Evaluation and validation of the efficacy of peroxyacetic acid and chlorine as antimicrobial treatments for agricultural surface water to be used post-harvest

by

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B.S., University of Nebraska Lincoln, 2020

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2022

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Abstract

The recent increase in food-borne illness cases associated with produce threatens public health, the produce industry, and the national economy. Water used in produce washing is one of the routes of microbial contamination, hence the increasing interest and importance of finding efficient antimicrobial disinfectants to treat wash water to ensure that it is potable and safe for produce washing. Objective one evaluated peroxyacetic acid and chlorine as treatments for simulated surface water inoculated with a non-pathogenic *Escherichia coli* cocktail (ca. 5 log colony forming units (CFU/ml) to determine their efficacy at achieving 'no detectable generic *E. coli* in 100 mL of post-harvest agricultural water'. Simulated surface water was prepared to turbidity levels of two and 100 NTU using PTI Arizona Test Dust. Each of the two turbidities was adjusted to both pH 6.5 and 8.4, and the turbid water was equilibrated to 32 and 12°C. Each sample was inoculated with ca. 5 log CFU/ml of non-pathogenic *E. coli* and treated with either free chlorine (C) 25±2 ppm, peroxyacetic acid (PAA) 75±5 ppm, or a sterile water control (W), with a 10 second (s) contact time. Following the 10 s contact time, samples were neutralized in Dey-Engley Broth, and *E. coli* was enumerated using *E. coli*/Coliform (EC) Petrifilm® at 0, 5, 10, 60, 1440, and 2880 minutes (min). At each time point, samples were also enriched in 2X brain heart infusion (BHI) broth to be streaked on MacConkey agar plates to confirm the absence of *E. coli*. All C and PAA treated samples were below the limit of detection on EC (5 CFU/ml) and negative for *E. coli* on MacConkey plates starting from the 0 min time point. This suggests the achievement of no detectable levels of *E. coli* by both C and PAA after a 10 s contact time. Objective two validated the efficacy of C and PAA at reducing *E. coli* in surface water sources to the 'no detectable generic *E. coli*' for agricultural water that will have post-harvest contact with the produce, as required by the Food Safety Modernization Act for post-harvest use. Rain barrel

and creek water sources obtained from two different farms were inoculated with ca. 5 log CFU/ml of a non-pathogenic *E. coli* cocktail and equilibrated at temperatures 32 and 12°C. Samples were treated as described in objective 1 and enumerated at 0, 5, 10-, 60-, 1440-, and 2880-min post treatment using the FDA- approved IDEXX Quanti-Tray/2000 Colilert method and EC Petrifilm. Samples were enriched in 2X BHI and streaked on MacConkey agar to test for the absence or presence of *E. coli*. Using Colilert, *E. coli* was detected until 60 min contact time in samples treated with C, while *E. coli* was not detected in samples treated with PAA at the 0 min (10 sec contact time) sampling point. When enumerating with EC Petrifilm, the W sample harbored 5 logs *E. coli*, which was statistically different than C and PAA ($P \leq 0.05$), both of which were negative for *E. coli* following enrichment in 2X BHI. Data collected during objectives one and two suggest that at the evaluated dosage levels and water conditions, chlorine and peroxyacetic acid effectively achieve no detectable *E. coli* in surface water sources in less than 60 min post-treatment.

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Acknowledgements

I want to thank my advisor, Dr. Sara Gragg, for taking a chance on me as a graduate student in her lab and for going through this research journey with me every step. I wouldn't have been able to do this research without her consistent advice, mentorship, and early mornings on trial days. I also want to thank my committee members Dr. Londa Nwadike and Manreet Bhullar, for their continuous, direct involvement, help, and guidance in making this project a success. Adding onto my advisor and committee members, this project was a team effort, and it wouldn't have been a success without the consistent expertise and advice shared by Dr. Don Stoeckel and Mr. Patrick Byers, so I want to extend my gratitude to them as well.

Additionally, I want to thank the undergraduate and graduate student workers in the K-State Food Safety and Defense Laboratory, Daniel Vega, Kellen Habib, Jimeng Bai, Erin Fashenpour, William Jaggers, Kellen Marcum, and Connor Horn, for all their assistance in the project preparation and sample analyses.

Finally, I would like to thank my mom, Pascasie Mugabekazi, and little sister, Chania Cyuzuzo, for being my inspiration and support system throughout my life. Thank you to my whole family and friends for motivating, celebrating, and seeing me through this journey.

Chapter 1 : Introduction and research questions

Fruits and vegetables are an essential part of the everyday diet, with proven health benefits that include lowering the risk of type 2 diabetes, cardiovascular diseases, and other chronic diseases (Griep et al., 2010; Slavin & Lloyd, 2012). The increasing demand for fresh fruits and vegetables has resulted in a rapidly growing horticultural trade commodity, serving as a source of income for several growers (Huang, 2004). Unfortunately, increasing produce consumption might have also contributed to the increase in food-borne illnesses associated with fruits and vegetables. In the US, there are an estimated 48 million cases of food-borne diseases every year, and produce and nuts are responsible for nearly half of the cases where an implicated food is known (Painter et al., 2013). This food-borne illness increase is a big concern for public health and the economy. Most produce is ready to eat and consumed raw, so these commodities are more prone to causing food-borne diseases after consumption. As an example, leafy greens, mainly romaine lettuce, were linked to 51 food-borne illness outbreaks from 2014 to 2018 (CDC, 2022b)

Some significant microbial pathogens associated with produce-caused food-borne illnesses include the following: pathogenic *Escherichia coli, Listeria* spp., *Salmonella* spp., norovirus, *Cyclospora,* and many more. Food-borne illness-causing organisms can be considered as enteric pathogens, which means that they cause Gastrointestinal (GI) diseases when they, or their toxins, are consumed by an individual (Kolling et al., 2012). Symptoms of food-borne illnesses range from a mild fever, nausea, and stomachaches to dangerous organ failures, such as hemolytic uremic syndrome caused by *E. coli* O157:H7, impaired cognitive development in children, and death (Gould et al., 2009; Kolling et al., 2012).

Water used in the irrigation and post-harvest handling of produce has been linked to numerous outbreaks of food-borne illnesses where produce was the implicated food vehicle. When contaminated water is used to irrigate plants, it can transfer the microorganisms to the produce, where they can attach to the product's surface or internalize through either natural openings or bruises (Warriner et al., 2003). If contaminated water was used for post-harvest washing of produce, this would be direct source of contamination to the product. Also, if contaminated produce contaminate the wash water during washing, it can lead to a mass cross-contamination event where produce that wasn't otherwise contaminated is now infected (Holvoet et al., 2012).

Chemical antimicrobial interventions such as chlorine (C) and peroxyacetic acid (PAA) are FDA-approved, affordable, and regularly used by growers in agriculture. Unfortunately, the efficacy of these chemicals is affected by the microbial and physicochemical characteristics such as turbidity, temperature, pH, and organic content of the water being treated (Davies et al., 1995; Huang et al., 2020).

The Food Safety Modernization Act Produce Safety Rule (FSMA-PSR) is a regulation that requires minimum operation standards that growers must abide by to supply produce for sale (FDA, 2020). The quality of agricultural water used for direct contact with produce is a critical aspect of the FSMA-PSR. More specifically, agricultural water that may directly or indirectly contact the produce (*i.e.*, post-harvest use) must have 'no detectable generic *E. coli*' (FDA, 2022a). Municipal/city-supplied water tends to be the safest potable water source, but it can be expensive, and not all growers have easy access to it. Despite its extended use and availability, the likelihood of surface water microbial contamination is high due to its exposure to environmental/human activities (Jamieson et al., 2004; La Borde, 2020). To be used in the post-

harvest activities, surface water sources must be treated to satisfy the FSMA-PSR requirements. Unfortunately, surface water sources reportedly have microbial levels greater than the maximum allowed by the FSMA-PSR, especially if they are to be used for post-harvest purposes (Haley et al., 2022).

Many small-scale farmers such as the Amish, Mennonites, and refugee growers in Kansas (KS) and Missouri (MO) require continuous guidance and education on properly implementing and abiding by the FSMA-PSR water standards. Hence there is an urgent need to identify and validate effective antimicrobial treatments for surface water to be used for post-harvest purposes that can: 1) effectively treat surface water that has varied composition in terms of turbidity, pH, temperature, etc., and 2) satisfy the FSMA-PSR requirements for agricultural water to have 'no detectable generic *E. coli*' when in direct contact with the produce or indirectly through post-harvest use. Thus, the two objectives of this thesis were: 1) evaluate the effect of turbidity, pH, and temperature on the efficacy of two FDA-approved antimicrobial interventions (chlorine and peroxyacetic acid) at reducing generic *E. coli* in simulated surface water, and 2) validate the efficacy of chlorine and peroxyacetic acid at reducing generic *E. coli* in two different surface water sources (creek and rain barrel sources).

Research questions

1. What time is required for chlorine and peroxyacetic acid to achieve the required ‘no detectable generic *E. coli* for post-harvest use’ in lab-simulated surface water?
2. Will turbidity, pH, and/or temperature significantly affect the time required for chlorine and peroxyacetic acid to achieve the required 'no detectable generic *E. coli*' for post-harvest use in lab-simulated surface water?

