 3.6-m-long stainless-steel flume tank (Heinzen Manufacturing, Inc., Gilroy, CA), by a 4.14 m-long, 10 cm-diameter hard plastic discharge hose and a centrifugal pump (Model XB754FHA, Sterling Electric, Inc., Irvine, CA) that circulate the water at a rate of approximately 10 L/s. The flume tank is equipped with two overhead spray jets (1 m from the start). A custom-made stainless-steel gate with 1.25 cm-diameter holes spaced 0.65 cm apart (Heinzen Manufacturing, Inc.) is secured to the end of the flume tank to prevent product flow while allowing continuous circulation of the wash water. The stainless-steel shaker table for partial dewatering was operated by a 1-HP Baldor washdown duty motor (Baldor Electric Co., Ft. Smith, AR) at 1760 RPM. Water was removed from the leafy greens during the mechanical shaking, passed through a mesh screen, and fed into the water holding tank by a water recirculation spout underneath the shaker table. A 50-lb capacity centrifugal Spin Dryer (Model SD50-LT, Heinzen Manufacturing, Inc.) with three internally timed spin cycles totalling 80 s was used for centrifugal drying (Figure 3.2).

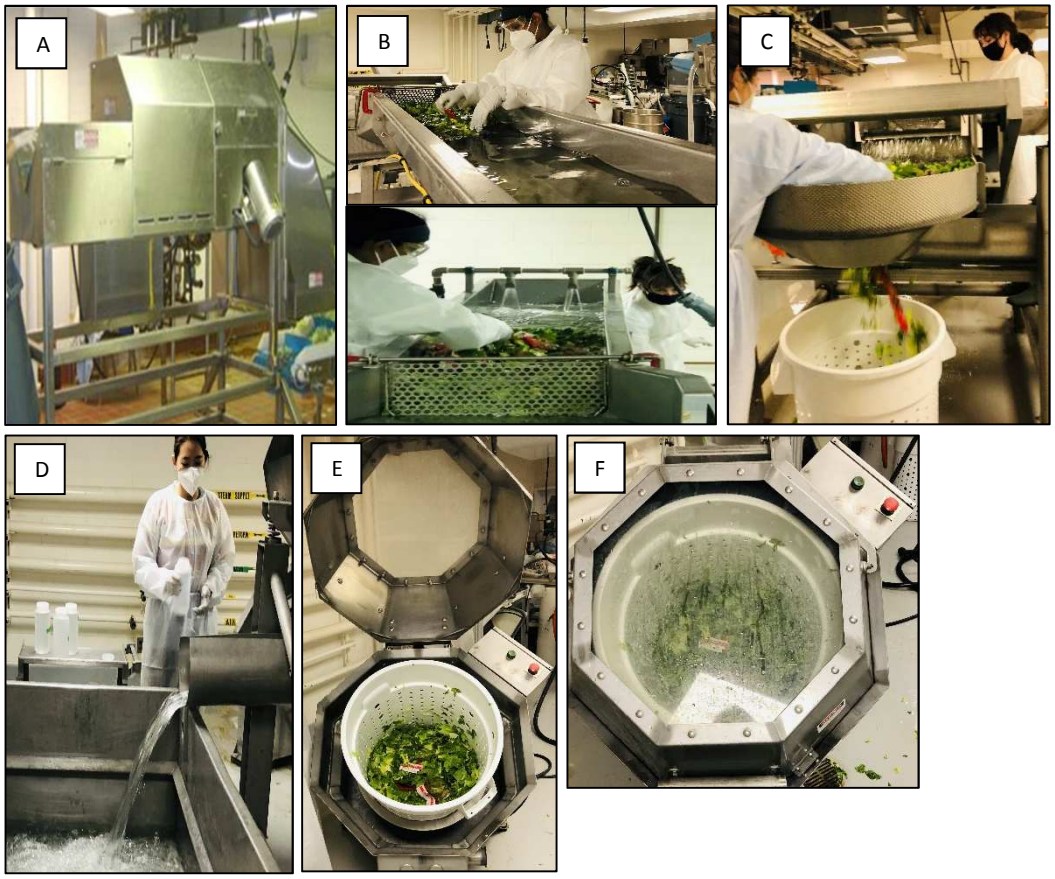


Figure 3.2 Pilot-scale processing line designed for fresh produce at Michigan State University. A. shredder; B. Flume tank; C. Shaker table; D. water recirculation tank; E-F. centrifugal dryer.

3.2.4 Wash water treatments

The following six wash water treatments were assessed for the batch experiments: (1) 100 mg/L available chlorine (XY-12, Ecolab) acidified to pH 6.5 with citric acid (Sigma-Aldrich, St. Louis, MO); (2) 80 mg/L peroxyacetic acid (Tsunami 100, Ecolab, St. Paul, MN); (3) 2.5% organic load with 100 mg/L available chlorine (XY-12, Ecolab) acidified to pH 6.5 with citric acid (Sigma-Aldrich, St. Louis, MO); (4) 2.5% organic load with 80 mg/L peroxyacetic acid (Tsunami 100, Ecolab, St. Paul, MN); (5) 2.5% organic load; and (6) sanitizer-free ultrapure water serving as the control. Free chlorine concentrations used in typical produce processing can range from 10 to 200 mg/L *(31)* and a concentration of 80 mg/L is the maximum allowable concentration for peroxyacetic acid to be used in produce processing *(105)*. Thus, 100 mg/L and 80 mg/L concentrations were used as concentrations for chlorine and peroxyacetic acid as sanitizers in this study.

For the pilot-scale produce processing experiments, the following two wash water treatments were assessed: (1) 100 mg/L available chlorine (XY-12, Ecolab) acidified to pH 6.5 with citric acid (Sigma-Aldrich, St. Louis, MO) and (2) sanitizer-free MSU tap water (< 0.6 ppm free chlorine) serving as the control treatments. Chlorine is the most commonly used sanitizer in commercial produce washing. Therefore, chlorine sanitizer was tested in this pilot plant study for the effectiveness of Ag removal with water used as the control for comparison.

A commercial chlorine sanitizer (XY-12) obtained from Ecolab, Inc. was used to prepare 100 mg/L chlorine solutions. The pH of the chlorine solutions was measured with a pH probe (pHTestr 30, Oakton, Vernon Hills, IL) and adjusted to 6.5 by acidifying with citric acid (600 µL of 96% (w/v) solution) (Sigma-Aldrich, St. Louis, MO). The chlorine concentration was measured by the N,N-diethyl-p-phenylenediamine (DPD) method using a DR300 chlorine meter (Hach,

Loveland, CO). Free chlorine was measured immediately before exposing romaine lettuce to the wash water using the DR300 chlorine meter. A commercial peroxyacetic acid sanitizer (Tsunami 100, Ecolab, St. Paul, MN) was used to prepare 80 mg/L peroxyacetic acid. Peroxyacetic Acid Test Kit 311 (Ecolab) was used to confirm the peroxyacetic acid concentration.

Organic matter in produce wash water in the batch experiments was represented by 2.5% organic load solutions. One hundred grams of romaine lettuce were blended for 2 min in 250–500 ml of ultrapure water using a household blender (Model BLC10650MB, Black & Decker, New Britain, CT) and added to the ultrapure water to achieve organic load levels of 2.5% (w/v) in 4 L of volume.

3.2.5 Lettuce washing/processing and sample collection

For the batch experiments, Ag NP-contaminated romaine lettuce pieces (25 g) were placed into the carboy jars. Romaine lettuce pieces were allowed to mechanically stir in the wash water for 5 min, and water samples (20 mL) were collected in 50-mL centrifuge tubes through the opened spigot at 30 s intervals during the washing. After the 5-min washing, the spout was closed, and lettuce leaves were then removed from the jars. The lettuce leaves were spun in the salad spinner by hand-pumping 5 times to drain excess water and air-dried under the fume hood in high flow for an additional 30 min. The washing process in the commercial produce processing generally lasts 90 s. However, a longer washing period (5 min) was used to allow for detectable Ag removal (Figure 3.1).

For the pilot-scale produce processing experiments, uncontaminated romaine lettuce (4.4 kg) was first hand-fed into the shredder. Contaminated lettuce in mesh bags (20) were also added to the flume tank. They were allowed to wash in 600 L of recirculating wash water for 90 s. The produce was then released from the flume tank and partially dewatered on the shaker table,

collected in a single centrifugation basket, and centrifugally dried. For each batch of the lettuce processed, 30-mL water samples were collected in 50-mL centrifuge tubes at 30 s intervals, and three bags of lettuce were collected at the flume gate at 30 s intervals during 90 s of flume washing. The wash water samples were collected to analyse Ag concentrations during flume washing and the lettuce samples were collected to analyse the remaining Ag concentration in lettuce after washing treatment. Three leaf samples collected from the flume tank at each time interval were combined, and then separated into two duplicate samples that were used for analysis. The rest of the lettuce leaves were placed in the centrifugal dryer. Water samples were collected from the centrifugal drain during the 80 s centrifugal drying cycle. After centrifugation, three contaminated bagged lettuce samples were collected from the centrifugation basket to analyse the Ag concentration in the contaminated lettuce after centrifugation (Figure 3.2).

3.2.6 Samples analysis for Ag

For the batch experiments, the wash water samples with organic load were centrifuged at 10,000 × g for 30 min at 4°C to remove coarse particles and the supernatant was then filtered through 0.45-µm filters. Then, all wash water samples (wash water treatments without organic load and filtrates of wash water samples with organic load) were analysed for Ag by ICP-MS. For the pilot-scale produce processing experiments, Ag concentrations were analysed for all wash water samples (without filtration) by ICP-MS. The ICP-MS analyses of all the water samples were performed at Michigan Elemental Analysis Lab (MEAL) at University of Michigan. In the batch and pilot-scale processing experiments, the leaf samples were freeze-dried and ground to a fine powder. The leaf samples were digested, followed by the measurement of Ag concentrations with ICP-MS at MEAL.

3.2.7 Statistical analysis

For the batch experiments, the mixed effect model with replicates and repeated measures were used to identify significant differences of Ag concentrations in wash water samples in washing treatments as compared to the control. Significant differences between fixed effects were tested using ANOVA and the mean comparisons (post hoc comparisons) for individual treatments were obtained using the Tukey test at a significant p value of ≤ 0.05.

For the pilot-scale produce processing experiments, the mixed effect model with replicates and repeated measures were used to identify significant differences in the chlorine treatment as compared to the control treatments for Ag concentrations in timed wash water samples and contaminated leaf samples. A two-way factorial design was used where the presence of sanitizer indicates 2 levels. Significant differences between fixed effects were tested using ANOVA and the mean comparisons (post hoc comparisons) for individual treatments were obtained using the Tukey test at a significant p value of ≤ 0.05. The t-test was used to identify significant differences in treatments for centrifuged wash water samples. A p value of ≤ 0.05 was considered significant. All statistical analyses were performed in R, version 4.1.0.

3.3 RESULTS

3.3.1 Batch experiments

Figure 3.4A and table 3.1 shows the changes in Ag concentrations in the wash water samples with time during the 5-min washing. With the exception of the treatment of organic load with peroxyacetic acid, Ag concentration was increased with time ($P \leq 0.05$) regardless of the sanitizer treatment or organic load. The Ag concentrations in the treatment of 2.5% OL with 80 mg/L peroxyacetic acid were very low, close to 0.

Ag concentrations in the wash water samples of each treatment was compared with that of the control treatment at each time point from 30 to 300 s. Ag concentrations in the wash water samples of the 80 mg/L-peroxyacetic acid treatment was significantly higher ($P \leq 0.05$) than that of the control treatment between 150-300 s (Table 3.1). The control treatment was not significantly different from chlorine treatment at any time point from 30 to 300 s (Table 3.1). This indicates there is no effect on Ag removal when chlorine is present in wash water as compared to water alone.

Ag concentrations in the wash water samples in 2.5% organic load treatment and 2.5% organic load with 100 mg/L chlorine treatment were significantly lower ($P \leq 0.05$) as compared to the control treatment starting from 120 s up until 300 s. Furthermore, Ag concentrations in 2.5% organic load treatment were not significantly different with Ag concentrations in 2.5% organic load with 100 mg/L chlorine treatment at any time point from 30 to 300 s (Table 3.1), indicating that chlorine did not influence the Ag removal in the treatments with organic loads.

