FOODBORNE AND ANTIBIOTIC RESISTANT PATHOGENS PREVALENCE DURING FRESH PRODUCE PRODUCTION USING NON-TRADITIONAL WATER SOURCES: FATE, TRANSPORT, AND RISK ASSESSMENT STUDY

A Thesis

by

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ABSTRACT

A growing population and demand for food force agricultural leaders to look for alternative water sources. Wastewater reuse could be introduced into commercial operations, if regulations and measures are in place to ensure food safety. The objective of this project was to cultivate lettuce with wastewater to track the fate of *Escherichia coli* and AP205 during and after cultivation to assess their fate and transport. Quantitative microbial risk assessment (QMRA) was performed to estimate risk of illness to the public. Contamination levels in foliage, leachate, and soil were directly ($P < 0.05$) related to initial concentrations of microorganisms in the irrigation water. *E. coli* concentrations during post-harvest storage (14 days at 4 ^{o}C) of foliage increased significantly, while AP205 concentrations decreased more than 2 logs. From randomly selected *E. coli* colonies, in all four biomass types, 81% and 34% showed resistance to ampicillin and cephalothin, respectively. QMRA revealed significant health risks associated with lettuce consumption. *E. coli* concentrations were used as a fecal indicator bacteria to estimate levels of 6 common pathogens in wastewater and AP205 concentrations were used to estimate norovirus and rotavirus levels. Norovirus and *Giardia* largely contributed to the 0.8 probability of illness developing from infection, while norovirus and rotavirus showed a 0.24-0.43 probability of illness developing from infection, when using *E. coli* and AP205 concentrations, respectively. Results show that non-traditional water usage for fresh produce cultivation can pose risks to humans, if standards are not in place to control pathogen contamination levels.

DEDICATION

To my parents, Newell and Kristi, who have supported me in every way. To my Mimi and Grandad, Carol and Harvey Summerlin, without them this extended journey would not have been possible. Finally, to my Granddaddy and late Grandmother, Darvin and Marilyn Hooker, who have helped shape me into the man I am today.

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CHAPTER I

INTRODUCTION AND RATIONALE

The conservation of water is becoming increasingly important with the ever-growing worldwide population. This is especially true for already arid climates such as the Lower Rio Grande Valley (LRGV) that is known for cyclic droughts. The LRGV is located adjacent to the Rio Grande River, which separates Mexico and the United States. (Robinson, 2002) There are many challenges involved in harnessing the use of this readily available water source. Many cities along the river have a population that is growing faster than the infrastructure can keep up with. Consequently, water treatment facilities simply cannot handle the volume, which leads to large amounts of untreated water draining directly into the Rio Grande River (Assadian, Di Giovanni, Enciso, Iglesias, & Lindemann, 2005b; Ribera & McCorkle, 2012). Without proper treatment, pathogens in the wastewater can ultimately contaminate the irrigation water that is being pumped out of the river downstream and used in agriculture.

The LRGV is a region that includes Cameron, Hidalgo, Starr, and Willacy counties, which produced approximately $820 million of crops in 2012 (Ribera & McCorkle, 2012). Spinach, onions, watermelons, cantaloupes, and cabbage are important crops that the LRGV supplies to the United States. This area amasses 475,000 acres of crop producing land, which requires approximately 615,000 ac-ft. of water from the Rio Grande each year. The demand of water is variable and depends on the amount of rainfall in this very dry climate. It is important to understand the quality of the water that

is being diverted from the Rio Grande River for agricultural use. The contamination of microorganisms such as fecal coliforms and *Escherichia coli* can pose a risk to the contamination of the crops being irrigated with the river water. This risk directly affects the population that will eventually consume the goods.

The water that is diverted from the Rio Grande was initially used for flood irrigation, in which nearby canals had to hold a large volume of water. This method of delivering water is still in practice today, but the irrigation practices have changed. Newer methods of irrigation such as drip and sprinklers require a lower volume and more frequent applications than the flood method (Knight, 2009). With technology evolving and more efficient irrigation practices being implemented, the old way of storing water is not as efficient and it introduces an environment for bacterial contamination and growth from wildlife, and other harmful microorganisms from the ground.

Contamination of food with foodborne pathogens is highly sensitive today and poses dangerous risks to public health. Outbreaks of contamination are often random and hard to predict, so it is important to monitor and detect the levels of indicator organisms, such as fecal coliforms and *E. coli* so that the risk level to consumers can be determined. Furthermore, antibiotic resistance in these indicator organisms is also important to monitor in the transport of irrigation water to fresh produce, since the rapid spread of antibiotic resistant bacteria (ARB) threatens human health and has significant social and economic impacts. The selective pressure exerted by the overuse and misuse of antibiotics has been considered one of the major factors in the emergence of bacterial

resistance to antibiotics (IFT, 2006). ARB can pose a significant threat to human health because society relies on the antibiotics to treat and prevent diseases. There is a limited number of antibiotics that doctors and scientists can use for treatment, so it is essential to minimize the growth and spread of the antibiotic resistant bacteria. These microorganisms are recognized by the industry, government agencies, and public health organizations to verify the effective implementation of Good Agricultural Practices (GAPs) (Hald & Baggesen, 2014; Tortorello, 2003; USFDA, 2008, 2013a). Coliforms and generic *E. coli* are used as indicators of fecal contamination and overall cleanliness. *E. coli* has been identified by the European Food Safety Authority (EFSA) to be suitable for hygiene criterion in the validation of GAPs and good hygiene practices (GHPs). Growers can adjust irrigation and handling process based on the levels of *E. coli* and fecal coliforms detected on their crops to reduce risks of diseases outbreak and ensure public health.

The rationale of this research project was to better understand the fate and transport of pathogenic microorganisms in the use of wastewater in food crop irrigation and to measure the levels of antibiotic resistance in these microorganisms to assess the risk to human consumption of contaminated fresh produce.

CHAPTER II

LITERATURE REVIEW

About 48 million people (1 in 6 Americans) get sick, 128,000 are hospitalized, and 3,000 die each year from foodborne diseases, according to recent data from the Centers for Disease Control and Prevention. FDA has compiled from CDC data information regarding produce associated outbreaks which occurred between 1996 and 2010 where contamination is likely to have happened early in the production chain, during growing, harvesting, manufacturing, processing, packing, holding, or transportation. This FDA data set demonstrates that from 1996 to 2010, approximately 131 produce-related reported outbreaks occurred, resulting in 14,350 outbreak-related illnesses, 1,382 hospitalizations and 34 deaths. These outbreaks were associated with approximately 20 different fresh produce commodities. This is a significant public health burden that is largely preventable (USFDA, 2012). Of the foodborne pathogens and illnesses the CDC keeps a record of, *Escherichia coli* spp. *(E. coli*), especially serogroups O157, O121, and O145 are some of the more common typically associated with beef and fresh produce (CDC, 2013a, 2014). In 2009-2010, Shiga toxin-producing *Escherichia coli* (STEC) caused 58 confirmed outbreaks, with 53 being caused by serogroup O157 (CDC, 2013).

Pathogenic strains of *Escherichia coli* can cause illness, either diarrhea or illness outside of the intestinal tract. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or through contact with animals or

person. The most recent outbreaks associated with pathogenic *E. coli* in 2016 were reported in flour and alfalfa sprouts (CDC, 2016). In fresh produce alone, outbreaks involving *Escherichia coli*, including *E. coli* O157:H7 have been reported in clover sprouts (CDC, 2015b), chicken salad (onions and celery)(CDC, 2015c), ready-to-eat salads (CDC, 2013b), spring mix blend (CDC, 2012b), romaine lettuce (CDC, 2012a), and spinach (CDC, 2012b) over the past 5 years. Food and water supplies can become infected with *E. coli* due to contamination from fecal matter introduced by food handlers, cross contamination, improper washing of raw vegetables, and undercooked ground beef (Adams, 2007; WHO, 2011).

Foodborne virus outbreaks are also of significant concern in recent years. For instance, since August 2016, there have been 1,037 norovirus outbreaks reported by nine states in the U.S. who use the National Outbreak Reporting System (NORS) (CDC, 2017a). Norovirus is among the most abundant pathogen found in primary and secondary wastewater (McBride, Stott, Miller, Bambic, & Wuertz, 2013). Rotavirus has become more prevalent in tests conducted by participating laboratories that report to the National Respiratory and Enteric Virus Surveillance System (NREVSS), testing positive in over 20% of samples in April 2017 (CDC, 2017b). Norovirus and rotavirus were selected to perform the risk assessment because of their similar chemical composition, shape, and size to AP205, which is a direct surrogate to the MS2 bacteriophage. These viruses' capsids are made up of proteins, enclose positive sense single stranded RNA, and have T=3 symmetry (180 proteins) (Shishovs et al., 2016). Though, rotavirus is

twice as large, it was assumed that observed AP205 concentration represented maximum viral load in the sample materials (CDC, 2015d).

Contamination can be prevented through basic good manufacturing practices in the industry and food hygiene at home (WHO, 2011). The FDA has several guidance documents and regulatory information available online to producers. Contained within these documents are methods to help mitigate microbial food safety hazards in fresh-cut fruits and vegetables, from farm to table. Maintaining water quality is the first step toward achieving safer produce. Furthermore, FDA recommends the use of antimicrobial chemicals to help minimize the potential for microbial contamination of processing water and subsequent cross contamination of the product (USFDA, 2012). It is critical that the water supply for fresh-cut produce be of adequate quality, i.e., little to no *E. coli* colony forming units (CFU) per 100 mL water. Water is a capable carrier for bacteria and pathogens and should be tested regularly at its source and at the furthest distance from its source to comply with federal, state, and local requirements. If the quality does not meet requirements, methods can be implemented to negate the presence of bacteria and pathogens. Water quality should always be tested before and after treatment to ensure the antimicrobials are effective in killing the contaminants.

In 2013, the FDA proposed rules for agricultural water standards in part of the FDA Food Safety Modernization Act (FSMA). The new rules were based upon practices already being implemented in farms that follow the standards of the California and Arizona Leafy Greens Marketing Agreement. The proposal considers the large diversity of growing conditions and practices so that the rules are adaptable and can make a

practical impact on food safety. Two standards for testing irrigation water have been proposed: no detectable *E. coli* present per 100 mL of water. This standard applies when using water for an activity both during and after harvest when there is a high likelihood that pathogens would survive. The second standard states: a statistical threshold value (STV) of no more than 410 CFU generic *E. coli* per 100 mL out of at least 20 samples over the first 2-4 years, providing a microbial water quality profile (MWQP) and a geometric mean (of five samples) of no more than 126 CFU/100 mL for the irrigation water. In subsequent years, five additional samples are collected and added to the most recent 15 samples to calculate a new MWQP. This standard applies to water used during growing produce covered by the proposed rule (other than sprouts) when it is applied in a manner that results in direct contact with the harvestable portion of the crop. Moreover, after testing in either case, if it is found there is more generic *E. coli* than the numerical standard prescribes, one would be required to immediately discontinue use of that source for the use subject to the standard and take specific follow up actions, including visually re-inspecting the water source and distribution systems, making changes to the system and re-testing; or treating the water to acceptable levels (USFDA, 2015).

Untreated surface water is the most vulnerable to external sources and contamination. Under the proposed rules, untreated surface water must be initially tested using a minimum of 20 samples. These samples must be taken as close to harvest as possible over a span of two to four years. After the initial survey, five samples per year are to be taken and combined with the latest 15 samples to comprise an ongoing dataset

of 20 samples. This is done to confirm that the water being used is safe to use and within the single point and average thresholds (USFDA, 2013b).

Antibiotic resistant bacteria (ARB) also pose a threat to public health through the continuum of fresh produce production. One of the many vehicles for bacteria to become immune to antibiotics is the reuse of human wastewater. ARB can develop from the overuse of antibiotics in humans through wastewater systems. These effluents may have ARB and associated antibiotic resistance genes (ARG) along with other chemical contaminants, and if not treated properly, ARB, ARG and other contaminants may enter the food chain posing human health risk. It is important to quantify the risks to human health regarding the presence of foodborne pathogens and ARB in wastewater irrigation to be used in food crops, as well as their fate and transport to fresh produce, so that strategies to mitigate these risks can be implemented by disinfection methods, rules, regulations and good agricultural practices.

In wastewater plants, there are several processes to effectively remove most contaminants from the water. The first process is often activated sludge treatment. In this process, air is pumped through pipes at the bottom of a large tank which the raw wastewater flows into. The air bubbles provide agitation and adequate oxygen to the bacteria in the water that eat the organic matter, which produces cellular biomass. In the next process, the cellular biomass is separated from the clear water in clarification tanks. These tanks allow the heavier biomass to fall to the bottom, and clear water flow on to the next phase. Wastewater treatment plants often use one of two methods for final sanitation and removal of microorganisms such as bacteria, fungi, and other pathogens.

Chlorine-based treatment can be used effectively against gram-positive and negative-bacteria. Ultra violet (UV) bulbs are also a popular mitigation strategy, which effectively inactivate bacteria. In this process, water is passed through a chamber equipped with UV bulbs to supply adequate dosage, which destroys microorganisms' nucleic acid and disrupts their DNA (Rao, 2012). However, only generic *E. coli* concentrations are required to be tested in accordance with Title 40 Code of Federal Regulations (USEPA, 2017). There are currently no regulations to monitor pathogens.

Quantitative microbial risk assessment (QMRA) is a framework and approach that brings information and data together with mathematical models to address the spread of microbial agents through environmental exposures and to characterize the nature of the adverse outcomes. Ultimately, the goal in assessing risks is to develop and implement strategies that can monitor and control the risks (or safety) and allow one to respond to emerging diseases, outbreaks and emergencies that impact the safety of water, food, air, fomites and in general our outdoor and indoor environments (Haas, Rose, & Gerba, 1999).