3. Will turbidity, temperature, and pH of the lab-simulated surface water significantly affect the microbial concentration over time?
4. Is chlorine or peroxyacetic acid more effective at achieving the required 'no detectable generic *E. coli*' for post-harvest use in lab-simulated surface water?
5. What contact time is required for chlorine and peroxyacetic acid to achieve the required 'no detectable generic *E. coli'* for post-harvest use in the rain barrel and creek water?
6. Is there a difference in the efficacy of chlorine and peroxyacetic acid to achieve the required 'no detectable generic *E. coli*' for post-harvest use in creek water compared to rain barrel water?
7. Does the temperature at which the creek and rain barrel water are stored impact the efficacy of chlorine and peroxyacetic acid to achieve the required 'no detectable generic *E. coli*' for post-harvest use?

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Chapter 2 : Literature review

2.1Pathogens associated with produce and wash water food-borne outbreaks

2.1.1 Description

Several bacterial, mold, protozoan, and viral microorganisms are often linked to food-borne outbreaks. Microorganisms can be classified as pathogenic and non-pathogenic. Pathogenic microorganisms and/or their secreted substances can cause illness in their host (people), while non-pathogenic microorganisms won't cause disease. Some major pathogens associated with food-borne infections include *Salmonella* spp, *Listeria monocytogenes*, *Escherichia coli*, *Vibrio* spp, *Campylobacter* (first source of infection), *Cyclospora*, *Clostridium* spp, norovirus, and Hepatitis A (CDC, 2019c; Pigłowski, 2019). *Salmonella*, *Escherichia coli, Listeria*, *Cyclospora*, Hepatitis A, and norovirus are the most linked to produce-associated outbreaks (Beuchat, 1996; CDC, 2019c).

Bacteria are unicellular, prokaryotic microorganisms that can be classified according to their shapes: curve/comma-shaped, bacilli/rod-shaped, and spherical/coccus-shaped (Tong, 2019). They can also be classified as Gram-positive or Gram-negative based on the thickness of the peptidoglycan layer in their cell walls. Gram-positive bacteria have a thicker peptidoglycan layer, while Gram-negative have a thinner peptidoglycan layer (Tong, 2019). Gram-negative bacteria can also be differentiated from Gram-positive bacteria by their possession of an outer membrane mainly composed of lipopolysaccharides (LPS). LPS are molecules in the outer membrane of Gram-negative bacterial cells, and LPS structures enhances the reduction of permeability of the bacterial cell outer membrane, which increases the bacterial resistance to antimicrobials and the natural immune responses of the host (Maldonado et al., 2016). Most bacteria that cause food-borne diseases reside in the host gastrointestinal (GI) tract and are

known as enteric pathogens (Riley, 2020). Some enteric bacteria are beneficial to the host gut, aiding digestion, and decomposition of food. At the same time, some bacteriophages act to protect the gut flora against the attachment of opportunistic/infectious bacteria (Balali et al., 2020). *Salmonella* spp, *Escherichia coli*, *Campylobacter,* and *Vibrio,* are considered Gram-negative, enteric bacteria that are pathogenic to humans. *Clostridium* spp and *Listeria* spp are examples of Gram-positive food-borne pathogens.

Viruses are obligate intracellular microorganisms that require a vulnerable living host cell to reproduce and cause infections (Balali et al., 2020). These microorganisms are unable to procreate and proliferate outside the host cell, but they can survive for a short time on different surfaces and within the environment. However, a large knowledge gap remains in terms of understanding their behavior and survival in the environment or laboratory spaces (Bosch et al., 2018). The most common viral enteric pathogens are norovirus (the cause of most food-borne illness-associated deaths of all enteric pathogens) and Hepatitis A (Bosch et al., 2018). They are transmitted through a fecal-oral route that can happen directly from person-to-person interactions or indirectly from food and water contaminated with feces (Balali et al., 2020).

Protozoans are unicellular, eukaryotic microorganisms that are ubiquitous in the environment (Yaeger, 1996). They can be parasitic or free living. Parasitic protozoa require a living host to live, grow and reproduce, but they can survive for approximately 2-6 months outside the host (OSU Extension, 2012). The impact of enteric parasites will vary with the host's immunity, and the effect may be severe in immunocompromised individuals, such as those with AIDS (Yaeger, 1996). Yaeger (1996) also discussed that when immunity is strong enough to fight the parasitic infection, humans may asymptomatically carry the parasite with the ability to transmit it to another person. The major parasites that have been associated with produce food-

borne illnesses, according to the CDC and other food reports, are *Giardia lamblia*, *Cyclospora cayetanensis,* and *Cryptosporidium parvum* (Beuchat, 1996; CDC, 2019c; OSU extension, 2012). They are transmitted through a fecal-oral route or person to person contact (OSU Extension, 2012). Table 2.1 summarizes the symptoms and sources of different enteric pathogens associated with produce food-borne outbreaks.

Table 2.1 Symptoms and sources of some microbial pathogens that have been linked to outbreaks of produce-associated illness (Adapted from Harris et al. 2012)

Microorganism	Symptoms	Source
Bacteria		
Clostridium botulinum	Nausea, vomiting, fatigue, dizziness, dryness of mouth and throat, muscle paralysis, difficulty swallowing, double, or blurred vision, drooping eyelids, and breathing difficulties	soil, lakes, streams, decaying vegetation, reptiles
Escherichia coli O157:H7	Bloody diarrhea, abdominal pain. Can lead to hemolytic uremic syndrome and kidney failure especially in children and the elderly	animal feces, especially cattle, deer, and humans; cross-contamination from raw meat animal and human feces.
Salmonella spp.	Abdominal pain, diarrhea, chills, fever, nausea, vomiting	cross-contamination from raw meat, poultry, or eggs
Shigella spp.	Abdominal pain, diarrhea, fever, vomiting	human feces soil, food processing
Listeria monocytogenes	Febrile gastroenteritis in healthy adults. May lead to spontaneous abortion or stillbirth in pregnant women; severe septicemia and meningitis in neonates and immunocompromised adults; mortality may be 20 to 40%	environments
Parasites		
Cryptosporidium spp.	Profuse watery diarrhea, abdominal pain, anorexia, vomiting	Animal and human feces
Cyclospora spp.	Watery diarrhea, nausea, anorexia, abdominal cramps (duration 7 to 40 days)	others? specific environmental sources unknown currently
Viruses		
Hepatitis A	Fever, malaise, anorexia, nausea, abdominal pain, jaundice, dark urine	human feces and urine
Norwalk/Norwalk-like virus	Vomiting diarrhea, malaise, fever, nausea, abdominal cramps	human feces, vomitus

2.1.2 Sources, transmission route, and survival of produce-related pathogens

There are several sources for various of pathogens to contaminate produce and cause major food-borne illness outbreaks associated with produce consumption. Potential sources of enteric pathogen contamination include animal feces, agriculture watersheds, dust, water runoff, manure, post-harvest handling practices, and more (Mandrell, 2009). *E. coli* O157:H7, non-O157 Shiga toxin-producing *E. coli* (STEC), and other enteric pathogens, such as *Salmonella*, are associated with feces, which may either directly or indirectly come in contact with produce (Heredia & García, 2018).

The carriage of food-borne pathogens by wild animals, livestock, and their subsequent shedding fecal shedding, can depend on several things, including how the animal encountered the pathogen (grass, water, manure, etc.), the consumed pathogen dose, the viability and stability of the pathogen in the GI tract of the animal, the use of antimicrobials during livestock production, the handling/disposal of the animal feces, animal movement and contacts, or a combination of these, and more (Mandrell, 2009). Global prevalence of Shiga Toxin producing *E. coli* (STEC), both O157 STEC (0.2-28%) and non O157 STEC (2.1-70.1%) in beef cattle were reported by Hussein & Bollinger (2005), with 44 STEC strains of cattle origin being isolated from patients with hemolytic uremic syndrome (HUS). *Salmonella enterica* and *Campylobacter* spp. in the feces of livestock (cattle, sheep, pigs) and several wild animals (deer, ducks, and more) have also been documented (Mandrell, 2009).

The amount and type of enteric pathogenic species shed by animals are as substantial as their detectability when causing food-borne outbreaks. About 75% of cows that are positive for *E. coli* O157 in feces usually release *E. coli* O157 at concentrations of ≤100 CFU/g, which makes it difficult to quantify in a complex microflora sample. However, other cows are known as

super shedders because they can release 10,000-10,000,000 CFU/g of *E. coli* O157 in their feces. Super shedders might be of a small number on a farm but release the majority of the contamination and have been linked to an increase in on-farm transmission, a 100- or 1,000-fold increase in human exposure infection risks (Chase-Topping et al., 2008; Munns et al., 2015). Shedding of pathogens like *E. coli* O157:H7 can influence produce contamination by directly infecting other animals in proximity or through the environmental contamination caused by cattle, thus the increased contamination risk caused by super shedders is a potential threat to human health (Munns et al., 2015).