Out of the 6 wash water treatments, the highest Ag concentrations were detected in the treatment of 80 mg/L peroxyacetic acid (Table 3.1 and Figure 3.4A), showing 59 µg/L Ag

concentration in the wash water samples at 5 min. Thus, the 80 mg/L peroxyacetic acid treatment had the highest decontamination effectiveness for the lettuce leaves among the tested 6 treatments.

Ag removal percentages at the specific time points based on the Ag concentrations in the wash water samples were calculated for all 6 treatments (Table 3.2). The highest Ag removal was achieved at 5 min, except for the 2.5% organic load with 80 mg/L peroxyacetic acid treatment. The highest Ag removal percentage of the sorbed Ag in the contaminated leaves was 6.6% in the treatment of 80 mg/L peroxyacetic acid. Ag removal percentages for the control treatment, 100 mg/L chlorine treatment, treatment of 2.5% organic load with 100 mg/L chlorine, treatment of 2.5% organic load, and treatment of 2.5% organic load with 80 mg/L peroxyacetic acid were 4.7%, 3.5%, 3%, 2.7% and 0.1% respectively (Table 3.2 and Figure 3.4B).

3.3.2 Pilot scale produce processing line experiments

Ag concentration in the wash water samples in the 100 mg/L chlorine treatment were significantly higher ($P \leq 0.05$) as compared to that of the control treatment at 30, 60 and 90 s time points (Table 3.4). This indicates that the 100 mg/L chlorine treatment was more effective in removing Ag from the contaminated leaves, as compared to control treatment. Figure 3.5A displays the Ag concentrations in the wash water samples over 90 s washing, showing temporal variations and no clear pattern. Despite the statistically significant difference in the Ag concentrations with time for the 100 mg/L chlorine treatment ($P \leq 0.05$), the temporal variations and the difference between the two treatments are not considered as substantial due to the very low levels of Ag detected (0.03 – 2.78 μg/L). Ag removal percentages were calculated for all the tested time points based on the wash water sample results for both treatments (Table 3.5). Ag removal percentages were higher for chlorine treatment as compared to the control and the differences were statistically significant ($P \leq 0.05$). Figure 3.5B shows the percent Ag removal for each treatment

during 90 s washing. The highest Ag removal (1.7−3.3%) from contaminated leaves was found for the treatment of 100 mg/L chlorine.

For both the batch-system and pilot-scale processing experiments there were very high variations for Ag concentrations in contaminated leaf samples after washing (within replicates) (Table 3.3 and Table 3.6). It was not possible to assess the removal percentages based on the Ag concentrations in the contaminated leaves before and after washing. Therefore, the Ag removal was evaluated based on the Ag concentrations in the wash water samples.

Ag concentrations in the centrifuged wash water samples for 100 mg/L chlorine treatment and control treatment are 51.5 µg/L and 31.6 µg/L, respectively and there was no significant difference between treatments in centrifuged wash water samples (Figure 3.6).

Table 3.1 Ag concentrations in the wash water samples at different time points during simulated produce processing in the batch experiments.

Treatment	Ag concentration in wash water samples (μg/L, Mean ± standard deviation)									
	30 s	60 s	90 s	120 s	150 s	180 s	210 s	240 s	270 s	300 s
Control (ultrapure water)	*15.1±2.3*	*20.2±1.3*	*24.4±1.6*	*27.7±1.9*	*30.6±2.9*	*33.4±0.9*	*36.8±3.1*	*38.4±3*	*40.2±2.8*	*41.6±4.6*
100 mg/L chlorine	*12.7±1.2*	*17.3±2.4*	*18.5±3.2*	*19.5±3.4*	*27±4.7*	*27.2±5.9*	*28.1±6*	*30.4±2.4*	*30.4±3.6*	*31.5±2.2*
80 mg/L peroxyacetic acid (PAA)	*20±3.6*	*27.9±4.4*	*33.8±6*	*38.3±6.9*	*43.2±7.8**	*46.8±7.4**	*50.2±7.6**	*53.3±7.6**	*56.6±7.9**	*59±8.2**
2.5% OL	*8.2±2.6*	*10.7±4.5*	*13.6±6.3*	*15.7±7.4**	*17.3±6.6**	*19.1±6.6**	*20.4±4.5**	*19.6±5.2**	*24.3±3.2**	*24.4±0.9**
2.5% OL + 100 mg/L chlorine	*7.8±2.4*	*11.4±1.9*	*14.2±2.3*	*15.9±1.4**	*19±3**	*21.1±3.6**	*22.6±3.4**	*24.7±3.9**	*25.8±4.6**	*27.1±4.2**
2.5%OL + 80 mg/L PAA	0*	0*	0.1±0*	0.1±0*	0.1±0*	0.1±0*	0.1±0*	0.1±0*	0.1±0*	0.1±0*

Within a column, * shows the significant difference between a given treatment and the control treatment at that particular time point. The Ag concentrations in italics means there is statistical difference in mean concentration at a given time point compared to that of the previous time point in each row.

Table 3.2 Ag removal percentages from the contaminated leaves (based on wash water samples results) at different time points during simulated produce processing in the batch experiments.

Treatment	Ag removal percentage (Mean ± Standard deviation)									
	30 s	60 s	90 s	120 s	150 s	180 s	210 s	240 s	270 s	300 s
Control (ultrapure water)	1.7±0.3	2.3±0.1	2.7±0.2	3.1±0.2	3.4±0.3	3.7±0.1	4.1±0.3	4.3±0.3	4.5±0.3	4.7±0.5
100 mg/L chlorine	1.4±0.1	1.9±0.3	2.1±0.4	2.2±0.4	3±0.5	3.1±0.7	3.2±0.7	3.4±0.3	3.4±0.4	3.5±0.2
80 mg/L PAA	2.2±0.4	3.1±0.5	3.8±0.7	4.3±0.8	4.8±0.9	5.3±0.8	5.6±0.9	6±0.9	6.4±0.9	6.6±0.9
2.5% OL	0.9±0.3	1.2±0.5	1.5±0.7	1.8±0.8	1.9±0.7	2.1±0.7	2.3±0.5	2.2±0.6	2.7±0.4	2.7±0.1
2.5% OL + 100 mg/L chlorine	0.9±0.3	1.3±0.2	1.6±0.3	1.8±0.2	2.1±0.3	2.4±0.4	2.5±0.4	2.8±0.4	2.9±0.5	3±0.5
2.5%OL + 80 mg/L PAA	0	0	0	0	0	0	0	0	0	0

The Ag removal percentages were calculated by dividing the released Ag mass in wash water by the initial sorbed Ag in lettuce leaves.

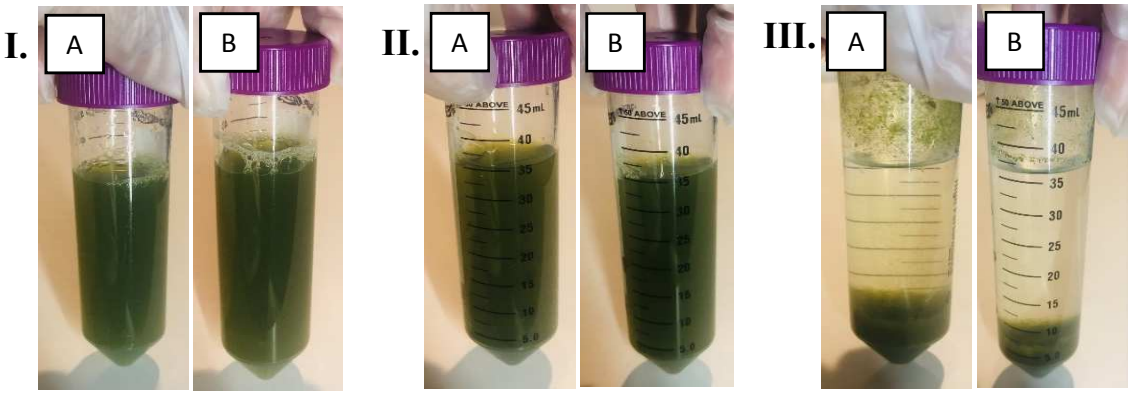


Figure 3.3 Wash water samples containing OL in the batch experiments. I. 2.5% OL; II. 2.5% OL with 100 mg/L chlorine; III. 2.5% OL with 80 mg/L PAA; A. The suspension contains no Ag NPs; B. The suspension contains Ag NPs.

Table 3.3 Ag concentrations in contaminated romaine lettuce leaves after washing in the batch experiments.

Treatment	Ag concentration in contaminated leaves (μg/g, Mean ± Standard deviation)
Control (ultrapure water)	105.5±66.1
100 mg/L chlorine	141.5±93.5
80 mg/L PAA	146.1±49.9
2.5% OL	80.6±34.4
2.5% OL + 100 mg/L chlorine	205.2±97
2.5%OL + 80 mg/L PAA	105±24.1

Table 3.4 Ag concentrations in wash water samples at different stages during produce processing in the pilot-scale processing experiments.

Treatment	Ag concentration in wash water samples (μg/L, Mean ± Standard Deviation)				
	0 s	30 s	60 s	90 s	Centrifuge
Control (water)	0.1±0	0.8±0.7	0.2±0.1	0.6±0.5	31.6±19
100 mg/L chlorine	*0.4±0.2*	*2.4±0.3**	*1.2±0.3**	1.5±0.6*	51.5±18.9

Within a column, * shows the significant difference between a given treatment and the control treatment at that particular time point.
The Ag concentrations in italics means there is statistical difference in mean concentration at a given time point compared to that of the previous time point in each row.

Table 3.5 Ag removal percentage from the contaminated leaves (based on wash water samples results) at different time points during produce processing in the pilot-scale processing experiments.

Treatment	Ag removal percentage (Mean ± Standard Deviation)		
	30 s	60 s	90 s
Control (water)	1.1±0.9	0.3±0.2	0.8±0.7
100 mg/L chlorine	3.3±0.4	1.7±0.4	2±0.8

The Ag removal percentages were calculated by dividing the released Ag mass in wash water by the initial sorbed Ag in lettuce leaves

Table 3.6 Ag concentrations in contaminated romaine lettuce leaves at different stages during produce processing in the pilot-scale processing experiments.

Treatment	Ag concentration in contaminated leaves (μg/g, Mean ± Standard Deviation))				
	0 s (unwashed)	30 s	60 s	90 s	Centrifuge
Control (water)	475.3±104	362.8±56.9	368.8±29.4	329±79.9	402.3±91.1
100 mg/L chlorine	475.3±104	333.6±61.2	356.4±29.9	313±25.5	337.4±80.1