CHAPTER III

HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

The concentration of pathogens and antibiotic resistance bacteria contained in irrigation water transferred to foliage, leachate, and soil in colony and plaque forming units per unit volume, is correlated to the initial concentration of the pathogens in irrigation water, and to the risk posed to human consumption.

3.2 Objectives

Grow and irrigate lettuce with wastewater effluent to track the fate of *Escherichia coli*, AP205, and antibiotic resistance through irrigation, cultivation, and postharvest storage. Determine risk to humans when wastewater was used to irrigate lettuce based on quantitative risk assessment analysis. The main objectives were achieved by completing the following tasks:

1. Measure levels of *E. coli* and AP205 in wastewater to be used as irrigation water.
2. Compare uptake of *E. coli* and AP205 by the foliage, leachate, and soil during lettuce cultivation.

3. Measure levels of sustained contamination of foliage in post-harvest storage.

4. Observe antibiotic resistance of *E. coli* colonies found in wastewater effluent, leachate, soil and foliage.

5. Complete statistical analysis to determine quantifiable risk level to human's health from the consumption of leafy greens irrigated with wastewater effluent.

CHAPTER IV

PREVALENCE OF FOODBORNE AND ANTIBIOTIC RESISTANT PATHOGENS DURING FRESH PRODUCE PRODUCTION UTILIZING NON-TRADITIONAL WATER SOURCES: FATE, TRANSPORT, AND RISK-ASSESSMENT STUDY

4.1 Overview

High demand for food and water mean new water reuse programs are being explored including treated municipal wastewater usage. However, these sources could contain high contaminant levels, and consequently pose risks to public health. The objective of this research was to grow and irrigate a leafy green with wastewater from a municipal wastewater treatment plant to track *Escherichia coli* and antibiotic resistant microorganisms through cultivation and post-harvest storage to assess their fate and transport. Subsequently, quantitative microbial risk assessment was performed to estimate risk of illness to the public. Contamination levels found in the foliage, leachate, and soil were directly ($P<0.05$) related to *E. coli* concentrations in the irrigation water. Wastewater concentrations from 177-423 CFU/mL resulted in approximately 15-25% retention in the foliage. Leachate and soil had means of 231% and 116% retention, respectively. *E. coli* accumulation on the foliage was observed ($P<0.05$) and increased by over 400% during 14-days storage (4^{o}C). From randomly selected *E. coli* colonies, in all four biomass types, 81% and 34% showed resistance to ampicillin and cephalothin, respectively. Intermediary resistant colonies, 9% and 10%, were between the susceptible

and resistant thresholds, which could evolve to fully resistant organisms. The risk assessment revealed significant cumulative risk of illness from lettuce consumption (up to 0.8 probability) from 6 pathogens commonly found in municipal wastewater. Norovirus and *Giardia* contributed the most to illness risks. Results show that non-traditional water sources usage for leafy greens cultivation can pose risks to humans, especially considering the bacteria found have a high probability of being resistant to one or more antibiotics.

4.2 Introduction

As world population and demand for food increase, safe water for agricultural use has become increasingly scarce. The water footprint of humanity is estimated at 9087 km^3/year, of which agriculture accounts for 92% (Hoekstra & Mekonnen, 2012). In some areas, there may not be any readily available surface water, and other solutions, such as drilling a well are not a cost-effective. Many countries with arid climates are already forced to use treated wastewater. If farmers can safely use treated municipal wastewater for the irrigation of crops, it may be able to alleviate the growing concern for safe available water used in agriculture. There are several practices that already use irrigation techniques with treated wastewater to mitigate the risk of contamination, such as drip, flood, and subsurface irrigation (Pavione, Bastos, & Bevilacqua, 2013; Solomon, Potenski, & Matthews, 2002). Due to the morphology of plants, such as lettuce or spinach, though, commercial-scale production requires canopy (or spray) irrigation. This

process involves water coming into direct contact with the edible foliage, which poses a higher risk of contamination (Robinson, 2002). There is a lack of knowledge in the fate and transport of pathogens and antibiotic resistant bacteria in wastewater irrigation regarding precisely where pathogens accumulate during and after harvest, as well as their potential effect on future crops along with risks posed to human health. It is important to know the complete and lasting effects of wastewater irrigation, as it is becoming an increasingly popular alternative.

Several studies have examined the effects and risks of using wastewater effluents to irrigate fresh produce such as lettuce, spinach, rocket, and tomato (Assadian, Di Giovanni, Enciso, Iglesias, & Lindemann, 2005a; Ribera & McCorkle, 2012). Throughout these studies, multiple factors have been tested to observe their effect on the prevalence of fecal indicator bacteria (FIB), which are often used to estimate the levels of harmful pathogens (Alam et al., 2014). However, it has been reported that there is a lack of correlation between FIB and pathogens in current microbiological monitoring standards (Alam et al., 2014; Orlofsky et al., 2016). Furthermore, many studies have reported contamination of crops, but there is no knowledge of how the entire system of foliage, soil, and leachate, is affected over time when using wastewater irrigation.

Consumption of fresh produce is on the rise, due to its associated health and nutritional benefits. At the same time, fresh produce is one of the leading causes of foodborne illnesses (Rai & Tripathi, 2007) with 377 outbreaks reported by the U.S. Center for Disease and Control from 2004 to 2012 (Callejon, 2015). Moreover, the overuse of antibiotics can be attributed to the propagation and occurrence of antibiotic

resistant bacteria (ARB), which have been increasing rapidly over the past several decades (Bitton, 2010; Edberg, Rice, Karlin, & Allen, 2000; WHO, 2006). Livestock production provides a direct path for antibiotics to watersheds and potential irrigation water via manure and rainfall runoff (Pepper, Gerba, & Brendecke, 1995). This issue has been identified by many global public health entities such as the World Health Organization (WHO) and U.S. Center for Disease and Control (CDC) as a critical concern (Bitsch, 2014). Conversely, there are no studies which quantify the risks to public health of using ARB contaminated wastewater as irrigation water. With a finite number of current antibiotics, it is important that practices be implemented to slow the advancement of resistant pathogenic bacteria. To help better understand ARB, analysis is needed to determine the fate of these bacteria and their potential effect on the environment and to the public safety.

To safely consider treated wastewater in agriculture, public health standards need be in place to monitor levels of contamination in irrigation water and fresh produce. The ability to quantify inherent risk of consumption of pathogenic and AR bacteria is vital to this process. Quantitative microbial risk assessment (QMRA) is a four-step process that can be used for hazard identification, exposure assessment, dose-response assessment, and risk characterization (Jones et al., 2008). QMRA has been applied to management strategies regarding water quality and public health (Pruden, 2014), and can be applied to assess the public health risk of fresh produce irrigated with contaminated water (Mena & Pillai, 2008). In this study, lettuce was irrigated with secondary treated wastewater to track the fate and transport of *E. coli* and antibiotic resistance throughout the entire

system (foliage, soil, and leachate) during cultivation, and postharvest storage. We then analyzed the risk of human consumption throughout the process using QMRA.

4.3 Materials and methods

4.3.1. Escherichia coli prevalence

4.3.1.1 Wastewater

Wastewater was obtained weekly from the Texas A&M Wastewater Treatment Plant, College Station, TX, USA. The wastewater was collected with a beaker affixed to a dipping pole, after solids removal and secondary clarification processes. Three liters were collected using a beaker affixed to a pole and placed into sterile plastic jugs for transport to the laboratory.

4.3.1.2 Leafy greens

Twelve young 15-cm romaine lettuce plants (*Lactuca sativa* var. longifolia, Bonnie Plants, Union Springs, AL, USA) were purchased from local nursery and placed into 20-cm diameter plastic pots and filled with EcoScraps moisture retaining potting soil (EcoScraps Co., South Jordan, UT), leaving a 2-cm lip to the top. The potting soil was sterilized in an autoclave for 90 minutes at 121 ºC and analyzed by the Texas A&M Department of Soil and Crop Sciences laboratory (College Station, TX) which generated

the following results: pH: 7.2, Nitrate: 0 ppm, Phosphorus: 95 ppm, and Potassium: 441 ppm. A suggested supplement of nitrogen was applied in the amount of 0.68 g/cm^2.

Lettuce plants were transplanted and grown using Reverse Osmosis (RO) water for 14 days prior to the irrigation experiment. Supplemental RO water was examined by aerobic plate counting method (J. B. Rose, Haas, & Regli, 1991) and determined to be free of any bacterial contamination. Each row of six plants were grown under two 2-Light T12 fluorescent shop lights (Lithonia Lighting, Conyers, GA) containing four 1.22 m 40-watt fluorescent tube light bulbs (General Electric, Fairfield, CT). The bulbs provided 2900 lumens each and consisted of two 6,500 K and two 3,000 K color temperature bulbs to more closely resemble natural daylight. The lighting fixtures were plugged into a wall outlet timer that allowed 14 hours of continuous light located 15 cm above the plants.

4.3.1.3 Inoculation

Once per week for three weeks, approximately 15 mL of wastewater was applied directly to each plant's foliage, thoroughly covering each side of all leaves from a 15-cm distance. This was carried out by transferring wastewater to a 150-mL sterile spray bottle (Apothecary Products, Inc., Minneapolis, MN) and setting the nozzle on mist position. Then, 150 mL of the wastewater was poured into each plant's pot, completely soaking all the soil. All wastewater application to the plants was carried out inside a biosafety cabinet following biohazard safety level 2 standard procedures. Plants were also

supplemented 50 mL of RO water each day the rest of the week to prevent drying out and wilting. Table 1 shows the timeline for sample pre-harvest irrigation and post-harvest storage at 4 ^{o}C.

Table 1: Timeline for sample pre-harvest irrigation and post-harvest storage at 4 ^{o}C.

Day 0	Day 7	Day 14	Post-Harvest Day 7	Post-Harvest Day 14
Foliage, Leachate, Soil, & Wastewater	Foliage, Leachate, Soil, & Wastewater	Foliage, Leachate, Soil, & Wastewater	Foliage	Foliage

4.3.1.4 Sample collection and analysis

A 10-mL sample of the wastewater was taken and placed into a sterile conical centrifuge tube (VWR International, Radnor, PA). After wastewater irrigation, leachate from each plant was immediately collected from the pot saucer by pipetting 10 mL into a sterile conical centrifuge tubes (VWR International).

Foliage samples were collected 1 hour after irrigation, by cutting the outermost leaves from their stems with sterile scissors. Leaf blades were removed from the vein and cut into 2.5 cm strips. From each plant, 5 g of foliage was weighed and placed into Whirl-Pak® bags (eNasco, Fort Atkinson, WI). Then, 10 mL of buffered peptone water (BPW, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was added to each foliage sample to create a 1:2 ratio of foliage to buffer suspension. The bags were then massaged by hand for 2 min to homogenize the material. Postharvest foliage samples

were collected at the same time as the Day 14 samples and stored at 4 ºC for 7 and 14 days where BPW was then added and samples were processed accordingly.

Soil samples were collected 6 hours after irrigation to allow adequate drainage. From each pot, a sterile 2-cm diameter core tube was inserted 5 cm deep to collect and place 2 g of soil into sterile conical centrifuge tubes (VWR International). Then, 8 mL of BPW was added to create a 1:4 ratio of soil to buffer suspension. The tubes were then vortexed for 30 seconds to homogenize the contents. Subsequently, these samples were allowed to settle for 10 min to separate the soil from buffer.

Aliquots of the samples were then plated on MacConkey Agar (Hardy Diagnostics, Santa Maria, CA) by spread plating method. MacConkey Agar is a media selective to lactose fermenting gram-negative and enteric bacteria. Several serial dilutions of all samples were plated to ensure samples were within the limits of detection. Plates were incubated overnight at 37 ºC. Two plates per dilution of each sample were plated, counted, and reported in CFU/g-mL of sample (Shuval, Lampert, & Fattal, 1997).

4.3.2 Antibiotic resistant bacteria

Ten presumed *E. coli* colonies from each of the four materials collected (i.e., wastewater, soil, leachate, and foliage) were randomly selected and inoculated for a total of forty *E. coli* isolates per pre-harvest sampling time, and ten *E. coli* colonies from foliage per postharvest sampling time. Individual colonies were collected with a sterile

inoculation loop, streaked on Tryptic Soy Agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and incubated overnight at 35 ºC in accordance with the Kirby-Bauer Method (USDA, 1998). Briefly, *E. coli* cell suspensions were then prepared by placing two isolates into tubes containing 5 mL of Tryptic Soy Broth (TSB, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubating at 35 ºC for three hours while shaking at 150 rpm in a 12 L water bath (VWR International). Tubes were checked for appropriate turbidity by comparing them to 0.5 McFarland standard, which corresponds to a $10^7 – 10^8$ CFU/mL bacterial cell count (USDA, 1998).

E. coli suspensions were then re-streaked onto Muller Hinton Agar (MHA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates using sterile swabs. Antibiotic resistance of the colonies was determined by the Kirby-Bauer method for antibiotic susceptibility. Eight antibiotic susceptibility discs (Becton, Dickinson and Company, Franklin Lakes, NJ) of ampicillin (10 µg), cefoperazone (75 µg), cephalothin (30 µg), ciprofloxacin (5 µg), gentamicin (120 µg), imipenem (10 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), and tetracycline (30 µg) were stamped onto each MHA plate using a BBL® Sensi-Disc® 8-place Dispenser (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The stamped MHA plates were incubated for 16-24 hours at 35 ºC. Then, the zones of inhibition (ZOI) were measured to determine resistance, intermediate resistance, or susceptibility to each antibiotic, according to the Clinical and Laboratory Standards Institute (CLSI) standards (Bauer, Kirby, Sherris, & Turck, 1966).