Humans with the potential of encountering produce, also known as food handlers, can also serve as a transmission route and source of contamination. Humans can asymptomatically carry pathogenic microorganisms in their GI tract and feces. A community-based study conducted by Solomon et al. (2018) using 387 food handlers found that about 41% of them had an intestinal parasite, and 35 primarily drug-resistant *Salmonella* isolates were found in the stools of the tested food handlers. The burden of these microbial diseases in these food handlers was related to raw meat consumption, no handwashing after using the toilet, and no handwashing after touching dirty objects. Food handlers were reportedly contaminating produce due to unsanitary practices, for example, either by directly touching the produce with unwashed hands or cross-contaminating produce by mixing raw and cooked foods (Ehuwa et al., 2021)

Agriculture watersheds and other environmental transmissions (through soil, manure, dust, and more) must also be mentioned as essential sources of pathogenic contamination. Pathogens can be transmitted to watersheds when animal feces are directly deposited, or pathogens shed into the soil are transported to watersheds by runoff or other shallow water flow (Devane et al., 2018). In response to leafy green outbreaks in California, different Salinas Valley

watershed sites were tested for *E. coli* O157:H7 to identify a possible transmission route(s) to nearby farms, mainly because they often flood during the winter months. An overall prevalence of 6.5% was reported in the study for all sites, with increased positivity associated with locations closer to a cattle grazing operation and during the heavy rainy season (Cooley et al., 2007). This suggests a water transport of *E. coli* O157 by watershed to the produce farm from cattle grazing operations. However, the study reported no definitive mechanism of contamination, or even transportation, due to inconsistent results found over the different sampling times. On the other hand, a 30% prevalence of at least one of the three major food-borne pathogens (*E. coli* O157, *Salmonella* spp*., and Listeria* spp.) was reported in livestock manure in Britain (Sharma & Reynnells, 2016). Also, a study conducted by Weaver et al. (2016) showed leaching of *E. coli* from soils treated with cow feces (raw manure), especially under flood irrigation, demonstrating one mechanism of groundwater contamination. For an outbreak to occur, it is unlikely that one source of the pathogen is sufficient; instead, it is more likely that the contamination event is the result of different sources, transmission routes, and other factors that led to the outbreak (Fan et al., 2009).

The virulence and genetic make-up of *E. coli* O157:H7, as well as the genetic make-up of various commensal *E. coli* released through the shedding of animal feces, favors their survival from the animal GI tract to the secondary reservoir (soil, water, manure) (Durso et al., 2004). The survival of different pathogens in the environment will also vary depending on environmental factors, such as temperature, pH, moisture, etc. The prevalence of *E. coli* O157, *Listeria* spp., and *Salmonella* spp in livestock is reportedly significantly influenced by seasonality, with the highest levels reported in warmer months of the summer (Barcocky-Gallagher et al., 2003; Likavec et al., 2016; Webb et al., 2017). A study conducted by Calle et al.

(2021) found higher *E. coli* O157, STEC non-O157, and *Salmonella* shedding in cattle feces during rainy months than in dry months, but with a significantly higher incidence of *Salmonella* on carcasses in dry months compared to rainy months. The prevalence of *E. coli* and coliforms in vegetables was found to be higher in rainy seasons compared to dry seasons when cucumbers, tomatoes, and lettuce were studied in Cambodia. *Salmonella*'s seasonal effect may vary with vegetable type, with the highest levels found on lettuce (5.7 logs of *S. enterica*) compared to cucumbers (4.2 logs) and tomatoes (4.3 logs) (Desiree et al., 2020). Higher fecal indicator generic *E. coli* levels in the water were observed in warmer seasons than in cold seasons (Baker et al., 2021). Simultaneously, the highest infection rates and outbreaks of enteric diseases caused by *Salmonella*, *Listeria*, and STEC were observed during the summer in Canada (John et al., 2022). The variable effects of the seasons on enteric pathogens suggest a necessity to maintain microbiologically safe environments (feedlot, water, household, etc.) year-round.

Overall, enteric pathogens are likely transmitted to produce by direct contact with animal or human feces or indirectly water, wind, soil, or manure. *E. coli* inhabit the intestinal tract of warm-blooded animals, and they spread through the fecal-to-oral transmission route (Odonkor & Ampofo, 2013). The potential risks associated with fecal exposure to produce, both directly and indirectly, emphasize the need for prevention in the produce industry at both the pre-and post-harvest stages.

2.1.3 Generic E. coli *as an indicator organism*

Escherichia coli are Gram-negative, rod-shaped, facultatively anaerobic bacteria that are residents of the human and animal intestinal tract, thereby classifying them as enteric bacteria (Riley, 2020). The *E. coli* species are diverse and classified into non-pathogenic and pathogenic *E. coli*. Most *E. coli* serovars are non-pathogenic; they are not harmful to people and

do not cause illness (CDC, 2019a). Pathogenic *E. coli* serovars, also known as pathovars, are the variants able to cause illness when humans ingest their cells (Croxen et al., 2013). The different variants of *E. coli* that cause intestinal diseases can be grouped into five classes based on their pathogenicity, including enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC). Their virulence characteristics and disease symptoms are shown in Table 2.2

Table 2.2 The five virotypes of *E. coli* that are known to cause intestinal diseases in humans and their target hosts (Adapted from Todar, 2020)

Virotype	Virulence determinants	Disease characteristics
Enterotoxigenic *E. coli* (ETEC)	- Fimbrial adhesins - Produce Enteroxin (ET) or Heat stable toxin (ST) - Noninvasive	Watery diarrhea in infants and travelers; no inflammation, no fever
Enteropathogenic *E. coli* (EPEC)	-EPEC adherence factor (EAF) moderately invasive	Infantile diarrhea; watery diarrhea with blood, some inflammation, no fever; symptoms probably result mainly from invasion rather than toxigenesis
Enteroinvasive *E. coli* (EIEC)	-Nonfimbrial adhesins, possibly outer membrane protein -Invasive	Dysentery-like diarrhea (mucous, blood), severe inflammation, fever
Enteroaggregative *E. coli* (EAEC)	-Noninvasive -Produce ST-like toxin and a hemolysin	Persistent diarrhea in young children without inflammation or fever
Enterohemorrhagic *E. coli* (EHEC)	- Moderately invasive - Produce Shiga toxin (STEC)	Pediatric diarrhea, copious bloody discharge (hemorrhagic colitis), intense inflammatory response, and may be complicated by hemolytic uremia

With their fecal origin generic *E. coli* are used as an indicator organism, whereby their presence in food, water, and the environment indicates the possible presence of enteric

pathogenic microorganisms that are very dangerous to humans. Generic *E. coli* are recommended as an indicator organism in the literature and by various regulations for several reasons. Generic *E. coli* are also a preferred indicator organism due to the availability of their many simple, inexpensive, and rapid techniques to detect them in a variety of environments and food matrices (Odonkor & Ampofo, 2013). Different publications have also reported that pathogenic *E. coli* can lose their plasmid in differential broths and might not grow well at 44°C and 45.5°C compared to non-pathogenic thermotolerant *E. coli* (Odonkor & Ampofo, 2013; Pachepsky et al., 2016; Rice et al., 1999). The total coliform test was the global gold standard for identifying fecal contamination in drinking water. However, these tests have increasingly been replaced by generic *E. coli* testing in revised global drinking water guidelines by the World Health Organization (WHO), the Australian government, the European Union, as well as the agricultural water guidelines by FSMA-PSR because some coliforms aren't of fecal origin (Standridge, 2008). However, the assumption that the only source of *E. coli* is from a recent fecal contamination is rebutted by a review done by Jang et al. (2017), which showed that some *E. coli* strains could survive for more extended periods in the environment and even reproduce and adapt to environmental conditions becoming part of the natural environmental community. The latter finding indicates a probable nonfecal source of *E. coli* over time, undermining their indication of recent fecal contamination.

The indicator organisms recognized by different regulatory organizations of various countries will also vary. Table 2.3 summarizes the other organizations worldwide and the respective indicator organism requirements for safe/potable water and for safe post-harvest agricultural water by FSMA-PSR. It is important to emphasize that the presence of *E. coli* in surface water does not confirm the presence of other microorganisms. For example, Cooley et al. (2007)

reported a lack of correlation of generic *E. coli* with *E. coli* O157:H7 during Salinas Valley watershed testing, except for one specific site. Generic *E. coli* levels were high (400 MPN/100ml) in areas where *E. coli* O157:H7 tests were negative. Recently though, Truchado et al., (2018) reported that generic *E. coli* concentrations of ≥ 2.35logs CFU/100ml generally were accurate for predicting the presence of a pathogen in surface water with a specificity of 66%. It is crucial to point out that some samples with *E. coli* as low as 1.0 log CFU/100ml also harbored a pathogen. This suggests that generic *E. coli* presence indicates potential fecal contamination, which also suggests a higher risk of the presence of other fecal pathogens, such as Hepatitis A virus and *Salmonella* spp. (Pachepsky et al., 2016).