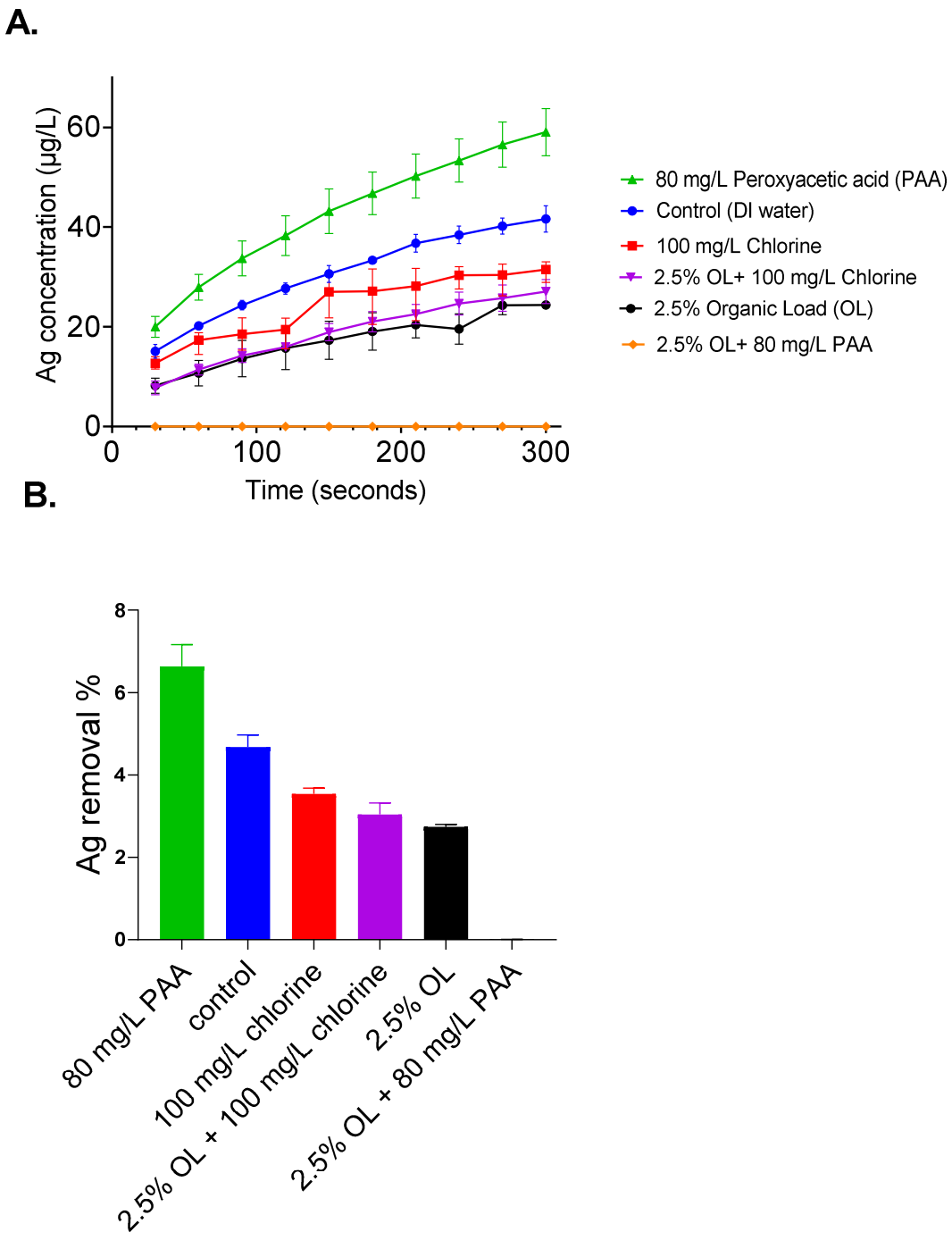


Figure 3.4 Ag concentrations in the wash water and Ag removal percentages in the batch experiment. A. Changes in Ag concentrations in wash water during 5 min washing; B. The percentages of the Ag removal from the sorbed Ag in the contaminated lettuce (calculated based on the Ag concentrations measured in the wash water) after 5 min washing.

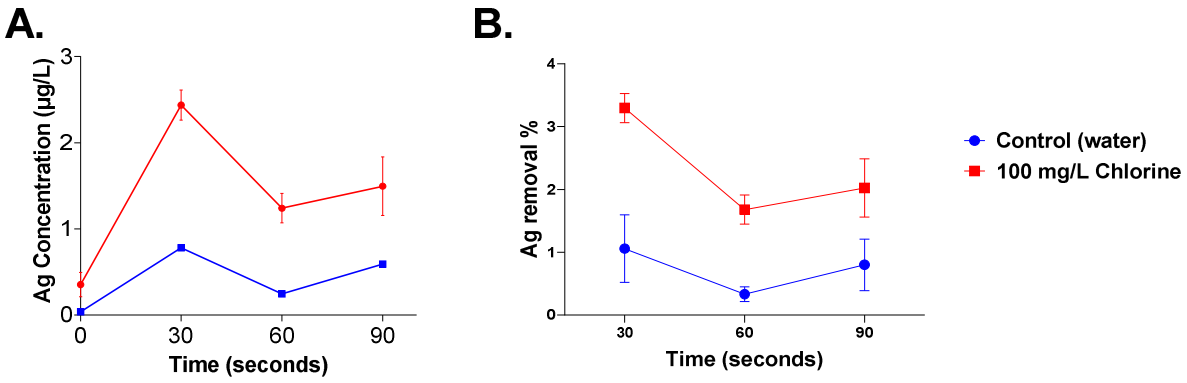


Figure 3.5 Ag concentrations in the wash water and Ag removal percentages in the pilot-scale produce processing experiment. A. Changes in Ag concentrations in the wash water during 90 s flume washing; B. The percentage of Ag removal for each treatment during 90 s flume washing based on the wash water sample results.

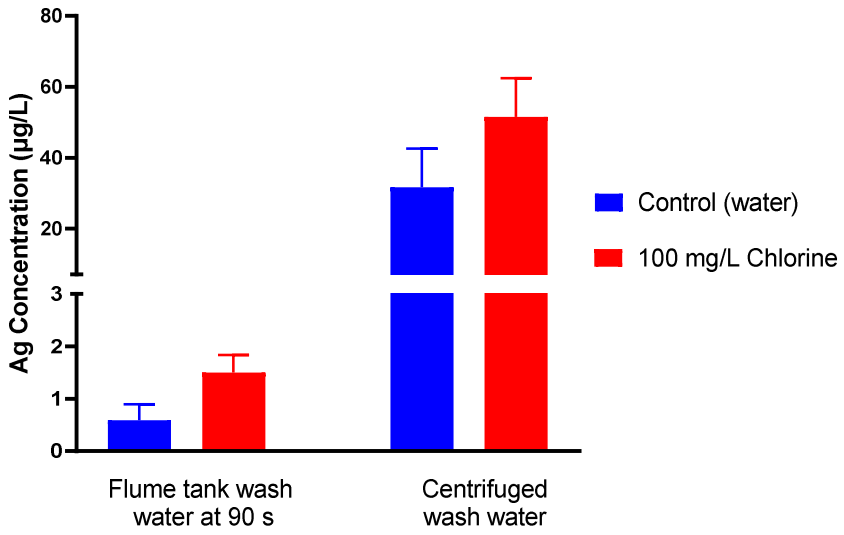


Figure 3.6 Ag concentrations in wash water at 90 s during flume washing and centrifuged wash water for control treatment and chlorine treatment in the pilot-scale processing experiments.

3.4 DISCUSSION

3.4.1 Batch experiments

The Ag concentrations in the wash water samples significantly increased over time during 5 min of washing contaminated lettuce for all the treatments except the organic load containing peroxyacetic acid treatment. This observation suggests that increasing the washing time could increase the removal of Ag NPs from fresh produce to certain degree. Nonetheless, Ag removal percentages of the sorbed Ag in the lettuce were low overall (3–7%) for all washing treatments in the study. Similarly, low percentages of Ag removal were also found in the previous studies. Larue et al. *(85)* observed merely the 2% removal of total Ag from lettuce after washing with 0.01 M acetic acid for 10 min and final rinse with DI water. Zhang et al. *(165)* reported that the removal of Ag NPs from contaminated spinach leaves was 5%, 21%, and 10% after washing with DI water, Tsunami 100 solution (80 mg/L), and Clorox bleach solution (200 mg/L NaOCl), respectively. Therefore, the removal efficiency of Ag NPs sorbed on the fresh produce is generally low, and could also vary with vegetable types. In fact, it was postulated that washing treatments would be ineffective in removing Ag NPs internalized into leaves. Ag NPs can penetrate into the leaf tissue through aqueous pores of the cuticle and stomata during contamination *(85)*. Further, Ag NPs could be oxidized *(15, 18, 50)* and further form Ag-thiol complexes and other Ag^+ species with Ag-thiol complex fraction, as thiol-containing ligands including cysteine, phytochelatins and metallothioneins can strongly bind with Ag^+ *(85)*. The two sanitizers used in this study (chlorine and peroxyacetic acid) are strong oxidants. But it is likely that these sanitizes could not penetrate inside of the leaves*(134)*. Indeed, other studies reported that the sanitizers could not inactivate microbial pathogens that were internalized into leafy greens*(81, 118, 121)*, again implying that the effect of sanitizers on the internalized Ag NPs is low.

Despite the low Ag removal, washing the lettuce with the organic load alone, organic load with chlorine, chlorine solution, ultrapure water (control), and peroxyacetic acid solution resulted in the removal percentage of 2.7%, 3%, 3.5%, 4.7%, and 6.6%, respectively. The Ag removal in the treatment of organic load with peroxyacetic acid solution were very low, close to 0. The treatment of 80 mg/L peroxyacetic acid had the highest decontamination capacity. Further, Ag removal by peroxyacetic acid was significantly higher than that of the control and all the other treatments. The oxidizing potential of PAA (1.81) is greater than the oxidative potential of chlorine (1.36) *(107)*. Peroxyacetic acid sanitizer exists as a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, PAA and water. Both peroxyacetic acid and hydrogen peroxide contribute to its higher oxidative potential as compared to sodium hypochlorite. The hydrogen peroxide oxidizes Ag NPs into Ag^+ ions *(49, 165)*:

$$Ag_{(s)} + H_2O_2 \longrightarrow Ag^+_{(aq)} + OH^{\cdot} + OH^-$$

$$Ag_{(s)} + OH^{\cdot} \longrightarrow Ag^+_{(aq)} + OH^-$$

The greater oxidation potential of peroxyacetic acid may explain the greater Ag NP removal with the peroxyacetic acid alone treatment.

Interestingly, the Ag removal percentage by the control treatment (ultrapure water) in our study was 4.7%. Similarly, Zhang et al. *(165)* reported the Ag NP removal of 5% from contaminated spinach leaves after washing with DI water.

There was no significant difference in the Ag removal by chlorine sanitizer (3.5%) as compared to that of the control treatment. It might be possible that the surface attached Ag NPs was oxidized into Ag^+ and AgCl were formed and immediately sorbed to lettuce leaves. This could be the reason for the low Ag removal by chlorine. Zhang et al. *(165)* reported the formation of AgCl (confirmed with EDS results) and the presence of AgCl particles on leaves when using

chlorine (Clorox) as the washing treatment. However, Zhang et al. found 10% of total Ag removal that is greater than 3.5% in our study, which may be due to higher concentrations of chlorine (200 mg/L) in their study.

Out of 6 washing treatments, the Ag removal was significantly lower, but not substantially different for the treatments with organic load (0-3%) as compared to the treatments without organic load (3.5-7%). The difference may result from the artifacts of the experimental procedures, as Ag-associated with organic matter in the wash water samples could be filtered during the sample pre-treatment and not analyzed by ICP-MS.

Further, out of the treatments containing organic load, organic load with peroxyacetic acid treatment has given much lower Ag concentrations (non-detectable amounts) as compared to the other 2 treatments containing organic load. It was noted that the organic particles were visually precipitated down to the bottom of the wash water samples collected from the treatment of organic load with peroxyacetic acid (Figure 3.3). Thus, any Ag sorbed to organic precipitates would have been excluded from the measurement by ICP-MS.

3.4.2 Pilot scale produce processing line experiments

In the control treatment, 0.2-0.8 μg/L of Ag was detected in the wash water samples, equivalent to the Ag removal percentage of 0.3–1%. In the batch experiments and the study of Zhang et al.*(165)* 2.7% (at 90 s) and 5% (at 5 min) of the Ag removal was found using DI/ultrapure water, respectively, and Larue et al.*(85)* observed 2% of the Ag removal after 10-min washing with slightly acidic water. Thus, the low Ag removal by the control treatment agreed with other laboratory studies that did not use processing lines.

The average Ag concentrations in the wash water samples in the chlorine treatment was significantly greater than those of the control treatment, but the difference is not substantial. The

slight difference might be because the strong oxidation potential of sodium hypochlorite *(165)*. Furthermore, the Ag removal percentages by chlorine during flume washing was still low (1.7–3.3%), which might result from the internalization of Ag NPs into leaf tissues (thus protected from chlorine) *(85, 134)* and formation of AgCl immediately sorbed to leaf surfaces. Importantly, the Ag removal percentage after 90-s washing with chlorine solution was similar in the batch experiments and the pilot-scale processing experiments (2-3%). This agreement indicates the validity of using the batch experiments to screen for promising washing treatments.

In the pilot processing experiments, the processing of produce was done in 2 steps similar to the commercial produce processing. First the produce was washed for 90 s period in recirculating wash water and then produce was drained in the shaker table and transferred to the centrifuge, followed by centrifugation for about additional 80 s to remove excess water out of produce. Interestingly, significantly higher Ag concentrations (32 to 52 μg/L) were detected in wash water for both treatments after centrifugation as compared to Ag concentrations in the bulk wash water after 90 s washing period (0.6 to 1.5 μg/L). Thus, additional Ag was released from the lettuce during centrifugation. It is possible that Ag in intracellular liquid from leaf tissue may have been released during centrifugation. Similarly, previous studies found significant differences in bacterial populations in centrifuged water samples as compared to the water samples collected during flume washing *(17, 31)*. This further suggests that centrifugations may help to increase removal efficiency for bacterial pathogens or chemical contaminants. Thus, it may be possible that repeated washing and centrifugation several times might help remove more Ag from contaminated produce during processing.