4.3.3 Quantitative microbial risk assessment

Quantitative microbial risk assessment (QMRA) was performed to determine inherent quantitative risk to human consumption of produce irrigated with contaminated irrigation water. QMRA is an evolving framework that has been used for the past 30 years to evaluate the relationship between humans and environmental microorganisms, including those associated with fresh produce, with the goal of developing management regimens that mitigate exposures to pathogens. The four-step QMRA paradigm of hazard identification, exposure assessment, dose-response characterization, and risk characterization can be applied to comprehensively review produce quality data and characterize the host–hazard relationship (Bauer et al., 1966).

For this experiment *E. coli* concentrations were measured, therefore, in order to determine health risk the *E. coli* concentrations were converted into a pathogen dose. Schoen and Ashbolt (2010) developed an equation to determine reference pathogen (*rp*) dose (number of pathogens) that is derived from the concentration of FIB in the waterbody from a specific source (*S),* expressed as (μ_{rp}^{S}). Equation 1 was modified to calculate the reference pathogen dose (μ_{rp}^{S}) in CFU for each sample time on the foliage.

Equation 1:

$$\mu_{rp}^{S} = \frac{C_{FIB}}{R_{FIB}^{S}\ x\ 100}\ x\ R_{rp}^{S}\ x\ M$$

where:

C_{FIB} is the concentration of *E. coli* (FIB) on the foliage (CFU/g)

R_{FIB}^{S} is the concentration of bacterial indicators in sewage (CFU/L)

R_{rp}^{S} is the concentration of pathogen species in sewage (number of pathogens or genomes/L)

M is the mass of foliage ingested (29 g)

Risk estimation was based on a potential situation where irrigation water contaminated with pathogen was directly applied to lettuce foliage, harvested, and consumed without any intervention (antimicrobial treatment) step. Average daily consumption of lettuce for the general public was estimated as 29 g (Chun, 2005). The calculated pathogen dose for each reference pathogen was then input into the corresponding dose response model (Table 2) to estimate the health risk.

Table 2: Reference pathogen dose response models.

Reference Pathogen	Dose-Response Model	Reference	Model Parameters	Parameter Values	Morbidity
Norovirus	Beta Binomial	McBride et al. (2013); Peter F. M. Teunis et al. (2008)	alpha beta	0.04 0.055	60%
Cryptosporidium	Exponential	McBride et al. (2013); USEPA (2005)	r	0.05	50%
Giardia lamblia	Exponential	Haas et al. (1999)	r	0.0199	45%
Campylobacter	Beta-Poisson	Medema, Teunis, Havelaar, and Haas (1996); USEPA (2010a)	alpha beta	0.145 7.59	60%
Salmonella	Beta-Poisson	Joan B. Rose and Gerba (1991)	alpha beta	0.3126 139.9	100%
E. coli O157:H7	Beta-Poisson	Teunis, Ogden, and Strachan (2008)	alpha beta	0.248 48.8	60%

4.3.4 Statistical analysis

A fractional factorial design with equal replications was used in this study. All experiments were performed in triplicate as independent experiments and results are expressed as mean ± standard deviation. Differences among variables were tested using

one-way analysis of variance ANOVA and statistical significances were expressed at the $P < 0.05$ level, significantly different means were separated by the Tukey HSD test. All data was analyzed using JMP®Pro statistical software (SAS, Cary, NC 27513). Due to the rain event prior to day 14, large input (wastewater) variability of *E. coli* contamination levels in foliage, soil, and leachate were normalized by transforming CFU/mL to percentage of *E. coli* retention. Response material *E. coli* concentration (CFU/mL) was divided by wastewater *E. coli* concentration (CFU/mL) to yield retention as a percentage. Beginning with pre-harvest data, statistical analysis was performed using a Tukey HSD Post-hoc test to compare *E. coli* prevalence over time among all materials (wastewater, leachate, foliage, and soil). Then differences in means were analyzed among materials for each sampling time. Finally, post-harvest foliage samples were analyzed to detect mean differences over storage time.

Antibiotic resistant bacteria (ARB) samples were analyzed, focusing on the three most common resistance patterns observed from ARB analysis: ampicillin (10 µg), cephalothin (30 µg), and ciprofloxacin (5 µg). According to (CAMRA, 2015; Haas et al., 1999), bacterial resistance to antibiotics is broken down into three categories: susceptible, intermediate , and resistant. An organism is categorized by its zone of inhibition (ZOI), which is unique to each antibiotic. This means the antibiotic was successful in preventing bacterial growth (USFDA, 1998). In this analysis, intermediate resistant and resistant bacteria were combined and expressed as 'resistant' to simplify the results. Resistance among samples was expressed as a percentage of each sampling population. ARB were compared over time among all materials. Then, mean differences

were analyzed among materials for each sampling time. All analyses were performed by using Tukey HSD Post-hoc test to separate differences in means, and Levene's test to test for equal variance.

4.4 Results and Discussion

4.4.1 Escherichia coli prevalence

The concentration of *E. coli* present in the irrigation water varied and was recorded in Log CFU/mL as 2.3 ± 0.0, 2.6 ± 0.2, and 5.1 ± 0.1 Log CFU/mL for days 0, 7, and 14, respectively (Figure 1). There was a large rain event of 32.8 mm (Underground, 2016) in the Bryan/College Station area on November 6th, 2016, one day prior to the collection of the Day 14 sample. It was also observed that the WWT plant was not operating at full effectiveness because of a failure in the aeration system, which provides oxygen to microorganisms in the solids removal tank. The WWT plant reported an *E. coli* concentration of 2.5 ± 0.6 Log CFU/100 mL in UV-treated effluent on day 14 and an average of 2.6 ± 2.0 Log CFU/100 mL in the four days following the rain event and system failure. These are significantly higher concentrations than the average for the rest of the month which was 1.0 ± 0.7 Log CFU/100 mL, which consequently affected the results by introducing very large concentrations of *E. coli* that were significantly different ($P < 0.05$) than the previous two irrigations. The EPA standard for final effluent discharge is a geometric mean of 2.1 Log CFU/100 mL (USEPA, 2017).

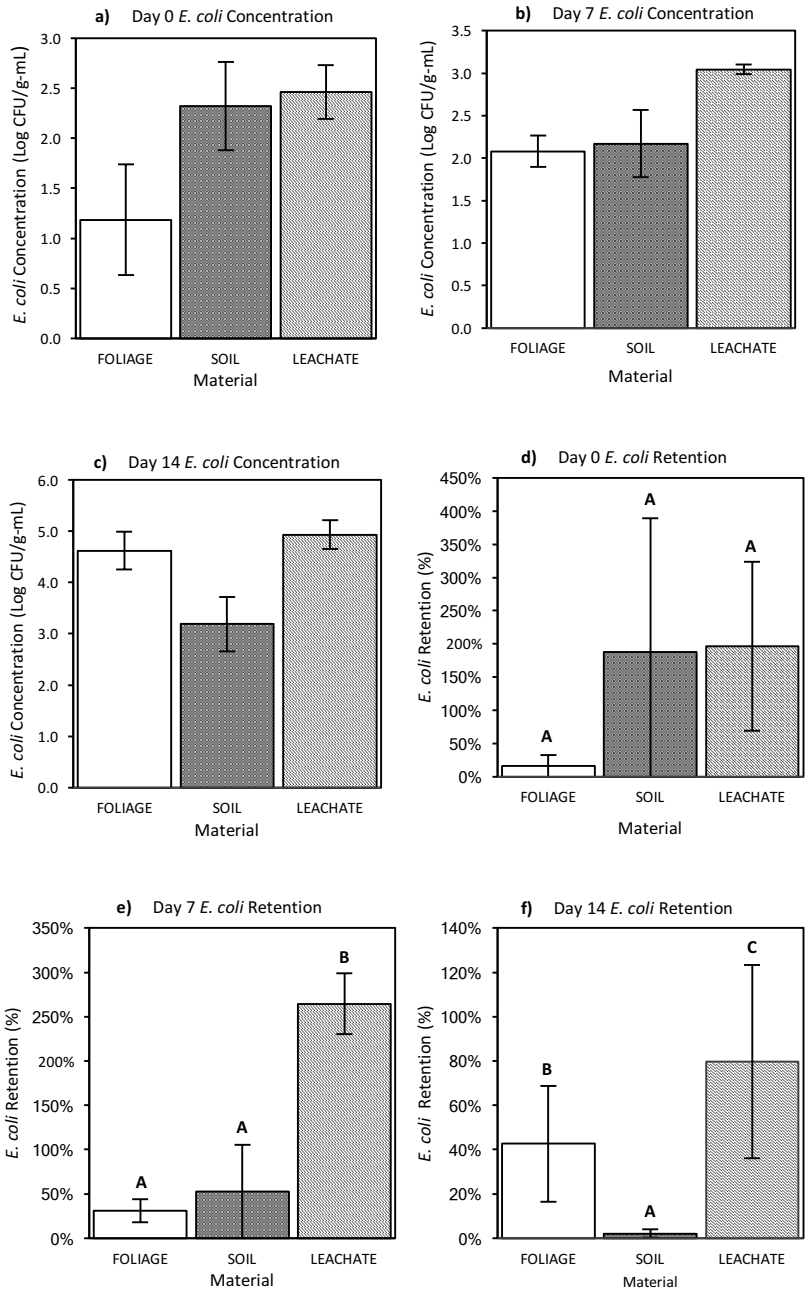

Figure 1: Comparison of *E. coli* concentration (a, b, and c) in Log CFU/g-mL, and retention (d, e, and f) in %, for sampling days per material. Retention was calculated by dividing *E. coli* sample concentration (CFU/mL-g) by weekly *E. coli* irrigation water concentration (CFU/mL). Error bars denote standard deviation for arithmetic mean (n=6 for day 0 foliage and n=12 for all others). Connecting letters reported using Tukey-Kramer HSD, α=0.05.

Initially, soil and leachate displayed higher concentrations of *E. coli* than foliage, until day 14 when foliage surpassed ($P < 0.05$) soil concentrations (Figure 1 c). Overall, each material showed an increase in *E. coli* concentration from the previous sampling time except for soil on day 7, as seen on Figure 2 a, b, & c. Foliage consistently increased concentration throughout cultivation and post-harvest storage. Leachate samples had the largest concentrations at each sampling time (Figure 1 a, b, and c). Foliage displayed a positive trend in retention with 16%, 31%, and 43% on days 0, 7, and 14, respectively (Figure 2 d). This shows that there was accumulation of *E. coli* on the foliage throughout the cultivation process. The bacteria were able to survive and persist on foliage for more than one week, similar to the findings of Alam et al. (2014), which studied cessation of irrigation prior to harvest, and how the elapsed time affected *E. coli* concentration.

Soil and leachate retention rates were often higher than 100% (Figure 2 e, f) which shows that the soil was not completely sterile prior to the first irrigation of wastewater on day 0. It is likely that the soil in the lettuce transplants was contaminated with *E. coli,* which propagated during the first two weeks of sterile irrigation prior to day 0. Orlofsky et al. (2016) studied the correlation of fecal indicator bacteria and pathogens found on fresh crops irrigated with different types of water, potable, secondary TWW, and tertiary TWW, and found *E. coli* in soil, which had only been irrigated with potable water. Contrary to Orlofsky et al. (2016) , soil concentrations in this study were relatively consistent, but displayed a negative trend in retention with 188%, 53%, and 2% on days 0, 7, and 14, respectively. *E. coli* concentration in soil plateaued, while input

concentration increased, yielding the negative retention trend (Figure 2 b, e). This could suggest that soil has a maximum contamination load and excess *E. coli* will stay in the irrigation water to become leachate. Contaminated soil could affect low growing crops that have direct contact with the ground, (Pavione et al., 2013) and future generation of crops.

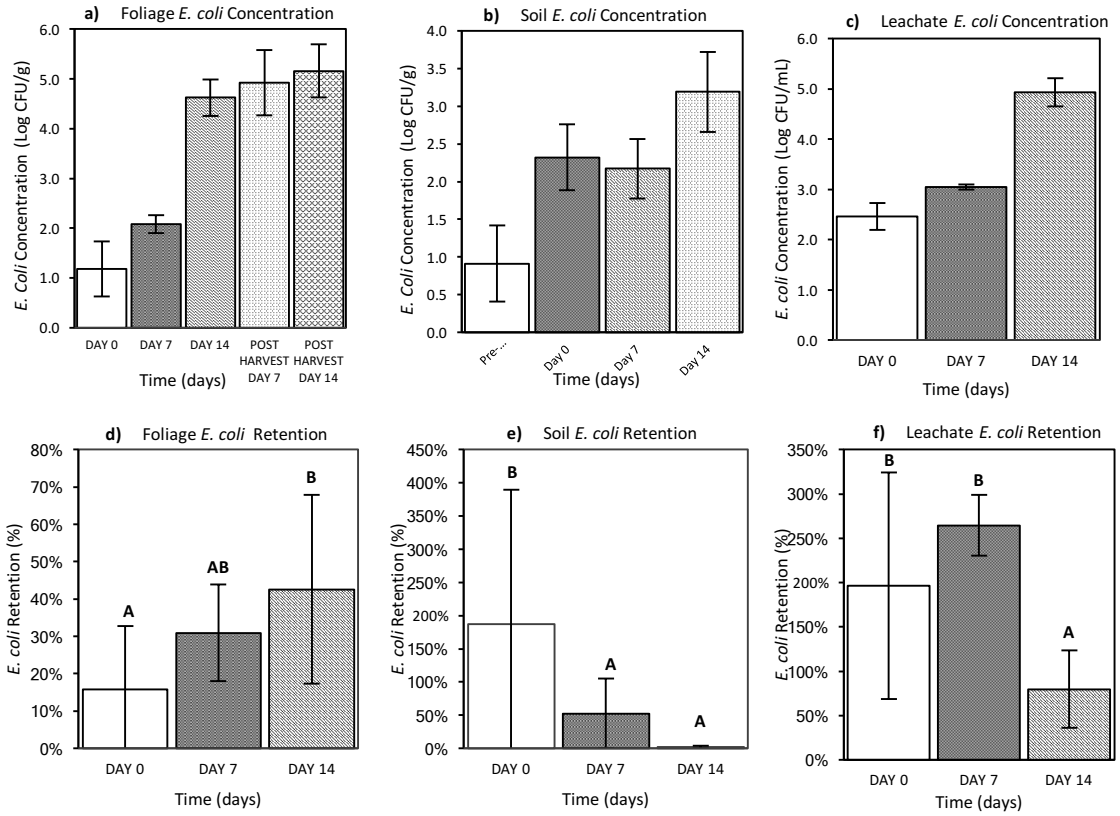

Figure 2: Comparison of *E. coli* concentration (a, b, and c) in Log CFU/g-mL, and retention (d, e, and f) in % for foliage, soil, and leachate samples over time. Retention was calculated by dividing *E. coli* sample concentration (CFU/mL-g) by weekly *E. coli* irrigation water concentration (CFU/mL). Error bars denote standard deviation for arithmetic mean (n=6 for day 0 foliage and n=12 for all others). Connecting letters reported using Tukey-Kramer HSD, α=0.05.