Table 2.3 Requirements for verifying the microbiological quality of water intended for human consumption, as established by the WHO, European Union, and Spanish legislation; and requirement for microbial quality of post-harvest agricultural water by FSMA-PSR. adapted from (Pichel et al., 2019)

Microorganism	**FSMA-PSR**	**WHO**	**European Union**	**Spain**
Escherichia coli	0 CFU/100 ml	0 CFU/100 ml	0 CFU/100 ml	0 CFU/100 ml
Thermotolerant coliforms		0 CFU/100 ml	–	–
Total coliforms		–	–	0 CFU/100 ml
***Enterococcus* spp.**		–	0 CFU/100 ml	0 CFU/100 ml
Clostridium perfringens		–	–	0 CFU/100 ml

2.2Surface water

2.2.1 Description and quality determining factors

Surface water sources significantly contribute to the aquatic, wildlife, and agriculture practices around them. According to the Kansas Department of Agriculture (KDA), 2021, 87.5 percent of all Kansas land is farmland. The continuous overuse of groundwater resources, such as the high plains aquifer (Ogallala) in Kansas could lead to a lowering of the water table, which threatens the future of groundwater sources (Pfeiffer & Lin, 2014; Steward & Allen, 2016). In

Kansas, about two-thirds to three-quarters of the total water moved by Kansas from their original source is groundwater, with the remaining being surface water (Lanning-Rush & Restrepo-Osorio, 2017). About 60% of the public municipal supplied water is from surface water, but municipal water only covers about 10% of the total water withdrawn for consumptive uses, while irrigation uses about 85% (USGS, 2014). The exploration of readily available surface water sources such as rain collected water, creeks, and ponds for produce rinsing and other agriculture activities would relieve the pressure from groundwater depletion. It might also reduce growers' water buying expenses.

Surface water sources are those exposed to the environment (*e.g.*, rivers, lakes, ponds, rain-collected cistern water). The use of surface water sources in either pre-harvest or post-harvest handling of produce is complicated because their openness to the environment makes them vulnerable to contamination from agricultural activities (animal waste, toxic metals, and organic compound erosion), precipitation, human activities (industrialization, cattle rearing), and other environmental pollutants (Ichor et al., 2014). Runoff and rain lead to sediment, pesticide, nutrient, and bacterial accumulation in surface water streams (Stamer et al., 1987; Korajkic et al., 2018). Rain barrels are often engineered to collect water runoff from a roof collection system. Rain barrel water quality is highly impacted by the rooftop material and accumulated environmental pollution, which could lead to the collection of unsafe/poor quality water that shouldn't be used for post-harvest purposes without treatment (Gwenzi et al., 2015; Rahman et al., 2014).

Microbial quality, physical quality, and chemical impurities indicate water quality. Water used for drinking and post-harvest purposes should be free from pathogens and other physical and chemical impurities at excessive or toxic levels. Groundwater is more likely to be free from

poisonous and microbial impurities, while surface water is exposed to the outside environment, and its quality may be highly influenced by the environmental/human activities in the immediate vicinity (Tucker & Hargreaves, 2004). Some of the most important physical characteristics of pond water and other surface waters are temperature, pH, dissolved oxygen, and turbidity, all of which will affect the viability of the water for different purposes (Christensen, 2001; Tucker & Hargreaves, 2004). Drought causes the surface water temperature to rise, and with the presence of nutrients, this temperature increase can lead to the growth of different phytoplankton, which in turn raises the water pH, lowers dissolved oxygen, and renders the water eutrophic (Christensen, 2001; Tucker & Hargreaves, 2004; van Vliet & Zwolsman, 2008)

Other physicochemical criteria of water quality are total dissolved solids, total suspended solids, total organic carbon, total organic nitrogen, nitrates, and other chemical nutrients that could have resulted from effluents of different agricultural/industrial activities (Christensen, 2001). Various environmental, human, agricultural, and industrial activities can produce assorted solids deposited directly in water streams or around them (e.g., in the sediments), which could eventually be carried to the water. When solids of greater than 2 microns are floating in the water, they are known as total suspended solids, and any other particle smaller than 2 microns are known as total dissolved solids (Fondriest Environmental Inc, 2014). Both total suspended and dissolved solids may be comprised of organic and inorganic material in the water. Another indication of water quality is turbidity, which is the measurement of water clarity as measured by sun penetration through the water. Turbidity values can be influenced by suspended solids and colored dissolved organic matter, such as humic acid stains. Total suspended solids (TSS) and turbidity indicate water dirtiness and could increase potential microbial water contamination due to the possible attachment of pathogens and toxic metals to the TSS (USGS water science school,

2018). Dissolved organic carbon (DOC) is another essential water quality indicator. Large amounts of DOC have been directly linked to the reduction of light absorption and water pH fluctuation, affecting the aquatic life in the water (Evans et al., 2003). DOC was also found to bind to bacteria, thereby protecting them from disinfectants and increasing their chance of survival on produce, ultimately increasing the risk to public health (Huang et al., 2020).

Surface water microbial quality is reportedly worse during high streamflow seasons but is also influenced by other human activities within the vicinity of the water sources (Davis & Bell, 1998). Once microorganisms are in the water, their survival could be enhanced by the physicochemical characteristics of the water. The survival of different microorganisms, such as fecal coliforms, *E. coli*, and other fecal indicators in the water, was studied by researchers who noted the survival of these microorganisms in the water in conjunction with availability of nutrients, dissolved organic matter, and micro sediments to which bacteria can attach (Davies et al., 1995; Gerba & McLeod, 1976; Howell et al., 1996). On the contrary, Maki and Hicks (Maki & Hicks, 2002) reported no influence of sediments in water on the survival of *Salmonella* Typhimurium.

Rattlesnake Creek, one of the significant, beneficial water sources for agriculture and wildlife in south-central Kansas, has pH levels ranging from 7.7-8.3 and nine other physical, chemical, and microbial characteristics, including turbidity, dissolved oxygen, dissolved solids, sodium, chloride, phosphorous, total coliforms, generic *E. coli*, and fecal coliforms were reported to not meet the established water quality standards at some point in the two years of monitoring (Christensen, 2001). Christensen (2001) attributed most of the fluctuation to the various agricultural activities around the creek basin and the seasonal variations throughout the year. This study is also consistent with the study done by Davis & Bell (1998), which reported

elevated amounts of nitrate, nitrite, organic carbon, fecal coliforms, and *E. coli* in the Ozark plateaus river basins (including Kansas and Missouri), especially in basins around agricultural land use compared to forest land use. The above various physical, chemical, and microbial characteristics of water are essential to control because they influence the quality, such as the color and flavor of the water. Still, they can also impact the efficacy of antimicrobial interventions used to improve water safety. Rattlesnake Creek is one example of many other surface water sources that would not be acceptable for post-harvest use without a controlled validated treatment, according to the FSMA-PSR.

2.2.2 Surface water and public health

Microbial contamination of surface water is an essential indicator of water quality. When untreated water comes into contact with plants or is used in a post-harvesting processing facility for fresh produce, it can contaminate everything, including food and nonfood contact surfaces (Davis & Bell, 1998). The microbial contamination of surface water used in agriculture is globally linked to various food-borne/water-borne illness outbreaks. According to the FDA (2019), a fall 2018 outbreak associated with romaine lettuce and *E. coli* O157:H7 contamination which caused a multistate food-borne illness outbreak, was traced back to an on-farm water reservoir in California. In 2006, a multistate spinach outbreak was caused by *E. coli* O157:H7 from a river near a farm in California (Gelting & Baloch, 2013). In Arizona (AZ), 11 cisterns holding rain-harvested water were tested, 30% were contaminated with *E. coli*, and 100 % harbored Enterococci with higher microbial numbers in the summer than in winter. Also, 40% of the cisterns had a lead concentration higher than the allowable standard (Jordan et al., 2008). In developing countries where surface water sometimes serves as a source of drinking water, the

impacts of surface water in causing major diarrheal infectious diseases, such as typhoid and cholera, are more critical (Yaya et al., 2018).

Surface water microbial contamination that results in food-borne and waterborne outbreaks originates from various non-point sources, including indirect fecal contamination from animals and humans, overflown sewage systems, runoff due to land overexploitation, and poorly handled manure (CDC, 2018a). There are also different point sources of produce microbial contamination like water used in irrigation, crop chemical spraying, and produce wash water (CDC, 2018a). Guan et al. (2001) and Ng et al. (2005) have shown that when contaminated water is used to mix with pesticides, growth of pathogens such as *E. coli* O157:H7 can be supported, and pathogens may subsequently contaminate the fruits, vegetables, and harvest of the sprayed plants. The pertinent regulation, intervention, and/or treatment strategy depends on the intended use of the water. The FDA recommends the use of water of adequate quality to minimize microbial contamination of final products, with the meaning of adequate changing based on whether the water is to be used before or after harvest and the chance of the water being in contact with the produce (FDA, 2008).

2.3Produce

2.3.1 Description and rationale

The Food Safety Modernization Act Produce Safety Rule (FSMA-PSR) defines produce as any fruits or vegetables (including mixes of any intact fruits and vegetables) and includes mushrooms, sprouts, peanuts, tree nuts, and herbs. The consumption and demand for fruits and vegetables has steadily been increasing in association with an increase in income and health concerns.

The increase in fruit and vegetable consumption has varied across the globe, with the largest and most steady increases reported for Asia, Europe, North America, and Oceania between 1990 and 2000. However, in more recent years, only Africa and Asia have reported a steady increase in consumption per capita, while North America, Europe and Oceania have reported inconsistent consumption rates that started with a sharp decrease from all 3 continents around 2008 (Balali et al., 2020). Conversely, the availability of fresh fruits per capita in the U.S. has increased by 21% between 1980 to 2016, replacing the declining demand for processed fruit (Minor & Perez, 2018).