Here, we studied the efficiency of typical commercial produce processing conditions on Ag removal from contaminated lettuce using different sanitizers currently used in the produce

industry. Large amounts of Ag retained in the leaves after washing treatments. This may be due to oxidation of Ag NPs to form Ag^+ and formation of Ag-thiol complexes or AgCl as well as internalization of Ag NPs into leaf tissue that protects Ag NPs from sanitizers. Therefore, relatively low Ag removal was observed in the in the batch experiment (3–7%) and in the pilot plant processing experiment (0.3-3% Ag). However, higher Ag concentrations were found in the centrifuged wash water (32-52 μg/L) as compared flume water at 90 s (0.6-1.5 μg/L), possibly due to release of some Ag-containing intracellular liquid from leaf tissue. Thus, it can be concluded that flume washing alone with or without sanitizers is inadequate in removing Ag NPs from contaminated romaine lettuce. Ag retained in produce from application of Ag NPs-containing nanopesticides to crops may possibly be transferred to humans. Repeated flume washing and centrifugation might help remove Ag NPs to a greater extent. Nonetheless, the low removal efficiency highlights the necessity to develop an efficient commercial washing methods for Ag NP removal from contaminated produce. Through optimizing produce processing conditions and washing treatments (types and concentrations of sanitizers approved by EPA and FDA), the efficiency of Ag removal may be improved. Future research is also needed to focus on evaluating the distribution of surface-attached and internalized Ag NPs in produce and what conditions could help remove both surface-attached and internalized Ag NP to a greater extent. It is also important to study the effect of different produce types on Ag removal as the morphology of leaves (veins, ridges, and wax present on produce) and chemical composition in produce can significantly affect the efficiency of the washing process.

3.5 CONCLUSION

In the batch experiments, Ag concentration significantly increased during the 5 min of washing for all treatments except peroxyacetic acid containing an organic load. Lower Ag removal (3-7%) was found overall when sanitizers with or without organic load were used to wash contaminated lettuce. This is because of the internalization of Ag NP into leaves, and washing treatments being not able to penetrate inside leaves to remove the Ag NPs. The order of the treatments for Ag concentrations detected in wash water from highest to lowest was 80 mg/L peroxyacetic acid, control (sanitizer-free ultrapure water), 100 mg/L chlorine, 2.5% organic load (OL) with 100 mg/L chlorine, and 2.5% OL. Ag removal by the organic load containing peroxyacetic acid was 0 because of the removal of Ag with organic particles during sample preparation and measurements excluded by ICP-MS. Out of all the treatments, 80 mg/L peroxyacetic acid showed the highest decontamination capacity of 7%. The strong oxidation potential of peroxyacetic acid increased the dissolution of Ag and the release of more Ag^{+} ions to the wash water. Washing with an organic load alone, organic load with chlorine, chlorine, control and peroxyacetic acid showed 2.7%, 3%, 3.5%, 4.7% and 6.6% Ag removal, respectively.

In the pilot-scale produce processing line experiments, Ag removal was significant when chlorine sanitizer was used to wash contaminated lettuce as compared to the control (tap water). However, Ag removal was very minimal. The small difference is due to dissolution of Ag NPs due to the higher oxidative potential of chlorine. Low Ag removal by both treatments (0.3-3%) occurred because of internalization of Ag NPs in leaves. This suggests that flume washing may be insufficient to remove Ag NPs effectively from contaminated produce, which further confirmed the findings of the batch experiment. However, releasing significantly higher Ag concentrations (32 to 52 µg/L) during centrifugation as compared to flume washing (0.6 to 1.5 µg/L) predicts that

repeated washing and centrifugation might increase the removal of Ag NPs from contaminated produce. This emphasizes developing efficient produce processing conditions to remove Ag NPs from fresh-cut produce.

CHAPTER 4:

CONCLUSIONS AND RECOMMENDATIONS

4.1 CONCLUSIONS

The overall goal of this research was to evaluate the effectiveness of produce washing practices on the removal of Ag NPs from contaminated fresh produce. Improved knowledge gained from this study will contribute to finding new control strategies for mitigating the risk of Ag NPs in produce.

The results from objective 1 showed that the AgCl-Ag NP aggregates form in the presence of chlorine with AgCl and Ag NP embedding in the DLE matrix. Therefore, both chlorine and lettuce organic matter significantly impact the behavior of Ag NPs. The results from objective 2 showed that 3-7% Ag was removed from the lettuce when chlorine and peroxyacetic acid sanitizers were used as sanitizers with or without an organic load during simulated batch type lettuce processing. In contrast, only 0.3% - 3% Ag was removed from the lettuce when water was used with or without chlorine sanitizer during small-scale pilot processing. Further, centrifugal drying after washing helped to release Ag from the lettuce. Overall, findings from these studies suggest that the current commercial produce processing conditions may not be effective in removing Ag NP from contaminated produce. This highlights the need to develop more efficient produce processing strategies for the removal of Ag NP from fresh produce.

4.2 RECOMMENDATIONS FOR FUTURE WORK

When the behavior of Ag NPs was evaluated in objective 1, we found that Ag may exist as AgCl or AgCl-AgNP aggregates in wash water and/or become embedded in plant organic matter. Since the fate of Ag NPs is greatly affected by both chlorine and the organic matter in lettuce wash water, future studies should evaluate the mobility and toxicity of the different forms of Ag during commercial washing of produce.

The efficacy of peroxyacetic acid sanitizer on Ag NP removal was evaluated using a cost-effective carboy system, but not in the pilot-scale produce processing line. Therefore, it is also important to evaluate the effectiveness of peroxyacetic acid sanitizer (with and without organic load) in removing Ag NPs from contaminated lettuce under pilot-scale produce processing conditions in future studies.

The effect of the chlorine sanitizer on Ag NP was evaluated in Chapter 3 of the dissertation. In commercial produce processing, water is recirculated, and a significant amount of organic load is released to the wash water during water recirculation. Therefore, it is also important to evaluate the impact of organic load on Ag NP removal using the produce processing line with and without sanitizers.

Commercial produce processing involves washing of produce in the flume tank followed by centrifugation. The washing period usually lasts 90 s. Therefore, chapter 3 in the dissertation followed this procedure using the pilot-scale produce processing line at MSU to represent commercial produce processing. The studies presented in chapter 3 showed that centrifugation removed a significantly higher amount of Ag as compared to 90 s of washing. Therefore, it can be predicted that repeated washing and centrifugation might contribute to higher Ag removal from contaminated lettuce. Therefore, an important area for future research is how efficiently Ag is

removed with repeated washing and centrifugation several times (2 or 3 times) during produce processing.

Relatively low Ag removal was observed in the batch experiment indicating that flume washing alone with or without sanitizers is inadequate in removing Ag NPs from contaminated romaine lettuce. Thus, it is important to develop an efficient commercial washing method for removal of Ag NPs from contaminated produce. The produce processing conditions need to be optimized and different washing treatments (types and concentrations of sanitizers approved by EPA and FDA) need to be tested to evaluate efficient removal of Ag NPs. Further, it is equally important to focus on evaluating the distribution of surface-attached and internalized Ag NPs in produce and investigating the produce processing conditions which would help to better remove both surface-attached and internalized Ag NPs.

Finally, it is important to evaluate different produce types (e.g., lettuce, spinach, tomatoes etc.) since produce surface, morphology (veins, ridges, and wax present on produce) and chemical composition can impact the efficiency of Ag removal during washing.

APPENDICES

APPENDIX A: Supplementary Materials to CHAPTER II

Table A1 Hydrodynamic diameters of Ag NPs at different time points for control treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	59.0	57.7	60.3	59.8	61.6	62.4
D0 2hrs	60.3	62.4	59.4	58.8	60.1	59.4
D0 6hrs	59.5	60.9	58.8	61.7	59.8	62.7
D0 12hrs	58.8	57.2	58.0	57.4	58.4	59.2
D1	57.6	58.5	58.6	57.0	57.9	62.7
D4	59.6	60.3	58.6	58.6	58.5	58.7
D7	58.1	60.5	59.2	58.5	58.9	59.8
D10	58.1	57.4	58.6	58.5	59.7	56.8

Table A2 Hydrodynamic diameters of Ag NPs at different time points for 2 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	302.3	308.2	379.7	375.2	379.0	377.7
D0 2hrs	410.6	429.4	424.9	407.7	461.9	452.0
D0 6hrs	439.3	409.8	412.5	398.6	438.9	403.8
D0 12hrs	412.5	386.8	424.5	390.5	417.4	409.9
D1	415.8	410.3	418.0	419.6	424.3	407.6
D4	425.4	427.1	442.2	374.4	436.0	429.2
D7	452.4	459.3	439.5	386.1	420.1	411.4
D10	428.7	422.1	356.0	428.5	417.2	392.5

Table A3 Hydrodynamic diameters of Ag NPs at different time points for 50 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	60.1	61.2	63.0	66.1	63.9	68.7
D0 2hrs	66.6	70.5	70.1	74.2	71.2	75.4
D0 6hrs	71.7	74.5	74.3	76.9	76.5	78.4
D0 12hrs	97.8	101.5	99.9	103.0	102.8	105.4

Table A3 (Cont'd)

D1	115.6	120.1	116.5	126.3	116.7	124.0
D4	331.5	327.7	326.1	348.9	329.5	354.3
D7	356.5	386.1	343.3	368.7	366.7	389.9
D10	364.8	375.3	375.7	375.5	364.4	380.3

Table A4 Hydrodynamic diameters of Ag NPs at different time points for 100 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	49.6	49.6	46.6	50.4	46.7	51.7
D0 2hrs	51.0	54.6	54.7	58.4	58.7	61.0
D0 6hrs	85.3	91.2	90.0	91.2	94.3	94.0
D0 12hrs	98.3	103.2	97.0	102.2	103.0	103.9
D1	157.2	164.7	155.5	162.5	165.2	161.4
D4	352.3	370.3	350.0	369.2	352.1	366.3
D7	375.2	378.9	350.5	385.2	350.7	387.7
D10	365.5	376.2	363.4	390.3	365.0	381.1

Table A5 Hydrodynamic diameters of Ag NPs at different time points for 0.1% DLE treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	84.2	86.2	84.2	85.7	83.2	81.0
D0 2hrs	84.5	85.4	81.3	84.7	81.0	86.4
D0 6hrs	85.9	89.3	81.4	81.7	85.8	82.3
D0 12hrs	89.3	80.7	115.3	114.6	115.9	123.9
D1	239.5	167.6	214.2	214.6	207.9	209.8
D4	324.6	274.8	243.6	224.0	312.2	260.9
D7	112.5	112.7	129.8	164.3	182.3	193.8
D10	199.6	155.4	102.8	99.4	235.2	277.5

Table A6 Hydrodynamic diameters of Ag NPs at different time points for 0.1% DLE with 50 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	91.2	90.3	89.3	89.1	86.3	87.4
D0 2hrs	125.7	124.5	87.9	92.4	86.5	87.7
D0 6hrs	93.6	93.9	92.7	91.8	93.9	92.8
D0 12hrs	108.7	103.4	101.2	111.0	103.2	99.9
D1	143.1	146.9	128.3	116.9	112.2	115.5
D4	169.7	149.1	269.9	288.4	296.0	229.6
D7	200.4	156.7	162.0	194.9	180.8	113.9
D10	113.5	151.0	118.0	135.7	106.2	125.4

Table A7 Hydrodynamic diameters of Ag NPs at different time points for 0.1% DLE only treatment (without Ag NPs).

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	142.1	143.2	131.8	135.0	131.3	134.7
D0 2hrs	130.5	134.8	130.5	132.3	132.7	134.8
D0 6hrs	130.7	139.0	130.8	139.3	136.6	138.7
D0 12hrs	146.9	147.9	143.6	141.9	133.5	135.0
D1	134.1	128.0	133.5	131.5	140.9	132.8
D4	122.8	136.0	741.5	385.8	382.8	498.5
D7	104.2	96.1	100.0	176.1	111.5	112.1
D10	90.2	88.4	103.6	279.2	98.3	195.6

Table A8 Hydrodynamic diameters of Ag NPs at different time points for 0.1% DLE with 50 mg/L chlorine only treatment (without Ag NPs).