Leachate exhibited the largest retention rates among response materials during the cultivation process (Figure 2 a, b, c). The leachate picked up existing *E. coli* in the soil in addition to the concentration introduced by the irrigation water, yielding a retention rate greater than 100%. The retention rate for days 0 and 7 were 197% and 265%, respectively. As stated by Dwivedi, Mohanty, and Lesikar (2016), the saturated water content of the soil is an important parameter in subsurface *E. coli* transport. The size of the pots could have been a contributing factor to soil saturation prior to wastewater irrigation, as they were required sterile supplemental water often to avoid drying out and wilting of the lettuce. Similar to soil, day 14 leachate retention was affected by large input concentration and was significantly less than the previous two sampling times, dropping to 80%, even though accumulation increased over time. Our results show that contaminated water can penetrate through 15 cm of soil, but further investigation is needed to determine *E. coli's* fate as water percolates down to groundwater reservoirs. It has been shown by Stall, Amoozegar, Lindbo, Graves, and Rashash (2014) that depth of soil has a positive effect on reducing *E. coli* concentrations in water.

E. coli concentration in foliage increased during post-harvest storage at 4 ºC (Figure 2 a), similar to what was found by the study of Lopez-Velasco, Davis, Boyer, Williams, and Ponder (2010), which studied the effect of post-harvest storage temperatures (4 ºC and 10 ºC) and times (5, 10, & 15 days) on *E. coli* contaminated spinach. *E. coli* counts increased from 4.6±0.37 Log CFU/g on the harvest day to 4.9±0.66 Log CFU/g after 7 days of refrigerated storage, a 200% increase. After 14 days

of storage, 5.2±0.54 Log CFU/g was observed, a 172% increase from day 7 of post-harvest storage. Days 7 and 14 were significantly different than day 0, but not significantly different from each other ($P < 0.05$, Figure 2 a). These results support the importance of fresh produce being free of any pathogenic microbial contamination during cultivation and processing, as *E. coli* left on the surface can quickly propagate at recommended storage temperature (4 ºC), and pose health risks to consumers (Lopez-Velasco et al., 2010).

4.4.2 Antibiotic resistant bacteria

A total of 140 *E. coli* isolates across all sampling times and materials were tested for antibiotic resistance against eight antibiotics. Ampicillin had the highest recorded resistance among isolates at 81%, followed by cephalothin at 34% (Figure 3). It has been found by Silva (2006) that wastewater treatment plants have generally been ineffective at removing certain strains of resistant bacteria, specifically *enterococcus* isolates resistant to the antibiotics ciprofloxacin, erythromycin, and tetracycline, and that the prevalence of ciprofloxacin resistance increased throughout the treatment process. Gentamicin and imipenem displayed the lowest rate of resistance, with 1% and 0%, respectively. Several antibiotics including, ampicillin, cefoperazone, cephalothin, and ciprofloxacin displayed larger intermediate rates of resistance, ranging from 8-9% of all isolates. These findings are important because there is a high probability that these organisms will adapt to their environment and become more resistant, as suggested by

Lagacé-Wiens et al. (2013). Because of this, isolates displaying intermediate resistance were categorized as resistant for the remainder of analysis, similar to Laird (2016).

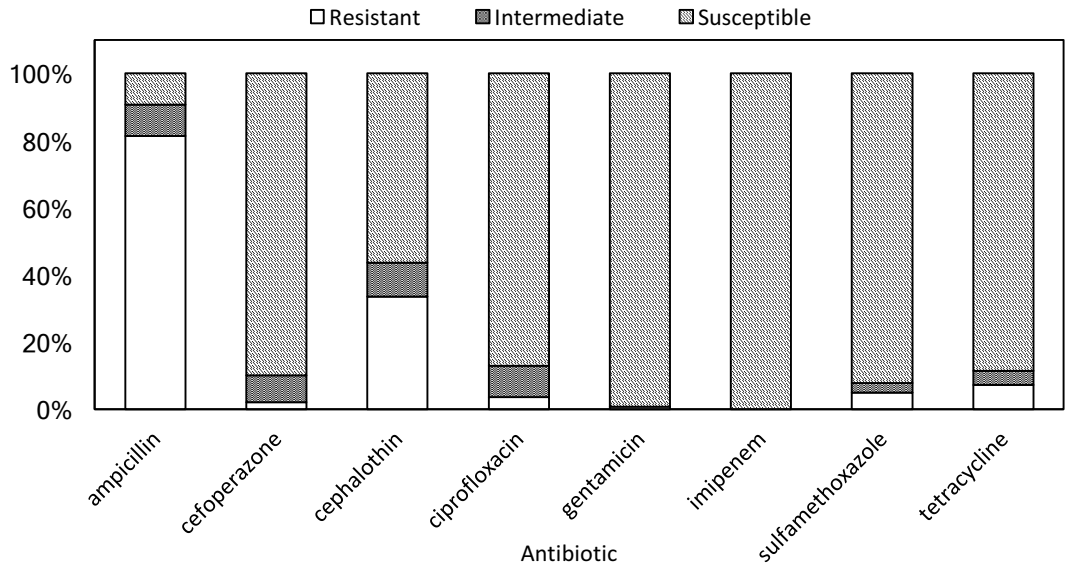

Figure 3: Distribution of antibiotic resistance (resistant, intermediate, and susceptible) among all materials (wastewater, foliage, leachate, and soil) for eight different antibiotics tested throughout fresh produce production (days 0, 7, 14, postharvest 7, and postharvest 14).

Three antibiotics with the highest combined prevalence of resistance and intermediate resistance were selected to further investigate their fate and transport throughout fresh produce production. These antibiotics were ampicillin, cephalothin, and ciprofloxacin with 90%, 44%, and 13% rate of resistance in all isolates, respectively. For an *E. coli* isolate to be categorized as resistant or intermediate resistant, the bacterial lawn on the Kirby-Bauer plate had to show little to no ZOI around a given antibiotic

disc. As seen in Figure 4 (a), ampicillin had the highest overall resistance prevalence in foliage. There was no distinct trend or significant differences in antibiotic resistance over the duration of the experiment, but one could hypothesize that antibiotic resistance would increase over time according to historical data and observations (Capita & Alonso-Calleja, 2013). Of the 20 postharvest foliage samples, 17 (85%) were resistant to ampicillin. Conversely, only 5% of postharvest foliage isolates were resistant to cephalothin and 0% for ciprofloxacin. In the U.S., ampicillin and ciprofloxacin are two of the top 5 antibiotics prescribed to adults (Shapiro, Hicks, Pavia, & Hersh, 2014). These antibiotics have been found in WWTPs in varying concentrations and treatment plant designs (Batt, Kim, & Aga, 2007) due to their frequent use in the past and today's society, which suggests that treatment plants may be contributing to the prevalence of antibiotic resistant bacteria found downstream.

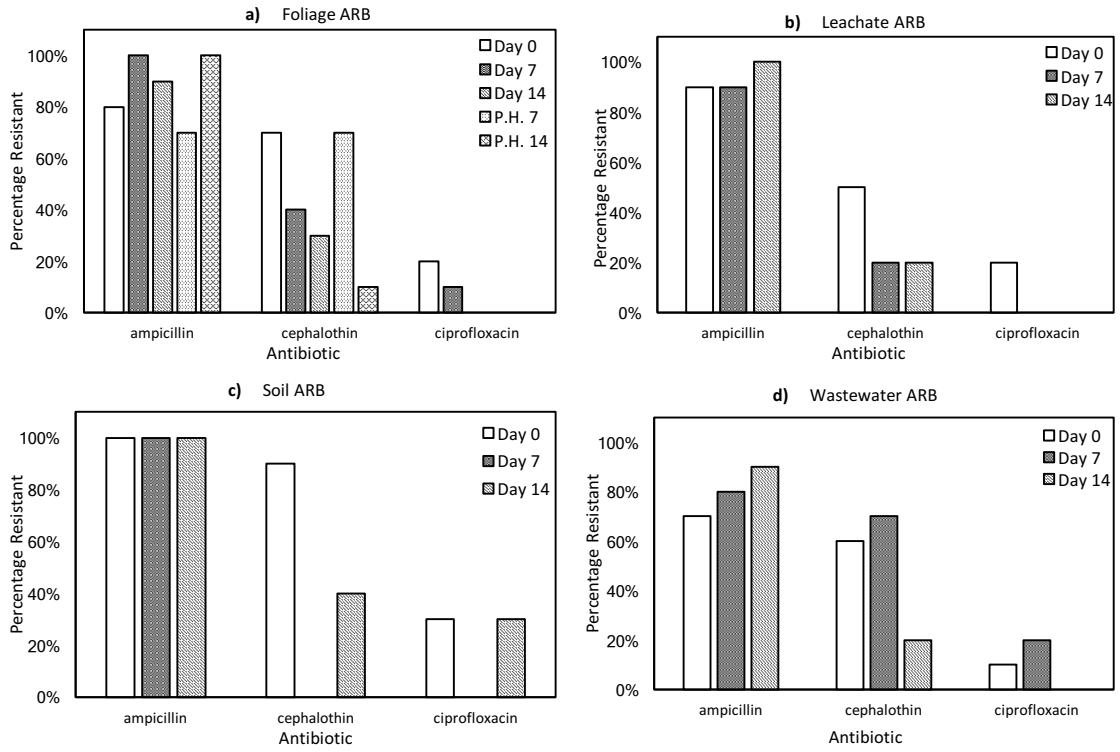

Figure 4: Distribution of antibiotic resistant bacteria (ARB) over time (day 0, 7, 14 and post-harvest (P.H.) day 7 and 14) for three antibiotics that displayed the highest prevalence of resistance (ampicillin, cephalothin, and ciprofloxacin) among response materials a) foliage, b) leachate, c) soil, and d) wastewater source.

Ampicillin is a commonly prescribed antibiotic used to treat illnesses such as bladder infections, pneumonia, gonorrhea, meningitis, and enteric infections (NRCP, 2017). Both leachate and soil displayed high levels of resistance to ampicillin with 28 out of 30 and 30 out of 30 isolates displaying resistance, respectively. It is equally important to track the level of antibiotic resistant bacteria in leachate and soil in addition to foliage because ultimately, those bacteria can make their way back into water sources

like rivers and creeks, via runoff and leaching. Prior to any wastewater application there were no detectable *E. coli* on the foliage, and the sterilized soil had 13 ± 10 CFU/g of *E. coli*, and 332 ± 358 CFU/g after first irrigation. Moist soil provides an optimal environment for resistant bacteria to propagate and pass along resistant genes (Orlofsky et al., 2016). The water systems are a key vehicle for these bacteria containing antibiotic resistant traits to propagate, multiply, and transfer their resistant genes (Pei, Kim, Carlson, & Pruden, 2006). There has been extensive research of the fate in transport of antibiotic resistant bacteria in water sources resulting from livestock production, but there is little information available on the risks involved of using water with human source antibiotic resistant bacteria for fresh produce production (Gunther, 1984), (Humphrey, 2005).