Fruit and vegetables have a variety of nutritional and health benefits, especially in terms of preventing chronic diseases due to their high content of vitamins, minerals, and dietary fiber. The World Cancer Research Fund has associated different fruits and vegetables, based on their nutritional or phytochemical contents, with the reduction of different cancers (Yahia et al., 2019). A diet rich in fruits and vegetables, mostly leafy greens such as spinach and kale, has been found to reduce the incidence and mortality rates of cardiovascular diseases (Huang et al., 2004; Wang et al., 2014). The increased consumption of whole fruits like grapes and berries was associated with a lower risk of type 2 diabetes, however, the increased consumption of fruit juices has been linked to an increase in type 2 diabetes (Muraki et al., 2013). People who replaced other food sources with an increased intake of fruits and non-starchy vegetables have been found to lose weight easier and faster than people with a reduced intake (Bertoia et al., 2015).

The consumption of raw produce (potentially contaminated) and current global widespread distribution system can lead to an increasing number of outbreaks since a single source of contaminated food can quickly be distributed globally. The different sources of

contamination mentioned above from farm to last consumer can either directly or indirectly lead to produce contamination, and the produce's water activity, pH and nutrient availability can aid in the growth of different pathogenic microorganisms (Wadamori et al., 2017). For example, the water activity of fresh produce ranges from 0.97-1, and the optimum water activity for *Salmonella* spp, EHEC and *Campylobacter* spp is 0.99. Most ready to eat produce also contains an adequate supply of nutrients (sugar, protein, iron, vitamins and more) needed by different microorganisms to grow effectively (Kotzekidou, 2016). Thus, when produce is contaminated with pathogens and consumed raw with no effective intervention, they may cause food-borne illnesses and outbreaks. Hence the crucial need to minimize microbial contamination of produce and identify effective interventions.

2.3.2 Produce contamination and public health

Most produce products are ready to eat, consumed raw, and their processing is minimal with no lethal heat treatment. This lack of treatment combined with increased consumption in recent years leads to a greater public health concern. An estimated 48 million food-borne illness cases are reported in the US annually with around 9.6 millions illnesses with a known implicated food and 38.4 millions with unspecified agent (Painter et al., 2013; Scallan et al., 2011). The most recent United States Department of Agriculture (USDA) estimates from a 2018 report that food-borne illnesses from the leading 15 pathogens cost the U.S. government around $17.6 billion annually (USDA ERS, 2018). Of the estimated 9.6 million annual food-borne illnesses with a known implicated food; fruits, vegetables, and nuts accounts for 46% of these illnesses (Painter et al., 2013; USDA ERS, 2019). Produce, as defined by the FSMA-PSR, represented a large percentage of the 15 foods that caused food-borne outbreaks in the U.S. (CDC, 2021).

From 2010-2017, an estimated 85 multistate food-borne outbreaks were linked to fresh produce (83 caused by bacteria), resulting in 4,658 illnesses, 1,187 hospitalizations, and 55 deaths (Carstens et al., 2019). Most of the outbreaks from fruits were caused by melons (10) followed by papaya (7), while in vegetables most outbreaks were caused by sprouts (16) followed by lettuce (8). Some of the most recent produce-related outbreaks included a packaged salad outbreak that caused 10 illnesses, 4 hospitalizations, and 1 death; a romaine lettuce outbreak from Salinas Valley, California that caused 167 illnesses, 85 hospitalizations, with 15 people who developed HUS; and many more that can be found at the CDC website (CDC, 2022a).

2.3.3 Internalization of pathogens by produce

The mechanism of microbial movement from the environmental source to the inside of free cell spaces within a plant is known as internalization (Bartz et al., 2015). Internalization of bacteria into the produce can happen either pre-harvest (in the field) or during post-harvest handling.

Pre-harvest contamination could originate from contaminated rainwater runoff, contaminated used irrigation water, animal or their excretion contact, seedling contamination, insect damage and contact, and many other pre-harvest conditions that will allow bacterial colonization of the plant/produce and could eventually lead to internalization of bacteria (Bartz et al., 2015; Mitra et al., 2009; Warriner et al., 2003). Pre-harvest produce contamination can enter through seeds, root uptake, leaves and stem (Bartz, 2009). Contaminated seeds can lead to growth of a contaminated plant with contaminated produce. For example, human pathogenic bacteria have been found to proliferate in seed sprouts, as the ideal growth conditions for sprouts are also ideal for human pathogen growth (Warriner et al., 2003). Seed contamination could

result from infiltration of bacteria into the seed, which has been reported with *Pseudomonas* (Gram-negative bacteria mainly found in soil, water and plants) (Kozlowski, 2012), or during germination. During germination, the seedlings release simple sugar and peptides that feed bacteria around their root system and allow bacteria entrance through the radicle and secondary roots. These bacteria can easily enter the xylem, as well as through growing leaves and fruits, because the Casparian strip that would protect the xylem is immature during germination (Howard & Hutcheson, 2003; Warriner et al., 2003). The infiltration of sprouting seeds would not represent other plant life stages because the growth environment of a plant, such as exposure to harsh sun and water stress, could lead to cuticle thickening (Bartz et al., 2015)

The uptake of bacterial contamination through the root to the plant vascular system and eventual contamination of leaves and fruits has been confirmed by a number of studies. Hintz et al. (2010) studied the ability of *Salmonella* in irrigation water to be internalized by the roots and transported to the shoot system (leaves and fruits). Of the 92 treated samples (20 roots, 20 stems, 20 leaves and 32 fruits) *Salmonella* was found in 36 (20 roots, 12 stems, 2 leaves and 2 fruits) after enrichment. This low number in the leaves and fruits shows weak transportation of bacteria into the vascular system. This is further supported by Mitra et al. (2009), who reported that nine plants grown in soil drenched with *E. coli* O157:H7 didn't result in internalization when using real time polymerase chain reaction (PCR) testing. The root uptake of bacteria from irrigation water is thought to be a passive process that is induced by hydrostatic pressure, which was tested using fluorescent polystyrene bacteria-sized beads in the irrigation that were eventually observed in the stem and leaves of plants (Deering et al., 2012).

Leaf and fruit contamination in the field as well as post-harvest during washing can lead to pathogen internalization through natural openings, natural lesions and/or artificially induced

wounds. Leaves and fruits have apertures such as stomata and lenticels that are used in respiration in respiration, when there is hydrostatic pressure caused by rain, overhead irrigation, spray washing these opening can flood in contaminated water providing a passive entrance to bacteria in their open apoplasts (Bartz et al., 2015). This mechanism would also apply in open wounds sustained by the fruits or leaves either due to natural lesions or caused by insects, produce rubbing on each other during washing and other damages. An active movement of bacteria deposited on leaves directly to the leaves' tissues and apoplast has also been found. For example, *E. coli* O157: H7 and some other non-pathogenic EHEC strains, once deposited on the leaves, were found to attach on the surface of salad leaves using the long EspA filament on the type III three secretion system (fT3SS) encoded by the locus of enterocyte effacement (LEE) (Shaw et al., 2008). The fT3SS is a complex, multiprotein organelle that aids bacteria in protein transportation (Shaw et al., 2008). After the attachment of bacteria, the internalization of *Salmonella* through stomatal opening was also found to be more correlated to the secretion of chemoattractant, such as sucrose during photosynthesis in the presence of light (which favored its motility), than it was to only the opening of stomata. When stomata were forced open in the dark, no internalization into the leaves was observed (Kroupitski et al., 2009).

The rate of bacterial internalization during produce washing depends on temperature differential as well as immersion time. Bacterial internalization into produce via infiltration has been found to increase with a negative temperature differential, which is when the produce is contaminated or dumped into contaminated water at a temperature lower than the temperature of the produce (Burnett et al., 2000; Zhuang et al., 1995). Immersion time has been found to be as influential, if not more, than temperature differential in terms of bacterial internalization. The internalization difference of *Salmonella* in tomato fruits was significant after 15 min of

immersion in a solution with a 30°F temperature difference, but wasn't significant in less than 2 min regardless of the temperature difference (Howard & Hutcheson, 2003).

The internalization of bacteria will not only damage or spoil the produce, but will also render the antimicrobial post-harvest wash inefficient at killing microorganisms already internalized because they are now protected within the interior of the plant (Zhuang et al., 1995). This emphasizes the importance of 1) using an antimicrobial (*e.g.*, chlorine) in the post-harvest wash water as a method for preventing contamination of the water and, thus, cross contamination, 2) preventing produce contamination, 3) using agricultural water of adequate and sanitary quality that satisfies all requirements of the FSMA-PSR, and 4) adhering to good agricultural practices (GAPs) and good manufacturing practices (GMPs).

2.4Food Safety Modernization Act Produce Safety Rule (FSMA-PSR)

2.4.1 Origin and Description

The Food Safety Modernization Act (FSMA) was signed into law in 2011, with the goal of strengthening the US food safety system by emphasizing prevention rather than reaction to public health hazards that contaminate food (FDA, 2020). Found in 21 CFR Part 112 is the Food Safety Modernization Act Produce Safety Rule (FSMA-PSR), the federal food safety legislation for fruit and vegetable farms. The FSMA-PSR established the regulatory standards for the production, harvesting, and handling of fruits and vegetables. This rule, as defined in 21 CFR Part 112-3, covers farms and farm-like facilities whose annual average income in the last three years, excluding the current year, from selling products is >$25,000. Farms that grow produce for personal or on-farm consumption, and other farms depending on the type of produce they grow (whether covered or not) and where they sell their produce, and the value of their food sales might be qualified for exemption as described in 21 CFR Part 112-4 and 21 CFR Part 112-

5. Produce that are not covered by the FSMA-PSR can be classified into three categories: produce rarely consumed uncooked (beans, potatoes, asparagus, etc.), produce grown for personal consumption, as well as produce that aren't considered raw agriculture commodities (Rogers, 2020).