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	140.0	142.1	140.2	142.6	141.1	141.7
D0 2hrs	140.9	142.5	139.3	142.0	142.6	142.8
D0 6hrs	192.6	183.9	204.5	186.7	165.5	185.3
D0 12hrs	192.4	178.6	179.6	172.1	223.2	193.7
D1	714.8	888.8	592.5	730.9	1037.0	1215.0

Table A8 (Cont'd)

D4	1529.0	1089.0	1846.0	1284.0	1120.0	1364.0
D7	1934.0	1309.0	1824.0	2046.0	1317.0	1239.0
D10	2527.0	3041.0	2083.0	1719.0	959.3	963.8

Table A9 Zeta potentials of Ag NPs at different time points for control treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-6.2	-7.2	-11.7	-15.3	-12.0	-9.2
D0 2hrs	-9.6	-9.4	-13.1	-17.8	-13.8	-16.2
D0 6hrs	-16.1	-17.3	-17.6	-16.7	-16.6	-18.7
D0 12hrs	-20.0	-17.1	-16.9	-17.0	-23.5	-21.6
D1	-15.3	-19.3	-19	-17.2	-16.3	-17.5
D4	-14.3	-11.4	-17.9	-18.9	-13.7	-18.0
D7	-20.4	-22.3	-16.9	-12.7	-18.7	-16.3
D10	-19.3	-18.3	-14.1	-13.7	-18.6	-20.1

Table A10 Zeta potentials of Ag NPs at different time points for 2 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-41.2	-40.5	-45.5	-46.5	-44.4	-42.0
D0 2hrs	-45.1	-45.5	-45.9	-44.9	-48.2	-48.5
D0 6hrs	-45.3	-42.5	-48.3	-47.2	-47.4	-47.1
D0 12hrs	-46.5	-46.7	-49.4	-48.3	-51.4	-48.9
D1	-46.7	-47.0	-49.0	-48.4	-47.9	-47.7
D4	-49.6	-49.7	-32.0	-35.0	-42.0	-43.0
D7	-45.0	-43.2	-41.5	-40.7	-48.4	-46.2
D10	-45.1	-42.6	-48.9	-49.0	-50.4	-49.8

Table A11 Zeta potentials of Ag NPs at different time points for 50 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-48.4	-46.3	-52.7	-51.1	-49.7	-51.0
D0 2hrs	-52.5	-53.9	-52.3	-55.6	-56.9	-56.3

Table A11 (Cont'd)

D0 6hrs	-58.2	-54.9	-60.1	-63.9	-62.1	-60.5
D0 12hrs	-72.3	-75.0	-71.6	-70.0	-64.8	-68.4
D1	-84.1	-80.6	-78.4	-77.5	-79.6	-79.2
D4	-96.0	-94.1	-91.5	-91.6	-96.0	-94.1
D7	-96.9	-98.5	-92.8	-91.6	-93.2	-92.7
D10	-96.2	-97.4	-92.9	-92.9	-96.4	-93.6

Table A12 Zeta potentials of Ag NPs at different time points for 100 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-35.0	-36.3	-40.2	-41.6	-40.2	-40.5
D0 2hrs	-41.9	-42.2	-47.0	-47.6	-44.6	-45.7
D0 6hrs	-61.3	-56.9	-66.4	-67.2	-66.7	-66.5
D0 12hrs	-71.5	-74.5	-72.5	-75.1	-72.5	-71.5
D1	-79.2	-80.1	-80.0	-79.3	-80.6	-80.1
D4	-95.5	-95.4	-93.6	-92.4	-91.7	-91.4
D7	-90.7	-92.3	-91.8	-94.8	-94.3	-94.5
D10	-92.6	-93.2	-94.6	-93.6	-93.1	-94.3

Table A13 Zeta potentials of Ag NPs at different time points for 0.1% DLE treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-25.5	-28.4	-25.5	-27.3	-32.3	-27.0
D0 2hrs	-29.8	-29.6	-26.2	-27.7	-28.7	-30.6
D0 6hrs	-29.6	-26.9	-29.8	-28.2	-28.4	-27.0
D0 12hrs	-26.9	-27.5	-29.6	-30.4	-33.1	-32.9
D1	-29.7	-29.8	-32.3	-28.6	-31.2	-31.2
D4	-30.8	-30.2	-30.5	-28.5	-28.2	-27.0
D7	-28.6	-30.3	-26.6	-26.7	-31.4	-28.5
D10	-27.8	-26.5	-25.9	-27.9	-28.6	-26.0

Table A14 Zeta potentials of Ag NPs at different time points for 0.1% DLE with 50 mg/L chlorine treatment.

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-27.6	-26.0	-26.8	-28.0	-30.3	-30.2
D0 2hrs	-29.8	-31.8	-30.9	-30.5	-33.4	-30.9
D0 6hrs	-30.1	-31.3	-28.5	-31.5	-34.6	-34.2
D0 12hrs	-33.0	-34.0	-30.1	-34.9	-31.0	-32.2
D1	-31.7	-31.8	-31.0	-32.8	-31.6	-30.7
D4	-30.0	-31.1	-32.1	-31.0	-30.7	-33.2
D7	-27.7	-29.1	-28.0	-30.4	-29.6	-30.0
D10	-31.7	-34.9	-30.0	-30.0	-31.1	-33.4

Table A15 Zeta potentials of Ag NPs at different time points for 0.1% DLE only treatment (without Ag NPs).

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-32.9	-29.5	-31.7	-25.3	-33.4	-31.6
D0 2hrs	-29.9	-30.6	-27.4	-28.1	-31.2	-33.9
D0 6hrs	-37.4	-36.0	-32.7	-31.3	-31.8	-30.7
D0 12hrs	-30.2	-28.5	-29.1	-29.8	-31.1	-34.8
D1	-33.7	-31.1	-26.4	-28.7	-28.4	-28.7
D4	-30.7	-29.9	-29.7	-32.2	-30.2	-27.3
D7	-31.1	-30.9	-31.7	-30.0	-33.6	-36.5
D10	-27.0	-25.1	-25.3	-24.4	-25.2	-20.5

Table A16 Zeta potentials of Ag NPs at different time points for 0.1% DLE with 50 mg/L chlorine only treatment (without Ag NPs).

Time	R1		R2		R3	
	A	B	A	B	A	B
D0 0hrs	-23.7	-24.3	-27.1	-27.8	-30.1	-31.3
D0 2hrs	-31.7	-33.6	-31.9	-33.1	-32.6	-31.1
D0 6hrs	-28.7	-30.0	-31.1	-30.5	-30.9	-32.2
D0 12hrs	-30.5	-31.4	-27.0	-25.7	-29.5	-30.8
D1	-29.0	-30.6	-30.3	-30.8	-29.8	-30.8

Table A16 (Cont'd)

D4	-26.4	-27.9	-28.8	-29.0	-29.2	-29.9
D7	-26.9	-27.7	-28.7	-29.2	-25.6	-26.5
D10	-27.2	-28.2	-26.5	-25.7	-27.7	-27.6

Table A17 Dissolved Ag concentrations at different time points for control treatment.

Time	R1	R2
D0 0hrs	0.5	0.6
D0 2hrs	0.6	0.5
D0 6hrs	0.8	0.6
D0 12hrs	0.8	0.8
D1	0.6	0.7
D4	0.6	0.7
D7	0.7	0.6
D10	0.5	0.7

Table A18 Dissolved Ag concentrations at different time points for 2 mg/L chlorine treatment.

Time	R1	R2
D0 0hrs	0.1	0.1
D0 2hrs	0.1	0.1
D0 6hrs	0.1	0.1
D0 12hrs	0.1	0.1
D1	0.1	0.1
D4	0.1	0.1
D7	0.1	0.2
D10	0.1	0.1

Table A19 Dissolved Ag concentrations at different time points for 50 mg/L chlorine treatment.

Time	R1	R2
D0 0hrs	0.0	0.0
D0 2hrs	0.0	0.0
D0 6hrs	0.0	0.0
D0 12hrs	0.0	0.0
D1	0.0	0.0
D4	0.0	0.0
D7	0.0	0.0

Table A20 Dissolved Ag concentrations at different time points for 100 mg/L chlorine treatment.

Time	R1	R2
D0 0hrs	0.0	0.0
D0 2hrs	0.0	0.0
D0 6hrs	0.0	0.0
D0 12hrs	0.0	0.0
D1	0.0	0.0
D4	0.0	0.0
D7	0.0	0.0
D10	0.0	0.0

Table A21 Dissolved Ag concentrations at different time points for 0.1% DLE treatment.

Time	R1	R2
D0 0hrs	0.0	0.0
D0 2hrs	0.0	0.0
D0 6hrs	0.0	0.0
D0 12hrs	0.0	0.1
D1	0.0	0.0
D4	0.0	0.0
D7	0.0	0.0
D10	0.1	0.1

Table A22 Dissolved Ag concentrations at different time points for 0.1% DLE with 50 mg/L chlorine treatment.

Time	R1	R2
D0 0hrs	0.0	0.0
D0 2hrs	0.0	0.0
D0 6hrs	0.0	0.0
D0 12hrs	0.0	0.0
D1	0.0	0.0
D4	0.0	0.0
D7	0.0	0.0
D10	0.1	0.1

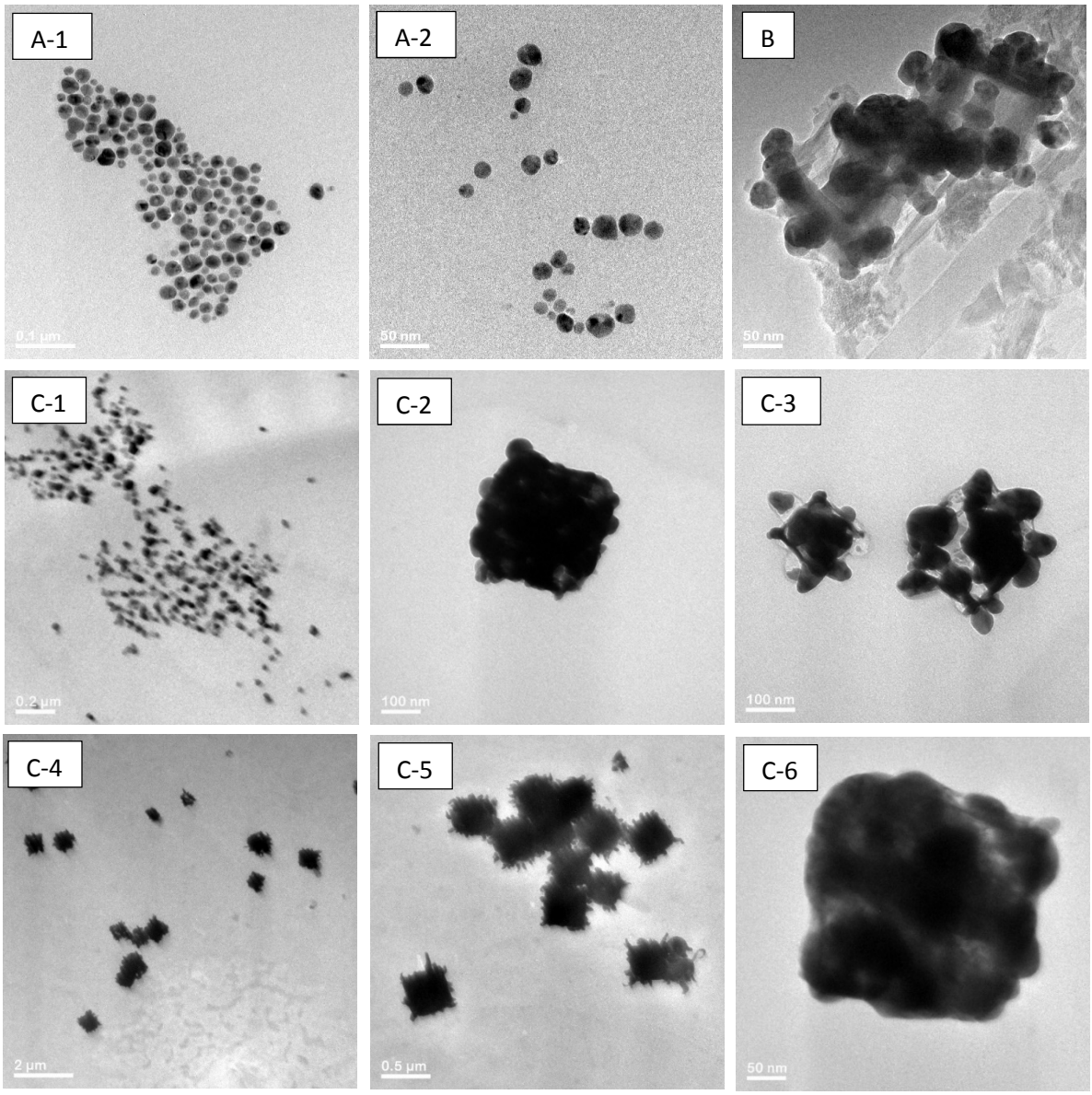
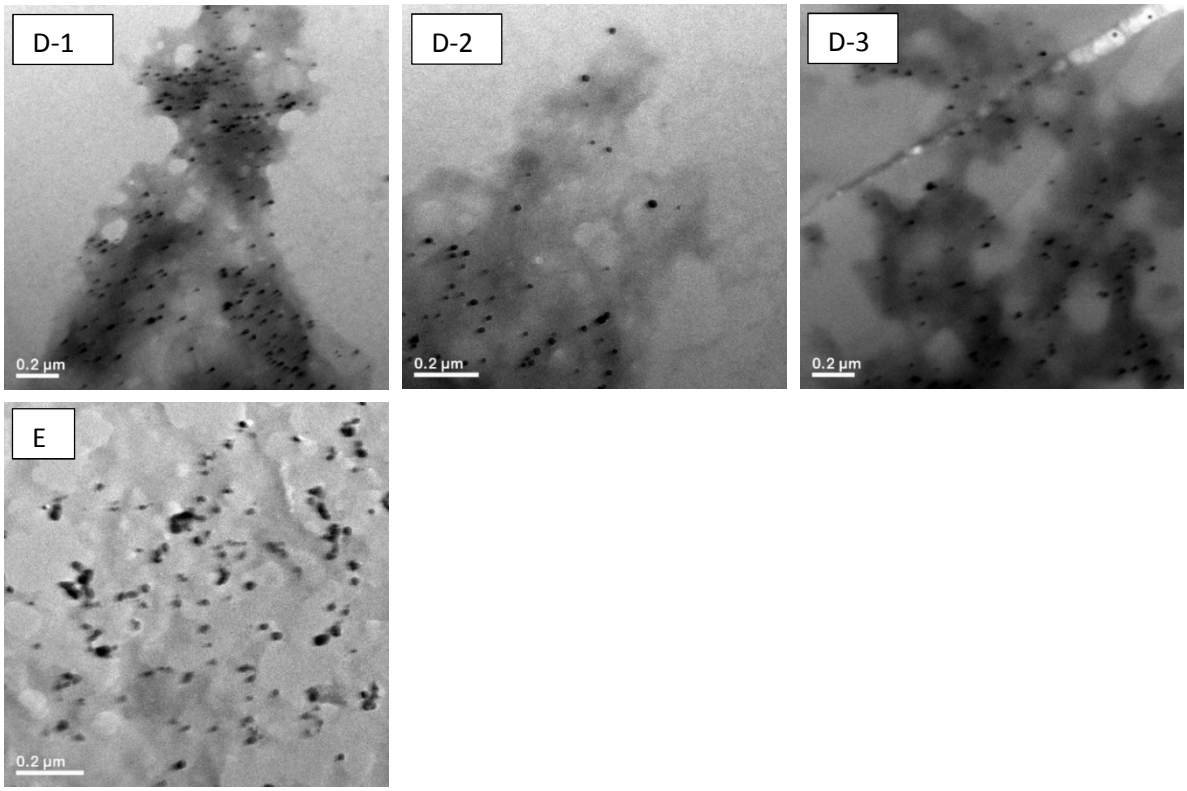