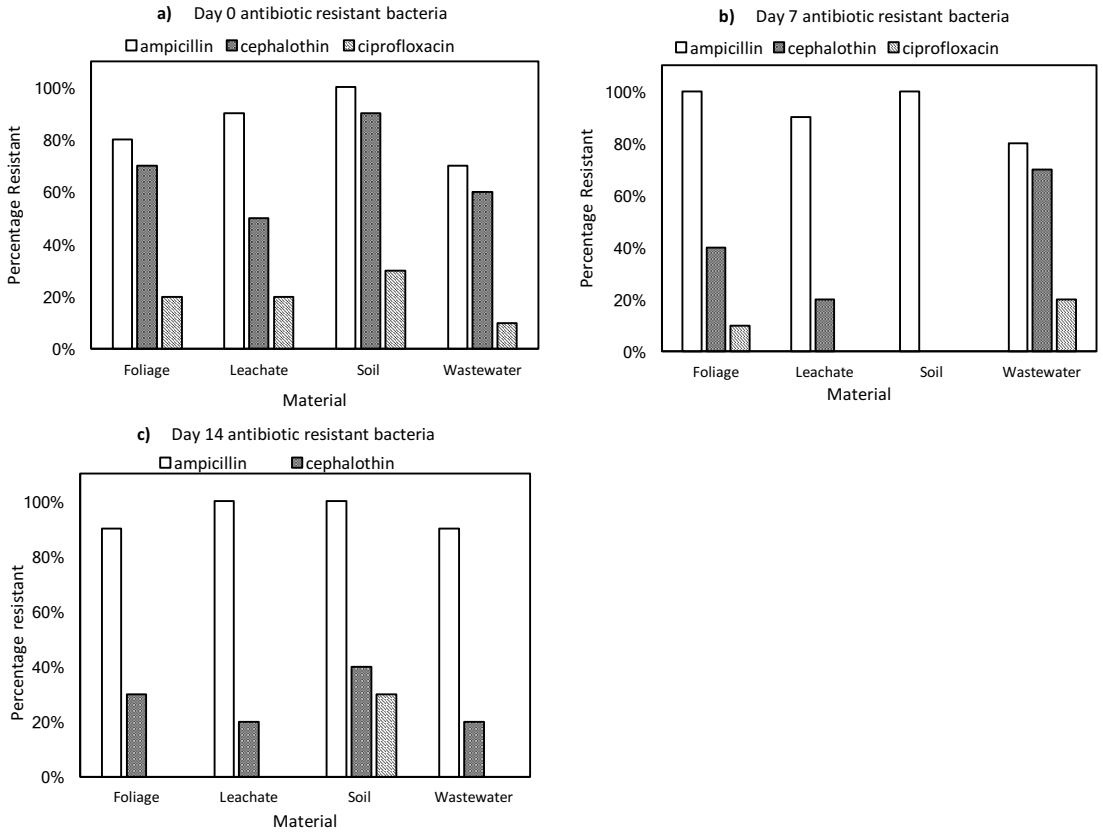

Figure 5: Comparison of antibiotic resistant bacteria (ARB) for three antibiotics that displayed the highest prevalence of resistance (ampicillin, cephalothin, and ciprofloxacin) among materials over sampling times during cultivation a) day 0, b) day 7, and c) day 14.

Day 0 sampling time displayed the largest resistance in isolates from ampicillin, followed by cephalothin and ciprofloxacin, for all four materials tested (Figure 5). There were no instances throughout sampling times, where ampicillin did not show the most prevalent isolate resistance among each material. Ciprofloxacin showed the least resistance, of the three selected antibiotics, for all materials for each sampling day,

except for day 7 for soil samples, where both cephalothin and ciprofloxacin showed 0% isolate resistance. For overall ampicillin resistance, soil and wastewater were significantly different from each other, however; no significant differences were observed from foliage nor leachate. There were no other significant differences among sample materials for cephalothin and ciprofloxacin.

Cephalothin was first introduced in 1964 and was a first-generation cephalosporin antibiotic and ciprofloxacin was introduced in 1987. Both antibiotics are still widely prescribed to stop bacterial growth and treat infections (NCBI, 2017). The mechanism of resistance to ampicillin is identical to that of penicillin (beta-lactamase), and has been evolving for as long as antibiotics have been used. Beta-lactamases are enzymes that cause antibiotic resistance in the beta-lactam family of antibiotics such as penicillins, including ampicillin and cephalosporin. These enzymes catalyze the hydrolysis of the amide bond of four-membered beta-lactam rings and render the antibiotic inactive against its original cellular target, the cell wall transpeptidase (Bajpai, Pandey, Varma, & Bhatambare, 2017). Penicillin was the first antibiotic discovered, and heavily overprescribed (Arendrup, Knudsen, Jensen, Jensen, & Frimodt-Møller, 2001). Consequently, penicillin resistance in bacteria rose quickly in the few decades after its discovery, in particular, 80% of Staphylococcus aureus became penicillin resistant by the late 1960's (Lowy, 2003). As previously studied, antibiotic resistant genes can be transferred among different bacteria species via transduction, conjugation, and transformation (Capita & Alonso-Calleja, 2013; Hleba, Kmeť, Tóth, & Kačániová, 2017). With a similar mechanism of resistance, it can be assumed that most of the

antibiotic resistance to ampicillin can be attributed to the phenomenon of widespread resistance to penicillin (Lobanovska, 2017).

4.4.3 Risk assessment

Six pathogens were chosen to perform the quantitative microbial risk assessment based on the eight waterborne reference pathogens established by the Environmental Protection Agency (EPA): norovirus, rotavirus, adenovirus, *Cryptosporidium* spp., *Giardia lamblia*, *Campylobacter* spp., *Salmonella*, and *E. coli* O157:H7. These pathogens are present in both human and animal fecal waste, and can be found in municipal wastewater (USEPA, 2010b). Furthermore, *E. coli* O157:H7 serotype is the most common cause of *E. coli* food poisoning, and is dangerous at low dosages (CDC, 2015a). Adenovirus was removed because there is currently no published dose-response relationship established for ingestion (Haas et al., 1999). Rotavirus was excluded from consideration for its lack of established concentration levels based on FIB (Soller, Schoen, Bartrand, Ravenscroft, & Ashbolt, 2010). These reference pathogens have been observed and concentrations recorded in primary sewage and secondary chlorinated effluents (Soller et al., 2010). A concentration level was estimated in secondary clarified wastewater (an intermediary step in WWTP) by taking the mean of primary sewage and secondary chlorinated effluents, which were obtained from (Soller et al., 2010). Three reference concentrations in microorganisms/L were created: low, mean, and high. These concentrations were used along with the ratio of *E. coli* (FIB) found on foliage and

original concentration in the secondary clarified wastewater to calculate dosage levels. An average consumption level of lettuce was concluded to be 29 g (Chun, 2005), which gave a final dosage levels for each pathogen (Table 1). Then, dosages were used in dose-response models, along with corresponding parameters as established by Haas et al. (1999) to yield risk of infection for each pathogen and sampling time (Table 3). Illnesses resulting from infection varied by pathogen and ranged from 45-100% with *Giardia* being the lowest, and *Salmonella* the highest (Soller, 2015), (McBride et al., 2013). The risks of illness to the public from all pathogens were combined within sampling time, to create a total risk of illness for each sampling time among low, mean, and high concentrations (Figure 6).

Table 3: Low, mean, and high pathogen dosages of microorganisms (Log Dosage), based on estimated concentrations (microorganisms/L) and average consumption of lettuce (29 g).

Log Dosage	Pathogen	Day 0	Day 7	Day 14	P.H. 7	P.H. 14
Low	Norovirus	0.4	0.6	0.7	1.2	1.3
	Giardia lamblia	-1.8	-1.6	-1.5	-1.0	-0.8
	Cryptosporidium spp.	-2.9	-2.7	-2.5	-2.0	-1.9
	Campylobacter spp.	-2.3	-2.1	-2.0	-1.5	-1.4
	Salmonella	-1.8	-1.6	-1.5	-1.0	-0.9
	E. coli O157:H7	-2.3	-2.1	-2.0	-1.5	-1.4
Mean	Norovirus	3.1	3.3	3.4	3.9	4.0
	Giardia lamblia	1.1	1.3	1.4	1.9	2.0
	Cryptosporidium spp.	-0.3	-0.1	0.1	0.6	0.7
	Campylobacter spp.	-0.3	-0.1	0.0	0.5	0.7
	Salmonella	0.1	0.3	0.5	1.0	1.1
	E. coli O157:H7	0.1	0.3	0.4	0.9	1.1
High	Norovirus	3.4	3.6	3.7	4.2	4.3
	Giardia lamblia	1.4	1.6	1.7	2.2	2.4
	Cryptosporidium spp.	0.0	0.2	0.3	0.8	1.0
	Campylobacter spp.	0.0	0.2	0.3	0.8	0.9
	Salmonella	0.7	0.9	1.0	1.5	1.6
	E. coli O157:H7	0.7	0.9	1.0	1.5	1.7

Among all concentration levels and sampling times, norovirus had the largest dosages, as it was far more abundant that the other reference pathogens in primary and secondary sewage, according to Soller et al. (2010). *Cryptosporidium* and *Campylobacter* had the lowest dosages largely because of their lack of abundance in secondary chlorinated effluent with means of 40 and 100 microorganisms/L. As levels of *E. coli* (FIB) increased over the sampling times, dosages of reference pathogens and their inherent risks increase, accordingly. The largest total risk came from day 14 of post-harvest storage total risk of illness at 30%, 70%, and 78% for low, mean, and high

concentrations, respectively. Conversely, the lowest levels of FIB were recorded on day 0 of pre-harvest, displaying total risk of illness at 27%, 42%, and 49% for low, mean, and high concentrations, respectively.

It should be noted that norovirus and *Giardia* accounted for the largest contributions to the total risk, ranking as the top two individual risks among all sampling days and concentration levels. In all low concentration risks, norovirus comprised nearly all the risk, ranging from 27-30%, with *Giardia* contributing 0.1% or less for each sampling day. All other pathogens were under 0.05% risk for all sampling days. It is apparent that total risk of consumption of contaminated food is directly related to levels of contamination in irrigation water. In this experiment, FIB was recorded and used as a reference to estimate pathogens potentially on foliage to be consumed. The baseline risk of illness at any sampling time was over 25%, with norovirus being the largest contributor.

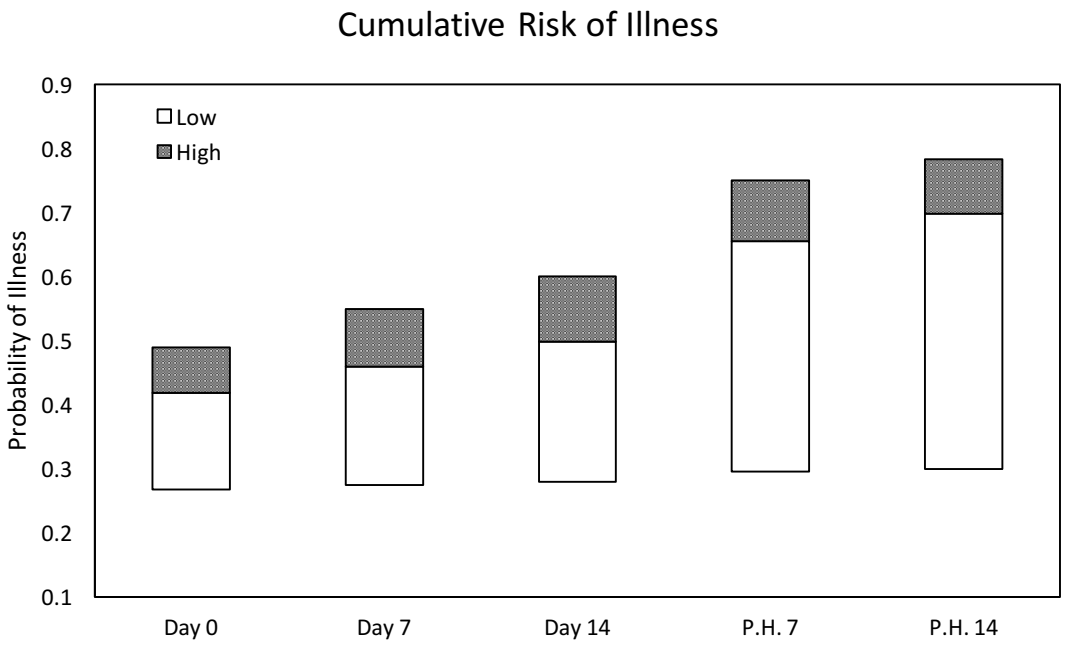

Figure 6: Low, mean, and high cumulative risks of illness from six reference pathogens (norovirus, *Cryptosporidium* spp., *Giardia lamblia*, *Campylobacter* spp., *Salmonella*, and *E. coli* O157:H7) over time, using average consumption of lettuce (29 g). The mean value is the line separating the low and high areas. Days 0, 7, and 14 are for pre-harvest lettuce samples, and P.H. 7 and 14 are the post-harvest lettuce samples kept at 4 ºC.

During post-harvest storage, risk increased as FIB concentrations increased, reaching nearly 80% on the final sampling day. This suggests that preemptive measures are vital to ensure the safety of fresh produce products, as without proper treatment, there is a serious threat to public safety. Municipal WWTPs effectively remove contaminants from human waste in accordance with the EPA to meet defined effluent standards for recreational water (USEPA, 2017). However, this water is not suitable to apply in fresh produce irrigation practices at the tested microbial load. If the

contamination level is reduced, the risks of illness will likely decrease accordingly. Furthermore, microflora in WWTPs is mostly from human sources, which poses a larger risk of infection and illness than the equivalent FIB level of contamination in water polluted by wildlife and other sources (Brooks JP, 2015). Pathogens in effluent derived from human wastewater can be especially dangerous, because WWTP sludge has been suspected to foster an ideal environment for the exchange and development of resistant genes, providing additional advantages not available to microbes in the natural environment (Nicholls, 2003). This potentially provides a quicker avenue for microorganisms to become multi-drug resistant, which poses a dangerous threat to society. These finding stress the significance of controlling overuse of antibiotics and other drugs, as they find their way to wastewater and contribute to adaptation of antibiotic resistant pathogens (Isturiz, 2000).

4.5 Conclusions

There is a direct relationship between the bacterial contamination of irrigation water and the contamination levels of subsequent biomass such as foliage, soil, and leachate. Contaminated soil and leachate can have health risks for future generations of crops, especially those with low growing foliage that have direct contact with the ground. Significant growth of *E. coli* on lettuce foliage occurred during 14 days of post-harvest storage at 4 ºC. Of the tested *E. coli* isolates, resistance to ampicillin displayed the largest prevalence, at 81%. Moreover, 75% of antibiotics tested showed intermediate

resistance. Herein, lies a dangerous situation where public health is placed at risk, if overall levels of resistance are not closely monitored. There are potential public health risks from using non-disinfected wastewater effluent to irrigate crops. Under the worst-case scenario, 8 out of 10 people consuming the crop have the potential to become ill, if reuse of wastewater, at the tested level of contamination, is present in fresh produce production. This is especially true if levels of antibiotic resistance continue to increase and spread, without being closely monitored and held in check. If wastewater effluent is to be used as an alternative source for fresh produce production, it is highly recommended that the water proceeds through disinfection processes with either chlorine or UV treatment to mitigate the public health risks.