2.4.2 FSMA-PSR on agricultural water quality

Agricultural water is water that has the potential of coming into contact with the produce (Mahmoud, 2018). The FMSA-PSR classifies agricultural water into two categories: production water and post-harvest water. Production water is the water used in the field, during produce growth, and can be in contact with the produce (*e.g.*, irrigation water, fertilization mixing water, and crops spray mixing water). Post-harvest water refers water used in the produce handling from harvest to packaging (*e.g.* water used in rinsing, washing, cooling, waxing, icing, or moving fruits and vegetables) (Mahmoud, 2018).

Microbial limits are defined by the FSMA-PSR based on the source of water and its subsequent use in the production or handling of produce. The original draft of FSMA-PSR establishes a limit of 126 colony forming unit (CFU) of *E. coli*/100 ml geometric mean (GM) for production water, and no detectable *E. coli*/100ml for post-harvest water (FDA, 2022a). However, due to confusion and complexity in the implementation of the initial preharvest water testing requirement by stakeholders, a new proposed revision of the FSMA-PSR on agriculture water has been proposed. This new proposed revision would change the pre-harvest required microbial analysis to system-based pre-harvest agriculture water assessment instead (FDA, 2022b). These proposed changes won't include pre-harvest water used for sprouts irrigation or any post-harvest used water microbial testing requirement. FSMA requires that untreated surface water may not be used as agricultural water during or after harvest (FDA, 2022a)

2.5 Good Agriculture Practices (GAP) and produce safety

In response to the surging microbial food contamination and food-borne illnesses, the FDA issued a guidance document containing foundational Good Agriculture practices (GAP) to help growers understand the most probable sources of food safety hazard risks on their farm and how to minimize them (FDA, 2008). This guidance covers all aspects of fruit and vegetable production, starting from production and harvesting on farm, involved personnel, buildings and equipment, sanitation operations, processing, all the way to shipping and traceback of produce (FDA, 2008). Even though food safety should be the main concern for farmers adopting GAP practices, having a GAP certification by the USDA or another auditing party may also give a farmer a bigger market to access (Bardsley et al., 2021).

Unfortunately, the practicing of GAPs, and abiding to FSMA-PSR and other federal regulations can be complex and not easy for farmers to understand. This leads to a necessity to provide community tailored extension services/education that will enable different growers to abide to safety regulations and supply safe produce.

2.6Water disinfection methods

With the increasing number of waterborne diseases and produce contamination caused by contaminated agricultural water, water quality is of great importance and there is a significant need for effective water disinfection methods. As mentioned above, the use of contaminated water, both pre- and post-harvest, causes a risk for pathogen internalization into plants and cross contamination (Bartz et al., 2015). Water disinfection is the practice of treating water to kill all microbial pathogens that might be present in the water. Different water disinfection strategies can be evaluated based upon their efficacy at killing the pathogens, cost, and the amount of

disinfection by-products they produce (DBPs) (Pichel et al., 2019). Disinfection methods can be classified as either physical or chemical.

2.6.1 Physical disinfection methods

Ultraviolet Radiation (UV)

Ultraviolet light at 254-260 nm wavelength is known to be one of the most efficient germicides against pathogens. Ultra violet radiation (UV) kills pathogens by directly acting on their DNA pyrimidine bases and preventing any further replication, as well as DNA transcription for protein formation (Bolton & Linden, 2003). The dose of UV required to disinfect water depends on the sensitivity of the microorganisms in the water. Bacteria are the most sensitive to UV radiation followed by non-enveloped virus and protozoa, with the enveloped viruses displaying the most, albeit variable, resistance (Kong et al., 2021). Bacterial spores are shown to be more resistant than vegetative bacteria (Hijnen et al., 2006). High scale UV disinfection constitutes the passing of water through tubes directly exposed to UV radiation at around 254 nm (Pichel et al., 2019). Like most other disinfection technologies, the efficacy of UV disinfection depends on its transmittance to the microorganisms, which is measured as a UV extinction coefficient. The UV transmittance may be hindered by different characteristics of the treated water, such as the presence of bacteria shielding particles (TSS) and turbidity while they are less affected by the organic matter and form little to no disinfectant by products (DBPs) (Friedler et al., 2021; Tak et al., 2020)

There are two UV disinfection systems, the low-pressure mercury and medium-pressure mercury vapor lamps. These two differ in that the low-pressure lamp can produce wavelengths of about 254 nm which is nearing the 260 nm level where bacteria are sensitive to UV, while medium-pressure lamps produce a wavelength of 200-380 nm (Kong et al., 2021). The high

wavelength produced by medium-pressure lamps makes them better disinfectants with about 25 times more treatment capacity than low pressure lamps because it helps them destroy more molecules such as proteins (Kong et al., 2021; Wolfe, 1990). The use of UV treatment in combination with chemical treatment such as chlorine has been found to lead to better microbial disinfection, especially against coliphages that are resistant to both treatments when used individually (Zyara et al., 2016). This practice would, however, increase the price of UV disinfection.

The UV radiation method is more viable in large scale farms/plants but is also not accessible for small holder growers due to expensive maintenance.

Advantages of UV disinfection

- No color, taste, or smell residue in treated water
- No health hazardous DBPs
- Fast disinfection time due to high microbial sensitivity to UV.
- Cheaper to maintain than Ozone application
- Least probability to lead to the development microbial resistance

Disadvantages

- Very expensive to install, use and maintain compared to most chemical disinfection
- Not very effective at killing viruses and some protozoans
- Leaves no residue disinfectant which makes it unsustainable in water with potential for recontamination
- Lamp disposal increases the hazardous waste management problem because they have mercury

Solar disinfection

The solar disinfection method involves the microbial decontamination of water by direct exposure of water to sunlight. This method is cheap, convenient, and used in developing countries. The majority of germicidal effect from solar disinfection system is due to the UVA light (320-400 nm) that is able to reach the Earth surface and kill bacteria in water, when the water is stored in clear containers under direct sunlight (Byrne et al., 2011). Even though the solar rays may act on the bacteria by directly being absorbed by the bacterial DNA, it has also been hypothesized that it mainly acts indirectly by exciting the photosensitizers either in the bacterial cells or surrounding environment, which then react with biomolecules or molecular oxygen, where the latter forms reactive oxygen species that are capable of directly acting on bacterial DNA and inactivating the cell (Reed, 2004).

Another hypothesized mechanism of action for solar disinfection is thermal inactivation, where water reaches a temperature of 50-60 °C, which was the temperature associated with the highest bacterial inactivation (Byrne et al., 2011; Reed, 2004). Small scale solar pasteurizers are commercially available and mainly made of opaque, dark materials to enhance heat retention (Reed, 2004). One important note on thermal inactivation of waterborne pathogens is the boiling of water disinfection method that is practiced in many developing countries, where water is brought to a boil for 1 min. Thermal exposure of pathogens to a temperature of 70 °C for 6 min is enough for bacterial disinfection but the lack of accessible thermometers have resulted in the recommendation to boil water (Pichel et al., 2019).

Like most studied disinfection methods, the efficacy of solar disinfection is also impacted by the presence of inorganic materials, enhanced by the presence of dissolved salts, affected by the temperature-oxygen solubility interaction of the water, as well as the type of container used for water storage, and reduced by a turbidity >300 NTU (Reed, 2004). Glass containers have

been shown to enhance inactivation because of better light transmission in comparison to plastic; however, glass is more easily broken than plastics, which is why plastics are more practical and frequently used even though plastics are associated with the formation of toxic photoproducts from the plasticizers leaching (Reed, 2004). It is important to note that Wegelin et al. (2001) has demonstrated that the plasticizers didn't lead to the leaching of photoproducts inside the water stored in plastic bottles.

2.6.2 Chemical disinfecting methods

Chlorine

Chlorine is one the most common, cheap, and effective water treatment method (Lee & Huang, 2019; Mathew et al., 2018). The major forms of chlorine used for disinfection are chlorine gas (Cl_2), hypochlorite ion (OCl-), chlorine dioxide (ClO_2), and hypochlorous acid (HOCl) (McDonnell, 2007). The HOCl form is the most effective form of chlorine due to its stability. Hypochlorite is the most used/safe source of chlorine, and HOCl can be found in either solid (calcium hypochlorite) form, or as a liquid (sodium and potassium hypochlorite), such as the bleach products commonly used in households (Lewis, 2010). The efficacy of chlorine at killing microorganisms depends on the amount of free available chlorine. As the name suggests, free chlorine refers to the unreacted chlorine available to kill microorganisms. Chlorine disinfectant efficacy is affected by numerous factors, including concentration, contact time, pH, temperature (Bitton, 2011; Chen & Hung, 2017), turbidity, and organic content (K. Huang et al., 2020; Waters & Hung, 2014).