Figure A1 TEM images of the Ag NPs in different treatments at different time points. A-1 and A-2: Control at Day 10; B: 2mg/L chlorine at Day 10; C-1: 100mg/L chlorine at Day 0; C-2 and C-3: 100mg/L chlorine at Day 7; C-4, C-5 and C-6: 100mg/L chlorine at Day 10; D-1 and D-2: 0.1% DLE at Day 7; D-3: 0.1% DLE at Day 10; E: 0.1% DLE + 50mg/L chlorine at Day 10.

Figure A1 (Cont'd)



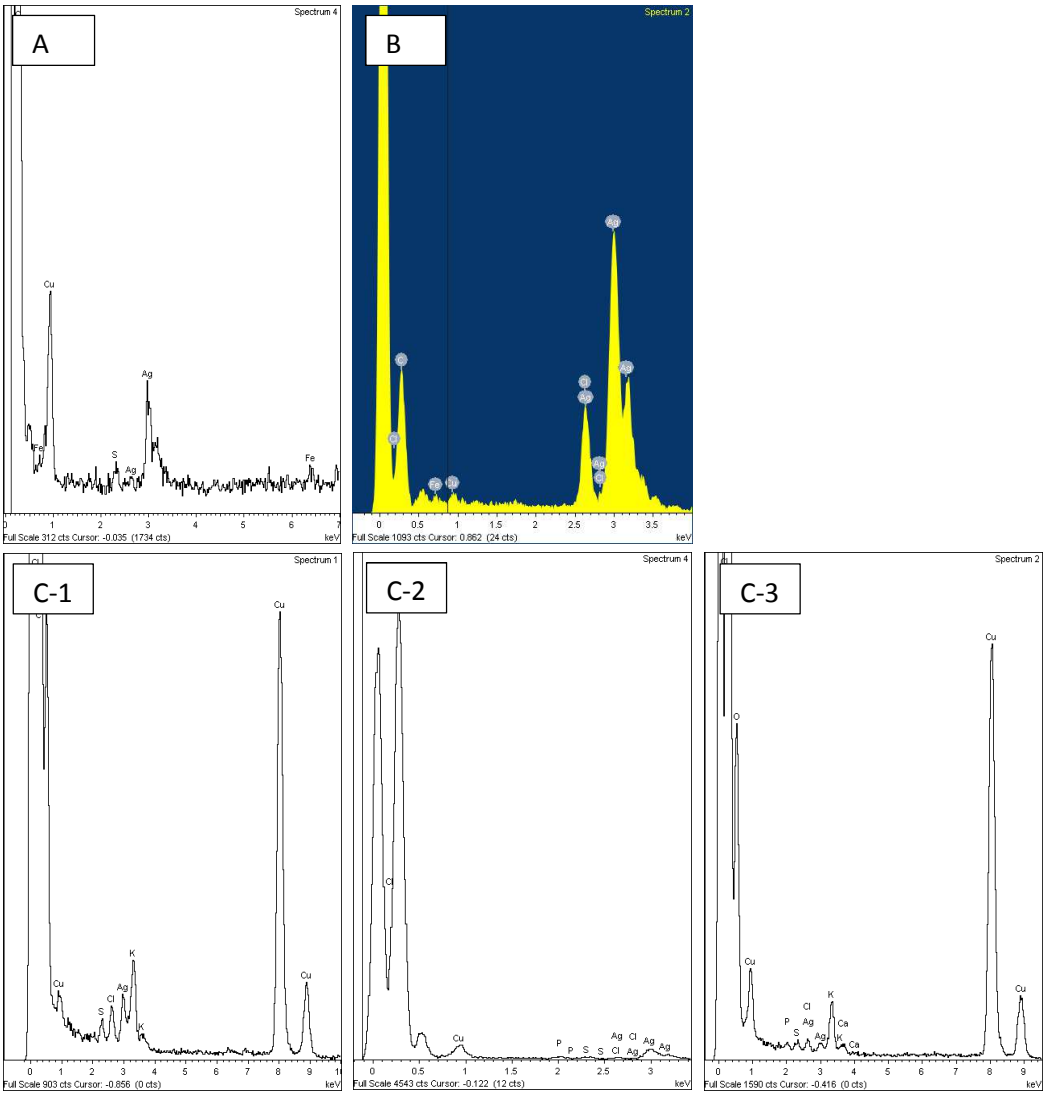
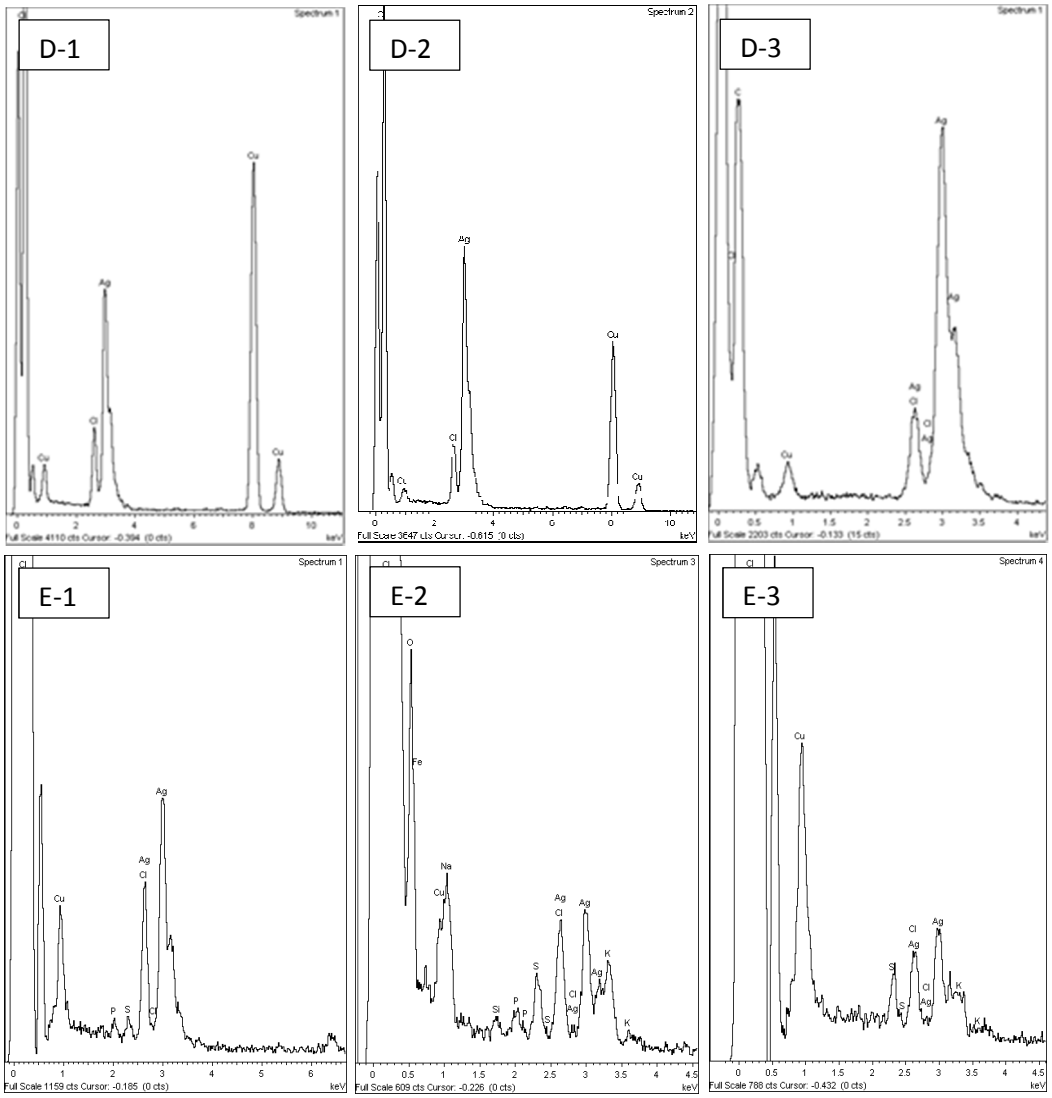


Figure A2 EDS spectra of the Ag NPs in different treatments at different time points. A: Control at Day 10; B: 2mg/L chlorine at Day 10; C-1: 100mg/L chlorine at Day 0; C-2: 100mg/L chlorine at Day 7; C-3: 100mg/L chlorine at Day 10; D-1: 0.1% DLE at Day 0; D-2: 0.1% DLE at Day 7; D-3: 0.1% DLE at Day 10; E-1: 0.1% DLE + 50mg/L chlorine at Day 0; E-2: 0.1% DLE + 50mg/L chlorine at Day 7; E-3: 0.1% DLE + 50mg/L chlorine at Day 10.

Figure A2 (Cont'd)



APPENDIX B: Supplementary Materials to CHAPTER III

Table B1 Ag concentrations in Ag NP suspension before and after contamination of leaves in the batch experiments.