CHAPTER V

ENTERIC VIRUS PREVALENCE DURING FRESH PRODUCE PRODUCTION USING NON-TRADITIONAL WATER SOURCES: FATE, TRANSPORT, AND RISK ASSESSMENT STUDY

5.1 Overview

High demand for food and water mean water reuse programs are being explored including treated municipal wastewater usage in agriculture. However, these sources could contain high contaminant levels, and pose risks to public health. The objective of this study was to grow and irrigate leafy greens with inoculated wastewater effluent to track AP205 bacteriophage prevalence through cultivation and post-harvest storage to assess fate and transport. AP205 is a bacteriophage that infects *Acinetobacter baumannii,* and was used as a surrogate for enteric viruses, norovirus and rotavirus. Subsequently, quantitative microbial risk assessment (QMRA) was performed to estimate risk of illness to the public. Low and high dosages of AP205 at 4.77 ± 0.39 Log PFU/mL and 6.63 ± 0.21 Log PFU/mL, respectively, were prepared to examine viral load influence on contamination levels and risk of illness. Foliage, leachate, and soil contamination levels were directly ($P < 0.05$) related to AP205 concentrations in the effluent. AP205 concentrations increased throughout cultivation for foliage and leachate, suggesting bacteriophage accumulation. During post-harvest storage (14 day at 4 ^{o}C), there was a significant decrease in AP205 concentration present on the foliage. QMRA

results revealed significant chance of illness for norovirus and rotavirus with a 30-40% probability of illness developing from infection. Risk of illness varied between dosages by only 5% and 1% for norovirus and rotavirus, respectively. Results show that non-traditional water usage for fresh produce cultivation can pose risks to humans, if standards are not in place to control pathogen contamination levels.

5.2 Introduction

Fresh produce production provides a direct vehicle for enteric pathogens such as norovirus (NoV) and hepatitis A virus (HAV) to human hosts via irrigation and minimal sanitation during the farm-to-fork continuum (Li, De Keuckelaere, & Uyttendaele, 2015). This issue is especially prevalent when treated wastewater effluent enters the irrigation system. Wastewater is becoming more commonly used in arid regions of the world to increase water sources for irrigation (Pachepsky, 2011). Finding alternative water sources provides more water for the ever-growing demand for food and leaves more potable water for direct human consumption. Links between enteric viruses in irrigation water and fresh produce have been studied and established as a health risk (Cheong, 2009; López-Gálvez et al., 2016). However, the complete fate and transport of enteric viruses during cultivation and post-harvest storage of fresh produce are still unclear.

Generally, bacterial indicators such as fecal coliforms are used as a guideline for the quality of irrigation water (Steele, 2004; USFDA, 2013a). Although, it has been observed that relationships of enteric viruses with indicator organisms, such as

Escherichia coli, are not correlated (López-Gálvez et al., 2016). This lack of direct relationship is apparent throughout the primary, secondary, and tertiary wastewater treatment stages, as reported by Hijnen, Beerendonk, and Medema (2006). Moreover, this study also showed that enteric viruses are very resistant to UV light, which kills 98.9% of *E. coli* and is part of the final stage in many wastewater treatment plants (La Rosa, 2010). This effluent is often discharged into creeks and tributaries of major rivers in which agricultural irrigation water is pumped out of. It is important to understand how these viruses impact public health when present in fresh produce irrigation water.

Viruses are resilient and can survive much longer in agricultural environments than bacteria. They are also much smaller, which enables them to matriculate down into the water table. There have been studies that track the prevalence of enteric viruses, in general, from irrigation water to crop surfaces (Cheong, 2009; López-Gálvez et al., 2016). These have been helpful in establishing that enteric viruses preside on crops after irrigation, and bear health risks to human consumption. To completely understand how these viruses persist and the risks they pose to public health, we need to know how the entire system, foliage, soil, and leachate, are impacted when using contaminated water for irrigation at different contamination levels.

Treated wastewater in agriculture creates new opportunities for water usage efficiency, but public health standards need to be established to monitor levels of contamination in irrigation water and fresh produce and ensure public safety. Being able to quantify inherent risk of consumption of enteric viruses is vital to this process. Quantitative microbial risk assessment (QMRA) is a four-step process that can be used

for hazard identification, exposure assessment, dose-response assessment, and risk characterization (Jones et al., 2008). QMRA has been applied to management strategies regarding water quality and public health (Pruden, 2014) and can be applied to assess the public health risk of fresh produce irrigated with contaminated water (Mena & Pillai, 2008). In this study, lettuce was cultivated with wastewater effluent with different initial concentrations of a surrogate enteric bacteriophage (*AP205).* The fate and transport of the virus was tracked in foliage, soil, and leachate, during irrigation and postharvest storage. We then analyzed the risk of human consumption throughout the process using QMRA analysis.

5.3 Materials and methods

5.3.1 Enteric virus prevalence

5.3.1.1 Microorganisms

All isolates in this study were obtained from Dr. Ry Young's (Department of Biochemistry and Biophysics, Center for Phage Technology, Texas A&M University) culture collection. Bacteriophage AP205 is a single stranded RNA bacteriophage that has a unique protein sequence among all known single stranded RNA phages.(Shishovs et al., 2016). AP205 was selected as surrogate organism for an enteric bacterial virus that infects *Acinetobacter* bacteria. Thus, *Acinetobacter baumannii* genotype 16 (ATCC

17988) was selected as a host organism to propagate AP205. *A. baumannii* is a gram-negative opportunistic *coccobacilli*. (Cherkaoui, Emonet, Renzi, & Schrenzel, 2015)

Initial concentration of the AP205 isolate was determined to be 10^9 PFU/mL (plate forming units per mL) by spot titration on a bacterial lawn containing Tryptic Soy Agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ) and the host bacteria (Klovins, Overbeek, van den Worm, Ackermann, & van Duin, 2002). Standard operating procedures (SOPs) were followed to produce enough lysate (raw phage) as provided by the Center for Phage Technology, Texas A&M University (CFPT, 2011a). Briefly, 100 µL of an overnight culture of *A. baumannii* and 100 µL AP205 was combined with 4 mL of molten TSA top agar in a sterile glass tube. The mixture was briefly vortexed and poured onto a TSA plate. The mixture was allowed to set for five minutes and then inverted and incubated at 30 ºC for 24 hours. Then, the top agar was scraped off the firmer bottom agar layer using buffered peptone water (BPW, Becton, Dickinson and Company, Franklin Lakes, NJ) as a lubricant and placed into sterile 50 mL Falcon tubes (VWR International) and centrifuged at 8000 g for 10 min. The supernatant was then filtered through 0.45 µm and 0.22 µm syringe filters (VWR International). The final concentration of the lysate stock after propagation was examined and determined to be 10^{10} PFU/mL using the same procedure as the initial concentration analysis. The AP205 stock (suspended in BPW) was stored under 4 ºC refrigeration throughout the experiment (for less than a month).

A. baumannii was cultivated by isolating a single colony from a stock TSA plate, and inoculating in 9 mL sterile tryptic soy broth (TSB, Becton, Dickinson and Company,

Franklin Lakes, NJ) and incubating at 30 ºC for 24 hours in a shaking water bath (12 L, VWR International,). *A. baumannii* culture tubes were then stored at 4 ºC for no longer than one week, in which a new culture would be cultivated by transferring 100 µL of the old culture into a new TSB tube and incubating as before.

5.3.1.2 Effluent

Effluent sample was obtained weekly from the Texas A&M Wastewater Treatment Plant, College Station, TX, USA. The effluent was collected after solids removal, two clarification processes, and UV light sterilization. Three liters were collected using a beaker affixed to a pole and placed into sterile plastic jugs for transport to the lab for further analysis.

5.3.1.3 Leafy greens

Ten young 15-cm romaine lettuce plants (*Lactuca sativa* var. longifolia, Bonnie Plants, Union Springs, AL, USA) were purchased from local nursery and placed into 20-cm diameter plastic pots and filled with EcoScraps moisture retaining potting soil (EcoScraps Co., South Jordan, UT 84095), leaving a 2-cm lip to the top. The potting soil was sterilized in an autoclave for 90 minutes at 121 ºC and analyzed by the Texas A&M Department of Soil and Crop Sciences laboratory (College Station, TX), which generated the following results: pH: 7.2, Nitrate: 0 ppm, Phosphorus: 95 ppm, and

Potassium: 441 ppm. It was recommended that supplemental nitrogen be applied in the amount of 0.68 g/cm^2, which was followed accordingly.

Plants were watered in and grown using Reverse Osmosis (RO) water for 14 days prior to testing. Supplemental RO water was examined and determined to be free of any detectable bacterial contamination by aerobic plate counting method (USDA, 1998). Each row of five plants were grown under two 2-Light T12 fluorescent shop lights (Lithonia Lighting, Conyers, GA) containing four 1.22 m 40-watt fluorescent tube light bulbs (General Electric, Fairfield, CT). The bulbs provided 2900 lumens each and consisted of two 6,500 K and two 3,000 K color temperature bulbs to more closely resemble natural daylight. The lighting fixtures were plugged into a wall outlet timer that allowed 14 hours of continuous light located 15 cm above the plants.

5.3.1.4 Bacteriophage inoculation

Once a week, for three weeks, AP205 stock was combined with freshly collected effluent to make two approximate dilution levels: 10^8 and 10^6 PFU/mL. Each dilution level of effluent was applied to the corresponding plants, first by spraying 15 mL of fine mist through sterile 147.9 mL spray bottle (Apothecary Products, Inc., Minneapolis, MN), completely covering all sides of the foliage from 15 cm distance. Then, 150 mL of the effluent was poured into each pot, completely soaking all the soil. Plants were supplemented 50 mL of RO water each day for the rest of the week to prevent drying out

and wilting. Table 4 shows the timeline for sample pre-harvest irrigation and post-harvest storage at 4 ^{o}C. All procedures were carried out inside a biosafety cabinet.

Table 4: Timeline for sample pre-harvest irrigation and post-harvest storage at 4 ^{o}C.

Day 0	Day 7	Day 14	Post-Harvest Day 7	Post-Harvest Day 14
Foliage, Leachate, Soil, & Effluent	Foliage, Leachate, Soil, & Effluent	Foliage, Leachate, Soil, & Effluent	Foliage	Foliage

5.3.1.5 Sample collection and analysis

A 10-mL sample of the effluent was taken after phage inoculation for further analysis. After effluent irrigation, a 10-mL leachate sample from each plant was immediately collected from the pot saucer. Each type of samples was collected by pipetting 10 mL into a sterile conical centrifuge tubes (VWR International, Radnor, PA).

Foliage samples were collected 1 hour after irrigation, by cutting the outermost leaves from their stems with sterile scissors. Leaf blades were removed from the vein and cut into 2.5 cm strips. From each plant, 5 g of foliage was weighed and placed into sterile Whirl-Pak® bags (eNasco, Fort Atkinson, WI). Then, 10 mL of BPW was added to each foliage sample to create a 1:2 ratio of foliage to buffer suspension. The bags were then massaged by hand for 2 min to homogenize the material (USDA, 2015). Postharvest foliage samples were collected at the same time as the Day 14 samples and

stored at 4 ºC for 7 and 14 days where BPW was then added and samples were processed accordingly.

Soil samples were collected 6 hours after irrigation to allow adequate drainage. From each pot, a sterile 2-cm diameter core tube was inserted 5 cm deep to collect 2 g of soil which was placed in sterile conical centrifuge tubes (VWR International). Then, 8 mL of BPW was added to create a 1:4 ratio of soil to buffer suspension. The tubes were then vortexed for 30 seconds to homogenize the contents. Subsequently, these samples were allowed to settle for 10 min to separate the soil from buffer.

All samples were then filtered through sterile 0.22 μm syringe filters (VWR International, Radnor, PA) to remove all bacterial agents per SOP's provided by the Center for Phage Technology, Texas A&M University (CFPT, 2011b). Then seven serial dilutions per sample were made by transferring 100 μL into micro centrifuge tubes containing 900 μL BPW. First, *A. baumannii (*100 μL) from an overnight culture, was combined with 4 mL molten TSA containing 0.5% (w/v) agar, poured onto TSA plates, and cooled to form a bacterial lawn. Next, 10 μL of each sample dilution was spotted on the corresponding label of the bacterial lawn. Two plates per sample, containing 7 dilution spots were counted. Plates were incubated overnight at 30 ºC, then plaques were counted and results were reported as PFU/g-mL of sample according to (CFPT, 2011b).

5.3.2 Quantitative microbial risk assessment

Quantitative microbial risk assessment (QMRA) was performed to determine inherent quantitative risk to human consumption of produce irrigated with contaminated irrigation effluent. QMRA is an evolving framework that has been used for the past 30 years to evaluate the relationship between humans and environmental microorganisms, including those associated with fresh produce, with the goal of developing management regimens that mitigate exposures to pathogens. The four-step QMRA paradigm of hazard identification, exposure assessment, dose-response characterization, and risk characterization can be applied to comprehensively review produce quality data and characterize the host–hazard relationship (NRC, 1983).

Specifically, bacteriophage concentrations at various points along the produce production chain were translated to potential human health infection risk(s) assuming different exposure scenarios constructed by incorporating information obtained from peer-reviewed literature. The measure bacteriophage concentration was assumed to correlate with a one to one ratio to the two reference pathogens, rotavirus and norovirus. The pathogen dose (D) in PFU for both rotavirus and norovirus were calculated from Equation 2.1.