An increase in pH reduces the antimicrobial efficacy of chlorine sanitizers by increasing the dissociation of HOCl to release OCl-; a less stable form that could easily react with other organic matters, which leads to less available chlorine for antimicrobial activity, and a decrease

in pH reduces the efficacy of chlorine by favoring the formation of harmful Cl_2 gas (Banach et al., 2015). The effect of pH doesn't only affect the efficacy of the chlorine treatment but also favors the formation of harmful disinfection by products (DBPs) and corrosion possibilities (Fatica & Schneider, 2009). More specifically, an increase in pH favors the formation of trihalomethanes (THMs) and a decrease of pH favors the formation of haloacetic acids (HAA). Very low pH also increases the corrosion potential in the processing plants. Efficacy in microbial treatment was evaluated by Hays et al., (1966), and at an average pH of 8.5, hypochlorite concentrations of 3, 6, and 12 ppm achieved complete inactivation of approximately 6 logs of *E. coli* in water in 30, 30, and 15 sec, respectively; the killing time was reduced to less than 15 sec when the pH was around 5 for all chlorine dosage levels. This study was done in water considered to be free of any organic matter or other contaminants that might influence the pH or impact the efficacy of treatment. The optimal pH activity of chlorine and its releasing agents was reported to be 4-7 by McDonnell, (2007), but was also estimated to be 6-8.5 by Shaw et al. (2013). One can hypothesize that increased efficacy of chlorine sanitizers would be in neutral pH waters which coincide with the pKa of HOCl of 7.5.

Like many other biocide activities, the activity/efficacy of chlorine as a disinfectant is believed to increase as temperature and contact time increase. However, it was noted that the efficacy of chlorine (25-200 ppm) at killing *Listeria monocytogenes*, *Staphylococcus* spp., *Bacillus* spp. and *Pseudomonas* spp., contrary to other sanitizers such as quaternary ammonium and iodophors, didn't significantly change and reduced equally the bacterial population at about 2°, 4° and 25°C (Overdahl & Zottola, 1991; Tuncan, 1993). These two studies are also in agreement with other research that showed the efficiency of sodium hypochlorite sanitizers slightly, but not significantly, increases as the temperature increases from 5 to 15°C , and that the

temperature impact is also highly dependent on the type and resistance of the microbial species and strains being treated (Grunert et al., 2018; Stefanello et al., 2021).

Water turbidity and organic content are some of the main hindrances of chlorine treatment efficacy. Turbidity is the measurement of the water clarity and is influenced by both inorganic and organic matter (LeChevallier et al., 1981). Both organic and inorganic matter in water reduce the efficacy of chlorine by creating a chlorine demand, which then reduces the available chlorine for microbial disinfection purposes. More specifically, total organic carbon (TOC) is positively related to turbidity of the water; it is also the primary chlorine demand creator in water, reducing the efficacy of chlorine (LeChevallier et al., 1981). Chemistry can explain this efficiency reduction of chlorine by organic content, where the organic chemicals will react with the available hypochlorite to form THMs, which are potential carcinogens (Fan & Sokorai, 2015). The microbial masking caused by organic matter can also reduce the ability of chlorine to access and kill microorganisms and enable cross contamination of produce during washing (K. Huang et al., 2020; Van Haute, et al., 2013).

As chlorine concentration increases so does the efficacy. Due to the formation of disinfectant by products (DBPs) such as THMs and HAA, the maximum concentration of chlorine treatment to be used in different sanitation medium (mainly in drinking water) and their acceptable produced DBPs are regulated by the EPA (71 FR 387, 2006). With the high sensitivity of chlorine to different water characteristics mentioned above, the use of chlorine treatment in new sources of water require efficacy validation research. Below are some advantages and disadvantages of chlorine (Bester, 2015; McDonnell, 2007; Shaw et al., 2013)

Advantages of chlorine

- Broad spectrum

- Cost effective
- Leave residues that provide the water with extra protection against recontamination

Disadvantages of chlorine

- Can promote corrosion on metal surfaces at very low pH and high concentrations
- Irritable and toxic at high concentrations
- Form strong smelling inorganic chloramines by reacting with ammonia and other N-compounds
- Can form potential carcinogenic by-products known as THMs when reacting with organic molecules

Peroxyacetic acids (PAA)

Peroxyacetic acid (PAA), also often referred to as peracetic acid, is one of the FDA-approved food-grade sanitizers (21 CFR Part 173.315). Peroxyacetic acid and hydrogen peroxide are strong oxidizing agents in the group known as peroxygens. Peroxyacetic acid is formed from a reaction of acetic acid and hydrogen peroxide in an aqueous solution (Bester, 2015). Peroxyacetic acid (PAA) is relatively unstable on its own, so its stability and reactivity are reinforced by the addition of hydrogen peroxide in the commercially distributed PAA sanitizers (McDonnell, 2007). Peroxyacetic acid (PAA) is one of the most commonly used sanitizers for produce wash water solutions to prevent microbial cross-contamination in produce (Singh et al., 2018). Peroxyacetic acid (PAA), is preferred for its stability to different water conditions, such as organic matter. Like chlorine compounds, PAA can only significantly reduce bacterial concentrations on produce at very high concentration (85-100 ppm) and on smooth surface produce (Singh et al., 2018), but PAA efficacy has been found to be more resistant to higher organic load of wash water than that of chlorine (Baert et al., 2009). However, PAA sanitizing

efficiency is can also be affected by initial concentration, pH, temperature of treated water, and the contact time.

Sánchez-Ruiz et al. (1995) demonstrated that in water with a pH of 5-8, PAA efficiency does not change; and is supported by the work of Shaw et al., (2013) who reported that PAA is effective at a wide range of pH from 5-9. PAA has a broad spectrum of microbial disinfection as much as it has a wide range of pH. When studied against inactivation of human norovirus and bacteriophages, PAA was found effective at pH 6.5-7.5 (Dunkin et al., 2019). The peroxyacetic acid dissociation value is a pKa of 8.2, so as the water pH increases above that, then PAA will change to its dissociated, less active form (Bester, 2015). Conversely, at a PAA concentration of 50-500 ppm, Kataria et al. (2020) demonstrated that the pH of the treatment wash solution had a negligible impact on the efficacy of PAA when used on chicken wing skin to kill *Campylobacter*, but that its efficacy was increased by the increase in contact time from 10 sec to 60 min.

Considering its high oxidation capacity, PAA can chemically react with organic matter, reducing the available sanitizing capacity, when all other factors are held constant (Fan et al., 2009). Peroxyacetic acid (PAA) demonstrated negligible reactivity to organic matter and was reportedly more stable to many water characteristics when used against *E. coli* and other enteric bacteria (Banach et al., 2015; G. Zhang et al., 2009). Davidson et al. (2017) also demonstrated that PAA-based sanitizers at a concentration as high as 50 ppm are not affected by the organic load present in the water.

Peroxyacetic acid (PAA) is an effective treatment for water sources, with studies reporting that 50 ppm PAA achieved ~4 log reduction of the non-toxigenic *E. coli* O157:H7 after 90 sec of contact time in wash waters containing up to 10% (wt/v) organic load (Davidson et al.,

2017)). Davidson et al. (2017) concluded that the sanitizer concentration might be a better direct indicator of PAA efficacy. Peroxyacetic acid (PAA) concentrations of as low as 6 ppm have achieved reductions 4.5 logs *E. coli* (ATCC 25922) within 5 min of treating river water, with higher resistance from environmental strains which were only reduced by 1.54 log in those 5 min (Bester, 2015). Based on the above sources, the dosages and contact time needed for PAA effectiveness will vary with different physicochemical characteristics of water sources, as well as the microbial species and variants present in the water.

Another advantage of PAA as a disinfectant is that it forms little to no harmful/toxic DBPs compared to those produced by other disinfectants, such as chlorine (Kitis, 2004; Monarca et al., 2002). Excess PAA and its byproducts can easily dissolve and decompose into unharmful acetic acid, oxygen and water (Banach et al., 2015). The EPA allows concentrations of PAA of up to 80 ppm in fruit and vegetable washing solutions (*40 CFR Part 180.1196)*. Below are some advantages and disadvantages of PAA (McDonnell, 2007; Shaw et al., 2013).

Advantages of PAA

- Broad spectrum of activity
- Non-toxic byproducts
- Greater activity in presence of organic materials and inorganic soils
- Non-corrosive

Disadvantages of PAA

- Strong vinegar smell
- Irritative and hypersensitive to skin at high temperature
- Explosive when stored in unvented areas
- Can cause nausea when used/opened in unventilated area.

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Chapter 3 : Evaluation of the Effect of Turbidity, Temperature, and pH of Agricultural Surface Water on the Efficacy of Peroxyacetic Acid and Chlorine Treatments.