Sample	Ag concentration	
	Rep 1	Rep 2
0 hr	44.6	43.3
1 hr	33.4	34.5

Table B2 Ag concentrations in wash water when 100 mg/L chlorine, 80 mg/L peroxyacetic acid (PAA), DI water (control), 2.5% organic load (OL), OL with 100 mg/L chlorine and OL with 80 mg/L PAA were used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Sample	Ag concentration (ppb=µg/L)		
Description	Rep 1	Rep 2	Rep 3
Chlorine 30	12.5	14.0	11.6
Chlorine 60	18.7	18.8	14.5
Chlorine 90	21.8	18.5	15.3
Chlorine 120	21.1	21.7	15.6
Chlorine 150	28.5	30.8	21.8
Chlorine 180	31.6	29.4	20.5
Chlorine 210	31.5	31.7	21.2
Chlorine 240	31.5	32.1	27.6
Chlorine 270	32.3	32.6	26.2
Chlorine 300	32.5	33.0	29.0
PAA 30	17.9	24.2	17.9
PAA 60	26.2	33.0	24.7
PAA 90	32.3	40.4	28.6
PAA 120	36.7	45.9	32.4
PAA 150	41.2	51.7	36.6
PAA 180	45.0	54.9	40.4
PAA 210	48.4	58.6	43.7
PAA 240	52.3	61.4	46.3
PAA 270	55.0	65.1	49.6
PAA 300	59.0	67.3	50.9

Table B2 (Cont'd)

Control 30	15.3	17.3	12.7
Control 60	21.4	20.3	18.9
Control 90	26.1	22.9	24.1
Control 120	29.9	26.1	27.1
Control 150	33.9	28.3	29.7
Control 180	34.3	32.5	33.2
Control 210	39.0	33.2	38.0
Control 240	41.7	35.7	37.9
Control 270	43.1	37.6	39.8
Control 300	46.6	37.6	40.8
OL+Chlorine 30	8.1	5.2	10.1
OL+Chlorine 60	11.6	9.4	13.2
OL+Chlorine 90	14.3	11.9	16.6
OL+Chlorine 120	16.5	14.4	17.0
OL+Chlorine 150	18.5	16.2	22.2
OL+Chlorine 180	20.6	17.8	24.8
OL+Chlorine 210	22.6	19.2	26.0
OL+Chlorine 240	25.2	20.5	28.3
OL+Chlorine 270	26.4	20.9	30.0
OL+Chlorine 300	28.6	22.4	30.3
OL+PAA 30	0.1	0.0	0.0
OL+PAA 60	0.0	0.0	0.0
OL+PAA 90	0.1	0.0	0.1
OL+PAA 120	0.1	0.1	0.1
OL+PAA 150	0.1	0.1	0.1
OL+PAA 180	0.1	0.0	0.1
OL+PAA 210	0.1	0.1	0.1
OL+PAA 240	0.1	0.1	0.1
OL+PAA 270	0.1	0.1	0.1
OL+PAA 300	0.1	0.0	0.1
OL 30	5.3	10.5	8.8
OL 60	5.9	14.6	11.7
OL 90	6.6	18.8	15.5
OL 120	7.3	21.4	18.5
OL 150	9.7	20.1	22.1
OL 180	11.9	24.7	20.7
OL 210	15.2	22.8	23.3
OL 240	15.6	17.7	25.5
OL 270	20.7	25.2	27.0
OL 300	23.3	24.8	25.0

Table B3 Calculated Ag removal % (based on wash water sample results) when 100 mg/L chlorine was used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 4 L (Y) µg	Ag removal % (Y/3560 µg)x 100
Chlorine 30 Rep 1	12.5	50.1	1.4
Chlorine 30 Rep 2	14.0	55.9	1.6
Chlorine 30 Rep 3	11.6	46.2	1.3
Chlorine 60 Rep 1	18.7	74.7	2.1
Chlorine 60 Rep 2	18.8	75.2	2.1
Chlorine 60 Rep 3	14.5	58.0	1.6
Chlorine 90 Rep 1	21.8	87.3	2.5
Chlorine 90 Rep 2	18.5	73.9	2.1
Chlorine 90 Rep 3	15.3	61.3	1.7
Chlorine 120 Rep 1	21.1	84.5	2.4
Chlorine 120 Rep 2	21.7	87.0	2.4
Chlorine 120 Rep 3	15.6	62.4	1.8
Chlorine 150 Rep 1	28.5	113.8	3.2
Chlorine 150 Rep 2	30.8	123.2	3.5
Chlorine 150 Rep 3	21.8	87.2	2.5
Chlorine 180 Rep 1	31.6	126.3	3.6
Chlorine 180 Rep 2	29.4	117.4	3.3
Chlorine 180 Rep 3	20.5	82.1	2.3
Chlorine 210 Rep 1	31.5	126.0	3.5
Chlorine 210 Rep 2	31.7	127.0	3.6
Chlorine 210 Rep 3	21.2	84.8	2.4
Chlorine 240 Rep 1	31.5	125.9	3.5
Chlorine 240 Rep 2	32.1	128.3	3.6
Chlorine 240 Rep 3	27.6	110.3	3.1
Chlorine 270 Rep 1	32.3	129.2	3.6
Chlorine 270 Rep 2	32.6	130.5	3.7
Chlorine 270 Rep 3	26.2	104.9	3.0
Chlorine 300 Rep 1	32.5	129.9	3.7

Table B3 (Cont'd)

Chlorine 300 Rep 2	33.0	132.0	3.7
Chlorine 300 Rep 3	29.0	115.8	3.3

Table B4 Calculated Ag removal % (based on wash water sample results) when 80 mg/L peroxyacetic acid (PAA) was used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 4 L (Y) µg	Ag removal % (Y/3560 µg)x 100
PAA 30 Rep 1	17.9	71.6	2.0
PAA 30 Rep 2	24.2	96.9	2.7
PAA 30 Rep 3	17.9	71.7	2.0
PAA 60 Rep 1	26.2	104.7	2.9
PAA 60 Rep 2	33.0	132.0	3.7
PAA 60 Rep 3	24.7	98.6	2.8
PAA 90 Rep 1	32.3	129.1	3.6
PAA 90 Rep 2	40.4	161.6	4.5
PAA 90 Rep 3	28.6	114.3	3.2
PAA 120 Rep 1	36.7	146.7	4.1
PAA 120 Rep 2	45.9	183.5	5.2
PAA 120 Rep 3	32.4	129.5	3.6
PAA 150 Rep 1	41.2	164.7	4.6
PAA 150 Rep 2	51.7	207.0	5.8
PAA 150 Rep 3	36.6	146.2	4.1
PAA 180 Rep 1	45.0	179.9	5.1
PAA 180 Rep 2	54.9	219.7	6.2
PAA 180 Rep 3	40.4	161.6	4.5
PAA 210 Rep 1	48.4	193.6	5.4
PAA 210 Rep 2	58.6	234.4	6.6
PAA 210 Rep 3	43.7	174.7	4.9
PAA 240 Rep 1	52.3	209.2	5.9
PAA 240 Rep 2	61.4	245.4	6.9
PAA 240 Rep 3	46.3	185.3	5.2

Table B4 (Cont'd)

PAA 270 Rep 1	55.0	220.1	6.2
PAA 270 Rep 2	65.1	260.3	7.3
PAA 270 Rep 3	49.6	198.2	5.6
PAA 300 Rep 1	59.0	235.8	6.6
PAA 300 Rep 2	67.3	269.1	7.6
PAA 300 Rep 3	51.0	203.6	5.7

Table B5 Calculated Ag removal % (based on wash water sample results) when DI water (control) was used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 4 L (Y) µg	Ag removal % (Y/3560 µg)x 100
Control 30 Rep 1	15.3	61.2	1.7
Control 30 Rep 2	17.3	69.3	2.0
Control 30 Rep 3	12.7	50.6	1.4
Control 60 Rep 1	21.4	85.6	2.4
Control 60 Rep 2	20.3	81.2	2.3
Control 60 Rep 3	18.9	75.6	2.1
Control 90 Rep 1	26.1	104.3	2.9
Control 90 Rep 2	22.9	91.7	2.6
Control 90 Rep 3	24.1	96.3	2.7
Control 120 Rep 1	29.9	119.4	3.4
Control 120 Rep 2	26.1	104.5	2.9
Control 120 Rep 3	27.1	108.5	3.1
Control 150 Rep 1	33.9	135.6	3.8
Control 150 Rep 2	28.3	113.2	3.2
Control 150 Rep 3	29.7	118.6	3.3
Control 180 Rep 1	34.3	137.2	3.9
Control 180 Rep 2	32.5	130.1	3.7
Control 180 Rep 3	33.2	132.9	3.7
Control 210 Rep 1	39.0	156.1	4.4

Table B5 (Cont'd)

Control 210 Rep 2	33.2	132.9	3.7
Control 210 Rep 3	38.0	152.2	4.3
Control 240 Rep 1	41.7	166.9	4.7
Control 240 Rep 2	35.7	142.9	4.0
Control 240 Rep 3	37.9	151.6	4.3
Control 270 Rep 1	43.1	172.6	4.9
Control 270 Rep 2	37.6	150.5	4.2
Control 270 Rep 3	39.8	159.2	4.5
Control 300 Rep 1	46.6	186.3	5.2
Control 300 Rep 2	37.6	150.3	4.2
Control 300 Rep 3	40.8	163.1	4.6

Table B6 Calculated Ag removal % (based on wash water sample results) when OL with 100 mg/L chlorine was used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 4 L (Y) µg	Ag removal % (Y/3560 µg)x 100
OL+Cl 30 Rep 1	8.1	32.3	0.9
OL+Cl 30 Rep 2	5.2	20.9	0.6
OL+Cl 30 Rep 3	10.1	40.3	1.1
OL+Cl 60 Rep 1	11.6	46.5	1.3
OL+Cl 60 Rep 2	9.4	37.8	1.1
OL+Cl 60 Rep 3	13.2	52.9	1.5
OL+Cl 90 Rep 1	14.3	57.1	1.6
OL+Cl 90 Rep 2	11.9	47.5	1.3
OL+Cl 90 Rep 3	16.6	66.3	1.9
OL+Cl 120 Rep 1	16.5	65.8	1.9
OL+Cl 120 Rep 2	14.4	57.4	1.6
OL+Cl 120 Rep 3	17.0	68.0	1.9
OL+Cl 150 Rep 1	18.5	73.8	2.1
OL+Cl 150 Rep 2	16.2	64.9	1.8
OL+Cl 150 Rep 3	22.2	88.9	2.5

Table B6 (Cont'd)

OL+Cl 180 Rep 1	20.6	82.3	2.3
OL+Cl 180 Rep 2	17.8	71.0	2.1
OL+Cl 180 Rep 3	24.8	99.3	2.8
OL+Cl 210 Rep 1	22.6	90.3	2.5
OL+Cl 210 Rep 2	19.2	76.7	2.2
OL+Cl 210 Rep 3	26.0	104.0	2.92
OL+Cl 240 Rep 1	25.2	100.8	2.8
OL+Cl 240 Rep 2	20.5	82.0	2.3
OL+Cl 240 Rep 3	28.3	113.3	3.2
OL+Cl 270 Rep 1	26.4	105.7	3.0
OL+Cl 270 Rep 2	20.9	83.7	2.4
OL+Cl 270 Rep 3	30.0	119.9	3.4
OL+Cl 300 Rep 1	28.6	114.2	3.2
OL+Cl 300 Rep 2	22.4	89.4	2.5
OL+Cl 300 Rep 3	30.3	121.4	3.4

Table B7 Calculated Ag removal % (based on wash water sample results) when OL with 80 mg/L PAA was used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 4 L (Y) µg	Ag removal % (Y/3560 µg)x 100
OL+PAA 30 Rep 1	0.1	0.3	0.0
OL+PAA 30 Rep 2	0.0	0.1	0.0
OL+PAA 30 Rep 3	0.0	0.1	0.0
OL+PAA 60 Rep 1	0.0	0.2	0.0
OL+PAA 60 Rep 2	0.0	0.1	0.0
OL+PAA 60 Rep 3	0.0	0.2	0.0
OL+PAA 90 Rep 1	0.1	0.3	0.0
OL+PAA 90 Rep 2	0.0	0.2	0.0
OL+PAA 90 Rep 3	0.1	0.3	0.0
OL+PAA 120 Rep 1	0.1	0.2	0.0
OL+PAA 120 Rep 2	0.1	0.2	0.0

Table B7 (Cont'd)

OL+PAA 120 Rep 3	0.1	0.2	0.0
OL+PAA 150 Rep 1	0.1	0.3	0.0
OL+PAA 150 Rep 2	0.1	0.3	0.0
OL+PAA 150 Rep 3	0.1	0.2	0.0
OL+PAA 180 Rep 1	0.1	0.2	0.0
OL+PAA 180 Rep 2	0.0	0.2	0.0
OL+PAA 180 Rep 3	0.1	0.3	0.0
OL+PAA 210 Rep 1	0.1	0.3	0.0
OL+PAA 210 Rep 2	0.1	0.3	0.0
OL+PAA 210 Rep 3	0.1	0.2	0.0
OL+PAA 240 Rep 1	0.1	0.4	0.0
OL+PAA 240 Rep 2	0.1	0.2	0.0
OL+PAA 240 Rep 3	0.1	0.3	0.0
OL+PAA 270 Rep 1	0.1	0.3	0.0
OL+PAA 270 Rep 2	0.1	0.2	0.0
OL+PAA 270 Rep 3	0.1	0.2	0.0
OL+PAA 300 Rep 1	0.1	0.3	0.0
OL+PAA 300 Rep 2	0.0	0.1	0.0
OL+PAA 300 Rep 3	0.1	0.2	0.0

Table B8 Calculated Ag removal % (based on wash water sample results) when 2.5% organic load (OL) was used as the wash water treatment (30 to 300 indicates different time points in seconds) in the batch experiments.