Equation 2:

$$D = C \; x \; V$$

where:

C is the concentration of pathogen species on the foliage (number of pathogens/g)

V is the volume of foliage ingested (29 g)

Risk estimation was based on a potential situation where irrigation water contaminated with virus pathogens was directly applied to lettuce foliage, harvested, and consumed without any intervention (antimicrobial treatment) step. Average daily consumption of lettuce for the general public was estimated as 29 g (Chun, 2005). The calculated pathogen dose for each reference pathogen was then input into the corresponding dose response model (Table 5) to estimate the health risk.

Table 5: Reference pathogen dose response models.

Reference Pathogen	Dose-Response Model	Reference	Model Parameters	Parameter Values	Morbidity
Norovirus	Beta Binomial	McBride et al. (2013); Peter F. M. Teunis et al. (2008)	alpha beta	0.04 0.055	60%
Rotavirus	Beta Poisson	Greenberg and Estes (2009)	alpha N_{50}	0.25 6.17	26%

5.3.3 Statistical analysis

A fractional factorial design with equal replications was used in this study. All experiments were performed in triplicate as independent experiments and results are expressed as mean ± standard deviation. Differences between variables were tested using one-way analysis of variance ANOVA and statistical significance were expressed at the $P < 0.05$ level, significantly different means were separated either by the Tukey HSD or student's t-test. All data was analyzed using JMP®Pro statistical software (SAS, Cary, NC). Beginning with pre-harvest data, statistical analysis was performed using a two-sample t-test test to compare bacteriophage contamination between the two effluent inoculation levels (high and low) of different materials (effluent, foliage, soil, and leachate) over time. Then, mean differences were analyzed among materials for each sampling time by performing a Tukey HSD Post-hoc test. Finally, post-harvest foliage samples were analyzed to test mean differences over storage time.

5.4 Results and discussion

5.4.1 AP205 bacteriophage prevalence

The concentration of *AP205* present in inoculated irrigation effluent varied slightly over time and was recorded in Log PFU/mL. The low dosage effluent was significantly different on day 14, from the previous two sampling times (day 0 and day 7), as shown in Figure 7. The inoculum concentrations were prepared based on

preliminary lysate testing data. The lysate was kept in four sterile Falcon tubes (50 mL), which may have caused the variance corresponding to 4.77 ± 0.39 Log PFU/mL for low dosage, and 6.63 ± 0.21 Log PFU/mL for high dosage. The high dosage effluent samples were not significantly different over time (Figure 7). Both dosage concentrations were lower than the theoretical concentrations of 6 and 8 Log PFU/mL for low and high dosages, respectively. This might have been due to natural reduction during storage (Cooper, Denyer, & Maillard, 2014) and after mixing with WW effluent. Even though final effluent dosages were lower than targeted concentrations, there was still a two logs difference between the dosages and the experiment could still be conducted with statistical significance as expected.

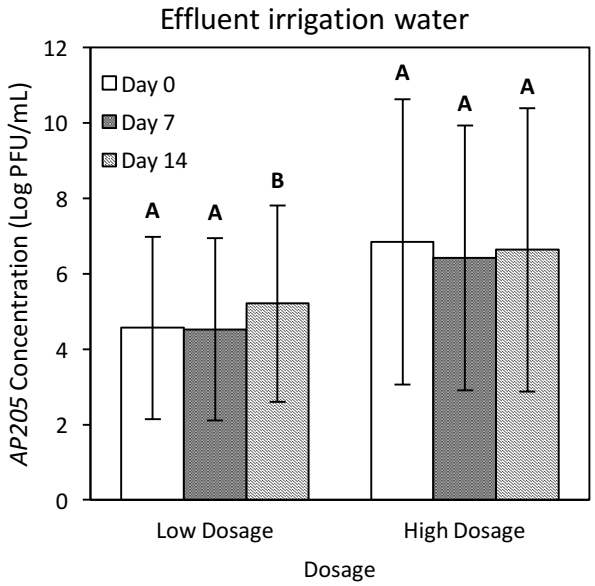

Figure 7: Comparison of *AP205* effluent concentration (Log PFU/mL) over sampling time. Error bars denote standard error for arithmetic mean (n=10). Connecting letters limited to comparison of each dosage and reported using Tukey-Kramer HSD, α=0.05.

Foliage and leachate contamination levels increased each sampling time among both dosage levels, while soil did not show any trend, nor have significant differences among any sampling time for both dosage levels, as seen in Figure 8**.** The low dosage treated foliage samples on days 7 and 14 were significantly higher than day 0, but not from each other (Figure 8). The high dosage foliage showed a steady increase in concentration, where days 0 and 14 were significantly different, but day 7 was not significantly different from either 0 or 14 (Figure 8). These results suggest that there is accumulation of *AP205* bacteriophage on the plant foliage throughout the cultivation process. The bacteriophage was able to survive and persist for more than one week. Although, alternative irrigation, such as subsurface drip, could prevent contamination of plant foliage, as previously reported in a study on bacteriophage transport in wastewater sub-irrigated soil during spinach production by Assadian et al. (2005b). This study also shows that bacteriophage did persist in the soil for 28 days, which suggests that virus inactivation strategies may be a necessary step in treating reclaimed wastewater for crop irrigation.

For the soil samples, both dosage levels did not display any accumulation over time, remaining at levels similar ($P > 0.05$) to the day 0 concentrations for the rest of the study. This lack of accumulation might suggest that there is a maximum viral load carrying capacity in the soil. It was concluded by Fongaro et al. (2017), which studied the fate and transport of enteric pathogens (*phiX174, mengovirus, Salmonella enterica* Typhimurium*,* and *E. coli* O157:H7) soil, that there are multiple factors including pH,

organic matter content, soil texture and moisture that affect the survival and persistence of viruses in soil.

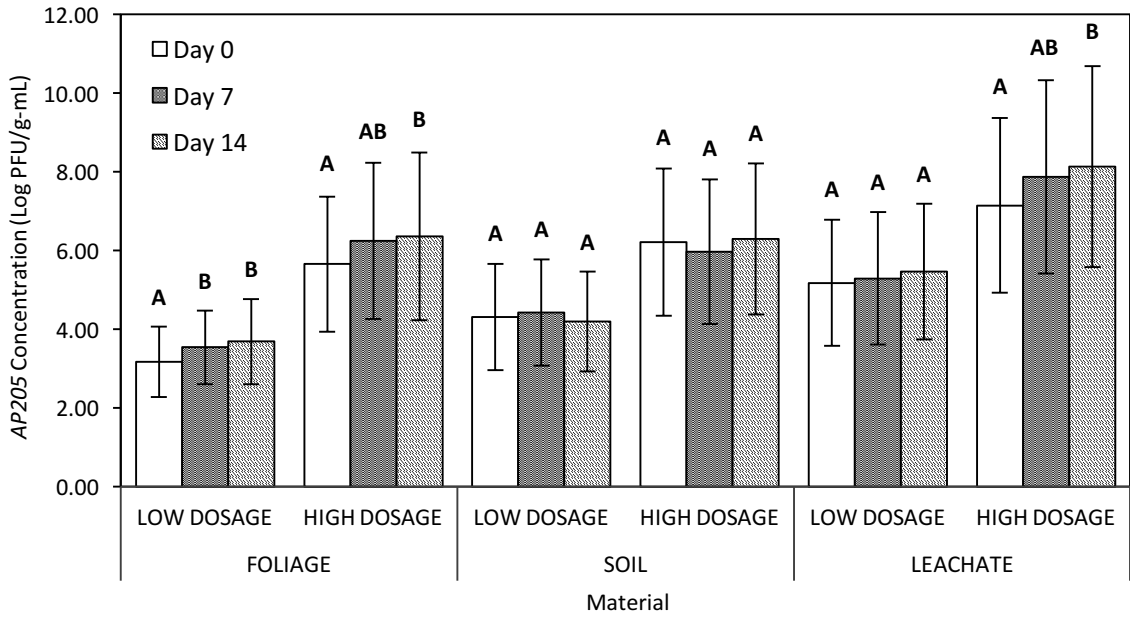

Figure 8: Comparison of *AP205* concentration (Log PFU/g-mL) within response materials (foliage, soil, and leachate) by dosage over sampling time. Error bars denote standard error for arithmetic mean (n = 10). Connecting letters limited to comparison of each dosage within response material and reported using Tukey-Kramer HSD, α=0.05.

Leachate did not show any significant difference in the low dosage samples, although mean values increased over time. Conversely, high dosage leachate sample means also increased over time, and significant differences similar to high dosage foliage, were observed (Figure 8). Day 0 and 14 high dosage leachate samples were different ($P < 0.05$) from each other, but day 7 was not significantly different from either day 0 or 7. It could be possible that the stability of low dosage leachate concentrations is

linked to the consistency and maximum carrying load of the soil. The irrigation effluent running through the soil was neither picking up nor depositing bacteriophage from the soil, which could be related to the soil being already at its upper limit of contamination, and the irrigation effluent was not significantly changing concentration over sampling time. As observed by Fongaro et al. (2017), the soil and leachate contamination levels of bacteriophage are dependent upon the characteristics of the soil. Assadian et al. (2005b) found that the coarseness of soil was a major factor in determining if using wastewater effluent in subsurface drip irrigation was microbiologically safe, concluding that coarser soil was less contaminated when compared to finer soils.

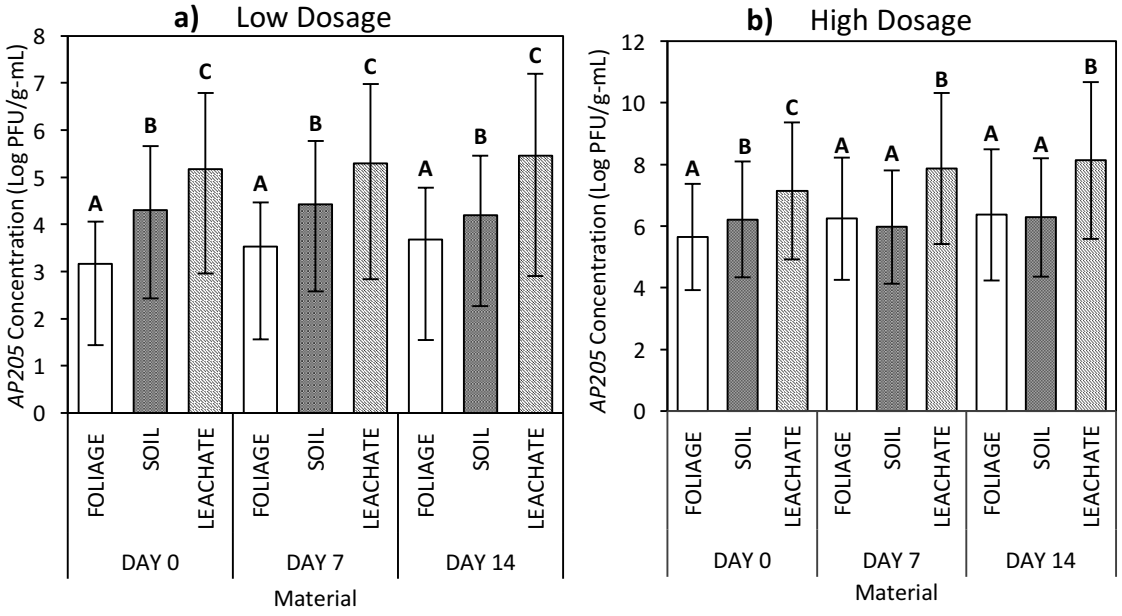

Figure 9: Comparison of *AP205* concentration (Log PFU/g-mL) for a) low dosage and b) high dosage among response materials (foliage, soil, and leachate) within sampling time. Error bars denote standard error for arithmetic mean (n=10). Connecting letters limited to comparison of response materials within sampling day and reported using Tukey-Kramer HSD, α=0.05.

There were significant differences in *AP205* concentrations observed in all low dosage response materials per sampling time, as shown in Figure 9 *AP205* concentrations were ($P < 0.05$) lowest in foliage, second highest in soil, and highest in leachate for each sampling time (Figure 9). These results could be explained by the potential load carrying capacities of the foliage and soil. Of the two, soil was able to retain more *AP205* than the foliage, but not enough to strike an equal balance between leachate. It was found by Seo (1999) that attachment of pathogens to leaf surfaces was often closer to the stomata and other cracks in the cuticle. In a separate study, DiCaprio et al. (2015) found that different pathogens (*norovirus* and *Tulane virus*) vary in localization patterns on fresh produce according to the surface roughness of the foliage, aggregating in and around stomata as well. These findings suggest that pathogens are more likely to attach to rougher portions of foliage, and not smooth waxy surfaces. Similarly, with more surface area, crevices, and places to attach, it is reasonable to conclude that soil retains more *AP205* than the smooth foliage of lettuce.

The high dosage samples for all response materials showed similar results to the low dosage samples on day 0 in terms of *AP205* concentration levels and significant differences. However, on days 7 and 14, the foliage and soil *AP205* concentrations were not significantly different, and soil was slightly less contaminated than foliage. Leachate's *AP205* concentration was significantly higher than foliage and soil on all three sampling days for the high dosage. As suggested by Iriarte (2007), there are several factors that affect bacteriophages' ability to survive, such as temperature, relative

humidity, and light within the UV spectrum. These and many other unaccounted factors may explain the difference in concentration levels between response materials.