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 4 L (Y) µg	Ag removal % (Y/3560 µg)x 100
OL 30 Rep 1	5.3	21.3	0.6
OL 30 Rep 2	10.5	41.8	1.2
OL 30 Rep 3	8.8	35.2	1.0
OL 60 Rep 1	5.9	23.5	0.7
OL 60 Rep 2	14.6	58.6	1.7

Table B8 (Cont'd)

OL 60 Rep 3	11.7	46.7	1.3
OL 90 Rep 1	6.6	26.4	0.7
OL 90 Rep 2	18.8	75.3	2.1
OL 90 Rep 3	15.5	61.9	1.7
OL 120 Rep 1	7.3	29.2	0.8
OL 120 Rep 2	21.4	85.7	2.4
OL 120 Rep 3	18.5	74.0	2.1
OL 150 Rep 1	9.7	39.0	1.1
OL 150 Rep 2	20.2	80.6	2.3
OL 150 Rep 3	22.1	88.2	2.5
OL 180 Rep 1	11.9	47.6	1.3
OL 180 Rep 2	24.7	98.9	2.8
OL 180 Rep 3	20.7	82.7	2.3
OL 210 Rep 1	15.2	60.8	1.7
OL 210 Rep 2	22.8	91.1	2.6
OL 210 Rep 3	23.3	93.1	2.6
OL 240 Rep 1	15.6	62.4	1.8
OL 240 Rep 2	17.7	70.7	2.0
OL 240 Rep 3	25.5	101.9	2.9
OL 270 Rep 1	20.7	82.8	2.3
OL 270 Rep 2	25.2	100.9	2.8
OL 270 Rep 3	27.0	107.9	3.0
OL 300 Rep 1	23.3	93.2	2.6
OL 300 Rep 2	24.8	99.3	2.8
OL 300 Rep 3	25.0	100.1	2.8

Table B9 Ag concentrations in romaine lettuce leaf samples after wash water treatments in the batch experiments.

Treatment	Ag concentration (μg/g)		
	Rep 1	Rep 2	Rep 3
100 mg/L chlorine	51.9	134.2	238.4
80 mg/L peroxyacetic acid (PAA)	104.8	201.6	131.8
Control	98.6	43.2	174.9
2.5% organic load (OL)	43.5	111.5	86.9
2.5% OL + 100 mg/L chlorine	133.6	166.5	315.7
2.5%OL + 80 mg/L PAA	86.4	132.3	96.3

Table B10 Ag concentration in contaminated leaves before wash water treatments in the batch experiments.

Replicate No	Ag concentration (μg/g)
Rep 1	277.7
Rep 2	326.1
Rep 3	197.6
Rep 4	317.2
Rep 5	117.5
Rep 6	134.0

Table B11 Calculated Ag removal % (based on leaf sample results) for all water treatments in the batch experiments.

Sample ID	Ag removal %
Chlorine Rep 1	0.8
Chlorine Rep 2	0.4
Chlorine Rep 3	0.0
PAA Rep 1	0.5
PAA Rep 2	0.1

Table B11 (Cont'd)

PAA Rep 3	0.4
Control Rep 1	0.6
Control Rep 2	0.8
Control Rep 3	0.2
OL Rep 1	0.8
OL Rep 2	0.5
OL Rep 3	0.6
OL+Chlorine Rep 1	0.4
OL+Chlorine Rep 2	0.3
OL+Chlorine Rep 3	-0.4
OL+PAA Rep 1	0.6
OL+PAA Rep 2	0.4
OL+PAA Rep 3	0.6

Table B12 Ag concentrations in Ag NP suspension before and after contamination of leaves for leaf samples 1 in the pilot-scale processing experiments.

	Ag concentration (mg/L)		
Time (hrs)	Rep 1	Rep 2	Rep 3
0	84.2	86.2	80.5
1	81.8	84.4	82.5

Table B13 Ag concentrations in Ag NP suspension before and after contamination of leaves for leaf samples 2 in the pilot-scale processing experiments.

	Ag concentration (mg/L)		
Time (hrs)	Rep 1	Rep 2	Rep 3
0	81.8	84.4	82.5
1	76.6	79.8	79.6

Table B14 Ag concentrations in Ag NP suspension before and after contamination of leaves for leaf samples 3 in the pilot-scale processing experiments.

Time (hrs)	Ag concentration (mg/L)		
	Rep 1	Rep 2	Rep 3
0	76.6	79.8	79.6
1	71.1	74.8	78.2

Table B15 Ag concentrations in wash water when MSU tap water (control) was used as the wash water treatment in the pilot-scale processing experiments (0 s, 30 s, 60 s, 90 s and centrifuge indicate different stages in produce processing).

Treatment	Ag concentration (ppb=µg/L)		
	Rep 1	Rep 2	Rep 3
Control 0 s	BDL	0.0	0.1
Control 30 s	0.0	1.4	0.9
Control 60 s	0.1	0.3	0.3
Control 90 s	0.1	1.2	0.5
Control Centrifuge	19.8	53.6	21.6

Table B16 Ag concentrations in wash water when 100 mg/L chlorine was used as the wash water treatment in the pilot-scale processing experiments (0 s, 30 s, 60 s, 90 s and centrifuge indicate different stages in produce processing).

Treatment	Ag concentration (ppb=µg/L)		
	Rep 1	Rep 2	Rep 3
Chlorine 0	0.3	0.6	0.1
Chlorine 30	2.2	2.8	2.3
Chlorine 60	1.2	1.6	1.0
Chlorine 90	2.1	1.4	0.9
Chlorine Centrifuge	32.5	51.5	70.4

Table B17 Calculated Ag removal % (based on wash water sample results) when MSU tap water (control treatment) was used as the wash water treatment in the pilot-scale processing experiments (0, 30 s, 60 s and 90 s indicate different time points in produce processing in seconds).

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 600 L (Y) µg	Ag removal % (Y/44400 µg)x 100
Control Rep 1 0 s	BDL	0.0	0.0
Control Rep 2 0 s	0.0	15.8	0.0
Control Rep 3 0 s	0.1	50.5	0.1
Control Rep 1 30 s	0.0	16.0	0.0
Control Rep 2 30 s	1.4	832.9	1.9
Control Rep 3 30 s	0.9	552.8	1.2
Control Rep 1 60 s	0.1	45.1	0.1
Control Rep 2 60 s	0.3	194.1	0.4
Control Rep 3 60 s	0.3	204.7	0.5
Control Rep 1 90 s	0.1	77.3	0.2
Control Rep 2 90 s	1.2	696.9	1.6
Control Rep 3 90 s	0.5	292.5	0.7

Table B18 Calculated Ag removal % (based on wash water sample results) when 100 mg/L chlorine was used as the wash water treatment in the pilot-scale processing experiments (0, 30 s, 60 s and 90 s indicate different time points in produce processing in seconds).

Treatment	Ag concentration ng/mL = ppb = µg/L	Ag in 600 L (Y) µg	Ag removal % (Y/44400 µg) x 100
Chlorine Rep 1 0	0.3	183.4	0.4
Chlorine Rep 2 0	0.6	375.0	0.8
Chlorine Rep 3 0	0.1	80.0	0.2
Chlorine Rep 1 30 s	2.2	1334.5	3.0
Chlorine Rep 2 30 s	2.8	1668.8	3.8

Table B18 (Cont'd)

Chlorine Rep 3 30 s	2.3	1385.4	3.1
Chlorine Rep 1 60 s	1.2	726.4	1.6
Chlorine Rep 2 60 s	1.6	930.5	2.1
Chlorine Rep 3 60 s	1.0	576.5	1.3
Chlorine Rep 1 90 s	2.1	1274.1	2.9
Chlorine Rep 2 90 s	1.4	856.2	1.9
Chlorine Rep 3 90 s	0.9	563.2	1.3

Table B19 Ag concentrations in romaine lettuce leaf samples for control treatment during produce processing.

Sample Description	Ag concentration µg/g					
	Rep 1A	Rep 1B	Rep 2A	Rep 2B	Rep 3A	Rep 3B
Control 30	403.1	401.0	305.1	290.1	376.1	401.7
Control 60	302.6	380.4	374.8	354.9	385.9	414.0
Control 90	420.0	386.2	368.9	310.5	253.2	235.5
Control Centrifuge	461.5	481.1	483.4	389.5	254.9	343.2
Control un-contaminated	0.6	0.6	0.6	0.6	0.6	0.6

Table B20 Ag concentrations in romaine lettuce leaf samples for 100 mg/L chlorine treatment during produce processing.

Sample Description	Ag concentration µg/g					
	Rep 1A	Rep 1B	Rep 2A	Rep 2B	Rep 3A	Rep 3B
Chlorine 30	307.3	291.1	327.1	267.8	369.8	438.7
Chlorine 60	404.6	364.4	304.9	414.7	398.5	251.3
Chlorine 90	389.8	294.7	268.6	322.2	266.1	336.7
Chlorine Centrifuge	497.4	328.6	316.7	374.9	222.0	284.8
Chlorine Un-contaminated	0.8	0.6	0.6	0.5	0.4	0.5

Table B21 Ag concentration in contaminated leaves before wash water treatments in the pilot-scale processing experiments.

Replicate no	Ag concentration µg/g
Un-washed Rep 1	367.4
Un-washed Rep 2	423.1
Un-washed Rep 3	600.5
Un-washed Rep 4	519.6
Un-washed Rep 5	365.6
Un-washed Rep 6	575.4

Table B22 Calculated Ag removal % (based on leaf sample results) when used 100 mg/L chlorine and control as the wash water treatment in the pilot-scale processing experiments (0 s, 30 s, 60 s, 90 s and centrifuge indicate different stages in produce processing).

Sample Description	Ag Concentration=S (µg/g)	Ag removal (S_0-S)	Ag removal % (S_0-S)100/S_0
Control Centrifuge Rep 1	471.3	4.0	0.8
Control Centrifuge Rep 2	436.4	38.8	8.2
Control Centrifuge Rep 3	299.1	176.2	37.1
Control 90 Rep 1	403.1	72.2	15.2
Control 90 Rep 2	339.7	135.6	28.5
Control 90 Rep 3	244.4	230.9	48.6
Control 60 Rep 1	341.5	133.7	28.1
Control 60 Rep 2	364.8	110.4	23.2
Control 60 Rep 3	399.9	75.3	15.9
Control 30 Rep 1	402.1	73.2	15.4
Control 30 Rep 2	297.6	177.7	37.4
Control 30 Rep 3	388.9	86.3	18.2

Table B22 (Cont'd)

Chlorine Centrifuge Rep 1	413.0	62.26	13.1
Chlorine Centrifuge Rep 2	345.8	129.49	27.3
Chlorine Centrifuge Rep 3	253.4	221.86	46.7
Chlorine 90 Rep 1	342.3	133.00	28.0
Chlorine 90 Rep 2	295.4	179.86	37.8
Chlorine 90 Rep 3	301.4	173.90	36.6
Chlorine 60 Rep 1	384.5	90.76	19.1
Chlorine 60 Rep 2	359.8	115.48	24.3
Chlorine 60 Rep 3	324.9	150.36	31.6
Chlorine 30 Rep 1	299.2	176.09	37.1
Chlorine 30 Rep 2	297.4	177.81	37.4
Chlorine 30 Rep 3	404.3	71.00	14.9

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