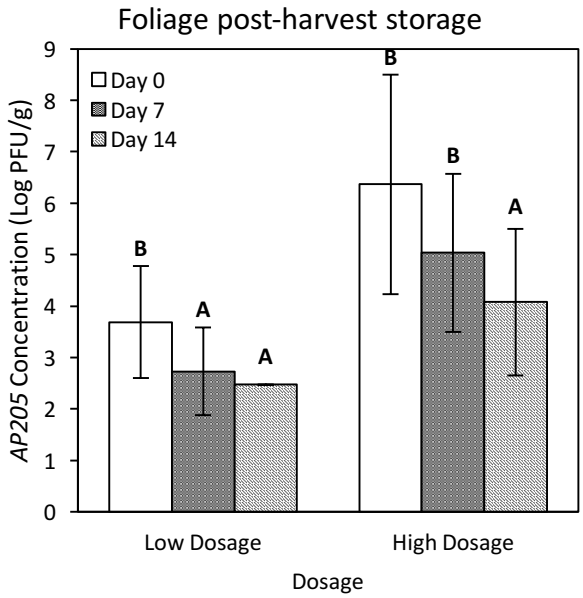

Figure 10: Comparison of *AP205* concentration (Log PFU/g) for foliage by dosage (high and low) over sampling time. Error bars denote standard error for arithmetic mean (n=10). Connecting letters limited to comparison of each dosage and reported using Tukey-Kramer HSD, α=0.05. On day 14, 90% of low dosage foliage samples were below the detection limit (297 PFU/g), thus value of 297 PFU/g was assumed for each instance to compare *AP205* concentration over time.

Overall, there was not significant difference in the contamination trends of the response materials expect for the high dosage in soil samples on days 7 and 14 (Figure 9 a, b). During the process, it was possible that soil had a maximum enteric virus load carrying capacity. The microbial integrity of the soil for future generations of crops can determine if it is safe to use or may contaminate new crops. A study by Pavione et al.

(2013) found that low growing crops could be more susceptible to contamination than taller growing crops. Furthermore, a fate and transport study by Vergine et al. (2015), who used different treatment levels of wastewater for subsurface drip irrigation, found that there was natural die-off and leaching of pathogens from the irrigation source, where less than 1% of initially 8.3 Log CFU/mL pathogens (*Escherichia coli*) were present in leachate at 36 cm below the soil surface. Leachate contamination also plays a major role as the runoff may enter water tables or tributaries where further propagation is possible and can pose public health concerns (Smolders, Rolls, & Ryder, 2015). Additionally, the low and high dosage samples displayed the same general contamination trends over time. This implies that the contamination trends between response materials is not affected ($P < 0.05$) by the initial concentration of *AP205* (2 order of magnitude difference) in the irrigation effluent. These results are important to begin understanding enteric viruses and pathogens fate and transport during crop irrigation.

AP205 concentration in foliage decreased during post-harvest storage at 4 ºC (Figure 10). Low dosage post-harvest foliage significantly decreased in *AP205* concentration from day 0 to 7. Day 14 data was supplemented due to limits of detection (100 PFU/g as tested), and worst-case-scenario concentration of 297 PFU/g was assumed. This was not significantly different from day 7. High dosage foliage samples also decreased in concentration, and were significantly different in days 7 and 14. Due to a large observed variance (6.4 ± 6.4 Log PFU/g), day 0 was not significantly different from day 7. These results are interesting, as they differ from typical behavior of bacterial

pathogens in the same circumstances (Lopez-Velasco et al., 2010). These results implicate that enteric viruses do not thrive and propagate on foliage during post-harvest storage. It is possible that there was inadequate host organisms for the bacteriophage to survive or that other, naturally present microorganism were propagating, which forced the competitive exclusion of *AP205* (Haerter, Mitarai, & Sneppen, 2014). It has also been observed that some bacteriophage (*phiXV3-16* and *phiXacm*) have natural die-off over time due to desiccation, which could have happened in this study, but that should not be assumed for all bacteriophage (Iriarte, 2007). There is still much to learn about how different pathogens, specifically bacteriophage and viruses, persist on fresh produce throughout handing and processing, and how they threaten human health.

5.4.2 Risk assessment

AP205 was used as a surrogate for other pathogenic viruses and its concentration throughout this study used to correlate with quantitative microbial risk assessment (QMRA) of the respective pathogenic viruses. Two viruses were chosen to perform the QMRA based on the eight waterborne reference pathogens established by the Environmental Protection Agency (EPA): norovirus, rotavirus, adenovirus, *Cryptosporidium* spp., *Giardia lamblia*, *Campylobacter* spp., *Salmonella*, and *E. coli* O157:H7 (USEPA, 2010b). These pathogens are present in both human and animal fecal waste, and can be found in municipal wastewater (USEPA, 2010b). Norovirus and rotavirus were selected to perform the risk assessment because of their similar chemical

composition, shape, and size to AP205, which is a direct surrogate to the MS2 bacteriophage. These viruses' capsids are made up of proteins, enclose positive sense single stranded RNA, and have T=3 symmetry (180 proteins) (Shishovs et al., 2016). Though, rotavirus is twice as large, it was assumed that observed AP205 concentration represented maximum viral load in the sample materials (CDC, 2015d). Consequently, norovirus and rotavirus concentrations throughout the analysis were taken from AP205 concentration. Low and high dosages were used to analyze if inherent risk of illness was affected by concentration. An average consumption level of lettuce was assumed to be 29 g (Chun, 2005), which gave us final dosage levels for each pathogen (Table 6).

Table 6: Low and high pathogen dosages (Log PFU/g) for norovirus and rotavirus based on observed concentrations (PFU/g) of AP205, and average consumption of lettuce (29 g). Post-harvest days written as P.H. 7 and 14.

Pathogens	Dosage (Log PFU/g)	Day 0	Day 7	Day 14	P.H. 7	P.H. 14
Norovirus and rotavirus	Low	4.8	5.0	5.2	4.2	3.9
Norovirus and rotavirus	High	7.1	7.8	8.0	6.5	5.9

Dosages were used in dose-response models, along with corresponding parameters as established by (Haas et al., 1999) to yield risk of infection for each pathogen and sampling time. Illness resulting from infection was 60% and 26% for norovirus and rotavirus, respectively (Greenberg & Estes, 2009; McBride et al., 2013). The risk of illness from each pathogen and sampling time were displayed as a bar graph, with the upper limit showing the high dosage risk, and lower limit for low dosage risk

(Figure 11 a, b). Worldwide, vaccines are used as preventive measures for many viruses, currently RotaTeq™ and Rotarix™ are the two available 2nd generation vaccines to prevent illness from rotavirus, with 3rd generation vaccines in development (Greenberg & Estes, 2009). There is currently no vaccine available for norovirus, though one has reached human trials, in its development process (Flynn, 2016).

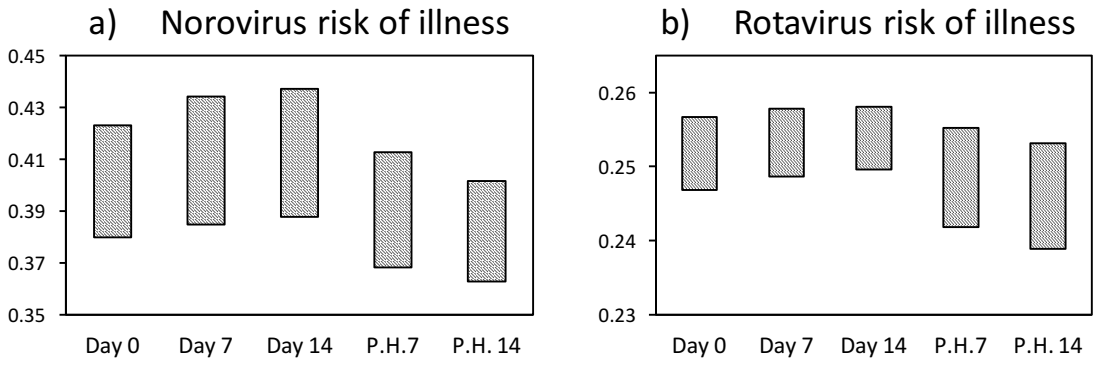

Figure 11: Risk of illness range for a) norovirus and b) rotavirus, with lower limit as low dosage risk, and upper limit as high dosage risk for all sampling days, including post-harvest (P.H. 7 and P.H. 14). Norovirus risk of illness is based on 60% of infection rate, whereas rotavirus risk is based on 26% chance of illness from infection.

Norovirus displayed more risk of illness than rotavirus for each sampling time. This difference is largely due to the rate of manifesting clinical symptoms post-infection. Since each pathogen has its unique dose response curve, probability of infection varied significantly between viruses, even though the dosage concentration was identical.

Norovirus displayed its largest probability of infection equal to 73% on day 14 for the high dosage scenario, whereas rotavirus was 99%. These findings show that while the probability of infection is larger with rotavirus than norovirus, the probability of an illness is less because of the available vaccines, which are 74% effective in preventing symptoms such as diarrhea (Greenberg & Estes, 2009).

Throughout the sampling days, risk of illness directly mirrored the trend in concentration of AP205, climbing to day 14 and descending during post-harvest storage. Even though, there was a 2-log decrease in concentration during post-harvest storage, the risk associated with each pathogen was not significantly affected. The largest difference in risk of illness for norovirus was from day 14 (pre-harvest) to post-harvest day 14, decreasing less than 4%. This lack of direct correlation can be attributed to the dose-response curve. In many cases, once a specific dosage is reached the rate at which risk increases eventually plateaus (Haas et al., 1999).

In accordance with EPA standards, only FIB concentrations are required to be monitored in effluents leaving the WWTP, not viral loads (USEPA, 2017). Water quality standards should be in place to evaluate pathogen prevalence in wastewater effluent and irrigation water if reused wastewater is used for fresh produce production. These tests are vital to ensure the safety of fresh produce products, as without proper treatment, there is a serious threat to public safety. Moreover, wastewater reuse for fresh produce should not be recommend as an alternative irrigation source, based on the observed results, unless steps are taken to ensure the water is safe to use and will not threaten public health.

5.5 Conclusions

A direct relationship exists between the viral contamination of irrigation water and the contamination levels of subsequent biomass such as foliage, soil, and leachate. Soil and leachate that become contaminated can pose health risks for future generations of crops, especially those with low growing foliage that have direct contact with the ground. Low and high dosages of effluent provided similar results, both causing foliage contamination to increase throughout cultivation, and decrease in post-harvest storage. However, the 2-log difference in dosages, only slightly decreased the risk of illness from norovirus and rotavirus by 5% and 1%, respectively. There was a significant drop in level of viral contamination of foliage during post-harvest storage at 4 ºC. There are potential public health risks from using non-disinfected wastewater effluent to irrigate crops. The EPA does not require WWTP's to monitor virus concentration in effluent, only FIB, which based on this study's results, could potentially compromise public health if similar viral concentrations were observed. Under worst-case scenario slightly more than 4 out of 10 people consuming the studied crop have the potential to become ill from norovirus, and just under 3 out of 10 can become ill from rotavirus. If wastewater effluent is to be used as an alternative source of fresh produce production, it is highly recommended that the water pass through disinfection processes with either chlorine or UV treatment and its quality tested to mitigate the public health risks. As more is learned about the fate and transport of viruses in crop irrigation, methods of water treatment may allow for wastewater irrigation to be a safe and viable alternative in the future.

CHAPTER VI

OVERALL CONCLUSIONS

In this thesis, generic *E. coli* and AP205 were used to track the fate and transport of related microorganisms in the wastewater irrigation of romaine lettuce. Levels of contamination in lettuce foliage were used to estimate the inherent risk of illness from related pathogens found in wastewater using QMRA analysis. Contamination levels of both *E. coli* and AP205 in foliage, leachate, and soil were correlated with the initial levels of contamination in irrigation water. Post-harvest storage displayed contrasting results, with *E. coli* concentrations increasing over time, while AP205 decreased. This shows that microorganisms behave differently when subjected to extended periods of storage at 4 ºC, and can increase the level of illness risk. Significant antibiotic resistance of *E. coli* isolates was observed to ampicillin, and 75% of antibiotics had levels of intermediate resistance. The levels of intermediate resistance in bacteria of antibiotics are important to monitor in the future, as these organisms can evolve to become fully resistant, and pass along resistant genes. This experiment proves that wastewater reuse for irrigation of lettuce and other ready-to-eat crops can pose a significant risk to humans if contamination levels are similar to the ones in this study. Contaminated soil and runoff can potentially influence future generations of crops and any use of water downstream. While FIB can provide a reference for other pathogens in wastewater, it is important to determine actual levels of microorganisms such as antibiotic resistant bacteria and norovirus, which displayed the greatest health risk in this study. To be considered safe to

use, stringent regulations should be in place to require the testing of reclaimed water for pathogen presence.

CHAPTER VII

FUTURE RECOMMENDATIONS

Future studies should consider the following:

- Grow lettuce from seed to minimize levels of initial contamination in soil.
- Control *E. coli* concentrations in irrigation water by inoculating specific concentrations into sterile wastewater effluent prior to each irrigation.
- Compare low and high concentrations of *E. coli* contamination for fate and transport to determine differences in fate, transport, and risk of illness from consumption.
- Evaluate effectiveness of microbial inactivation for different dosage levels of UV-irradiation and chlorine treatment to wastewater prior to irrigation.
- Irrigate with contaminated water several times per week to more closely simulate standard irrigation practices.
- Implement intervention strategies ("kill step"), such as a chlorine rinse or UV-irradiation treatment pre and post-harvest, to determine their effects on pathogen load and inherent risk of consumption of foliage
- Test the effect of direct presence of antibiotics in the water at different concentrations on the ARB fate and transport.
- Monitor how all experiment changes affect antibiotic resistance bacteria prevalence and transport.

- Select the most prevalent antibiotics and quantify the resistant microorganisms using metagenomics analysis and their fate and transport.

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