Abstract

Nearly half of food-borne illness cases are linked to produce and nuts, and water used in washing the produce can significantly contribute to their contamination. With the continuous use and extreme reduction of groundwater, surface water provides a good alternative source for agriculture activities such as post-harvest produce washing. However, exposure to the environment increases the susceptibility of surface water to microbial contamination and impacts its physical and chemical characteristics which in turn affects the activity of chemical disinfectants used to treat surface water. Peroxyacetic acid and chlorine were evaluated as treatments for simulated surface water inoculated with non-pathogenic *Escherichia coli* to determine their efficacy at achieving 'no detectable generic *Escherichia coli*' in post-harvest agricultural water at different temperatures, turbidities, and pH values. Simulated surface water was prepared to turbidities of 2 and 100 NTU, and adjusted to pH 6.5 or 8.4, then equilibrated to 32 or 12°C. Samples were inoculated with 5 logs of non-pathogenic *E. coli* and treated with either chlorine 25±2 ppm, peroxyacetic acid (PAA) 75±5 ppm, or a sterile water control (W). Samples were mixed with treatments for 10 sec (t=0) and then neutralized in Dey-Engley broth before *E. coli* enumeration on *E. coli*/Coliform Petrifilms at time points (t) 0, 5,10, 60-, 1440-, and 2880-min post-treatment. Neutralized samples were also enriched in 2X Brain Heart Infusion (BHI) broth and streaked on MacConkey agar plates (MAC) to confirm absence of generic *E. coli*. All C and PAA treated samples were below the limit of detection on EC Petrifilm

(<5 CFU/ml) and *E. coli* was not detected on MAC at t=0. In general, the efficacy of 75 ppm PAA and 25 ppm C at reducing *E. coli* in simulated surface water was not impacted by time, turbidity, temperature, or pH. This study provides important insights on chemical interventions that may be used by farmers for treating surface water to be used post-harvest.

3.1 Introduction

The increasing health awareness regarding the benefits of produce consumption has increased the interest of consuming these products. However, produce is also increasingly with a food-borne disease burden for both the public health and the food industry sectors, as about 46% of food-borne illnesses with a known food driver have been associated with produce and nuts, with leafy greens accounting for most of the illnesses (Painter et al., 2013). Food-borne illnesses cost the U.S. government around $17.6 billion annually (USDA ERS, 2018). Viruses such as norovirus and bacteria, mainly *Salmonella* spp,, Shiga toxin-producing *Escherichia coli*, and *Campylobacter* spp causes the majority of food-borne illnesses in global estimation studies (Scallan et al., 2011; Scallan Walter et al., 2021). These microbial pathogens have been reported to survive in soil, water, and other environments, thereby allowing them to eventually contaminate produce upon contact (Jacobsen & Bech, 2012; Mandrell, 2009).

Several produce-associated outbreaks have been traced to the use of agricultural water. One example is a 2018 California outbreak that was caused by *E. coli* O157:H7 in red leaf lettuce, green leaf lettuce, and cauliflower, and resulted in 62 cases, 25 hospitalizations, and 2 cases of hemolytic uremic syndrome (HUS), and was traced back to sediment in the agricultural water reservoir (CDC, 2019b). This and many more outbreaks and foodborne illness cases have been consistently associated with the use of contaminated water during irrigation, irrigating close to harvest, or post-harvest use. It is important to keep in mind that most of the recent produce

related outbreaks are of unknown source which calls for effective microbial contamination prevention and intervention strategies both preharvest and post-harvest since so many routes of contamination are possible.

Surface water, such as pond water and rainwater catchment, are readily available to farmers and are now exploited for different agricultural and industrial activities. In 2015, 74% of total water withdrawals in the US was from surface water, the majority of which was used for public supply, irrigation and industry (Dieter et al., 2018). Unfortunately, since these sources are exposed to the environment, they are unsafe for post-harvest use without treatment, as different studies link their microbial contamination risk to both environmental sources (floods, temperature raises, seasonal differences) and human activities, such as agricultural activities, cattle rearing, deforestation and more (Christensen, 2001; Cooley et al., 2007; Tucker & Hargreaves, 2004).

Because the production of fruits and vegetables is increasing (Balali et al., 2020), and there have been increased produce contamination patterns, the Food and Drug Administration (FDA) Food Safety Modernization Act (FSMA) established the Produce Safety Rule (PSR) to establish safe practices and minimum standards to be followed by produce farmers in order to proactively prevent produce contamination (FDA, 2022a). The FSMA-PSR requires no detectable generic *E. coli* (0 CFU/100 ml) for water used in the post-harvest handling of produce or other activities with potential contact to produce (FDA, 2022a). Most enteric pathogens causing food-borne illnesses have a fecal-oral route and generic *E. coli* is the indicator used by the FDA to represent the potential for fecal contamination and pathogens in water (FDA, 2022; Gerba, 2009)

Produce contamination during the washing process can result from contact with contaminated water and cross contamination from other contaminated produce (Huang et al., 2020; Smolinski et al., 2018). The use of contaminated water can also lead to microbial internalization by produce through natural pores or by wounds resulting from the exerted hydrostatic pressure on the produce during washing, or actively by the attachment of bacteria from water followed internalization favored by chemoattractant secretion (Bartz et al., 2015; Shaw et al., 2008). The contamination facilitated by wash water emphasizes the heightened risk that would result from using contaminated/untreated water in produce washing or for other post-harvest uses.

Chlorine and peroxyacetic acid (PAA) are the most common and affordable chemical interventions used in the food industry. To avoid contact and cross contamination, the treatment concentration and contact time with water used during produce washing and for other post-harvest uses is crucial to the reduction of produce outbreaks associated with contaminated water. Previous research has shown that temperature, pH, turbidity, total organic carbon (TOC), and bacterial count affect the survival and growth of bacteria in water, and also affect the efficacy of PAA and chlorine (Davies et al., 1995; Huang et al., 2020; Van Haute et al., 2013). Much of the research that focuses on water as a source of microbial contamination is directed at irrigation water and less on the water used post-harvest. Most post-harvest wash water studies don't reflect the use of surface water with its physicochemical characteristics in produce washing or post-harvest (Dandie et al., 2020; Machado-Moreira et al., 2019; Singh et al., 2018). A knowledge gap currently exists regarding chemical efficacy in treating surface water for post-harvest use in the produce industry. This study aims to address this knowledge gap by evaluating the effectiveness of chlorine and PAA at reducing generic *E. coli* populations in simulated surface water to no

detectable levels. The specific objective is to evaluate the effect of turbidity, temperature, and pH of simulated surface water on the effectiveness of chlorine and PAA at achieving the required 'no detectable generic *E. coli*' requirement as described by the FSMA-PSR for agricultural water used for post-harvest uses in the produce industry (FDA, 2022a).

3.2Materials and Methods

3.2.1 Bacterial strains

Generic *E. coli* strains isolated from feces and purchased from the American Type Culture Collection (ATCC) were used in this study, and included ATCC 8739, ATCC 13706, and ATCC 23631. These strains were recommended for water use and antimicrobial chemical efficacy studies (ATCC 8739, 2022; ATCC 13706, 2022; ATCC 23631, 2022). Frozen stock cultures were streaked for isolation on Nutrient Agar plates (NA; Difco™, Sparks, MD), incubated at 37°C for 24±2hours, and a single *E. coli* colony was used for inoculum preparation (described below). Prior to initiating the study, all pure cultures were plated on MacConkey agar (MAC; Thermo Scientific™, Remel™, Lenexa, KS) and *E. coli*/Coliform Petrifilm (EC Petrifilm™, 3M™, Saint Paul, MN) to document appearance and ensure proper colony counting during the inoculation study. Pure *E. coli* cultures grew pink on MacConkey agar (MAC), and blue to purplish colonies with gas bubbles grew on EC Petrifilm.

3.2.2 Inoculum preparation

The working inoculum cocktail of the three strains was prepared by transferring one isolated colony of each ATCC strain into 10 ml Brain Heart Infusion broth (BHI broth; Thermo Scientific™ Oxoid, Hants, UK) tubes separately. The three tubes (one tube per strain) were incubated at 37°C for 24±2hours to achieve a concentration of $10^{8\text{-}9}$ CFU/ml. The separate cultured BHI broth tubes were transferred to 15 ml conical tubes and centrifuged at 4300 x *g*, for

15 min at 4°C. The supernatant was discarded, and the pellets were then resuspended separately in 10 ml Phosphate Buffered Dilution Water (PBDW; EMD Millipore Corporation, Billerica, Massachusetts). The remaining equal volumes (≈27 ml total) of the three strains were then mixed in a 50 ml centrifuge tube to form a 3-strain cocktail. The inoculum was diluted 1:10 in PBDW to generate a final target concentration of ~$1.0x10^{7-8}$ CFU/ml. The cocktail concentration was enumerated at the beginning and end of the trial to ensure that the *E. coli* populations had not changed during the inoculation process. To ensure that strains were used in equal proportion in the working inoculum, the concentration of each strain was separately enumerated, and the working cocktail was also enumerated, by diluting in PBDW and plating in duplicate on EC Petrifilm. The EC Petrifilm were then incubated at 37°C for 48±4 hours. Colonies (blue and purplish with gas bubble) were counted on EC Petrifilm.

3.2.3 Water preparation

Simulated agricultural surface water was prepared in the lab targeting a turbidity of 100 NTU and 2 NTU according to the FDA/ Environmental Protection Agency (EPA) protocol for development and registration of treatments for preharvest agricultural water (EPA, 2022), with modifications. Briefly, 300 mg and 2 mg of PTI Arizona test dust (TD; PTI Powder Technology Inc, Arden Hills, Minnesota) were each added in 2 L of DI water to make 100 NTU and 2 NTU finished turbid water, respectively. The water turbidity was determined and verified using the Hatch 2100Q Laboratory Turbidimeter (Hatch Company, Loveland, Colorado). Next, 3.2 g of sea salt (Sigma Aldrich, Saint Louis, Missouri) was added [target 1350-1650 mg/L total dissolved solids (TDS)] in both 2 L turbid water batches and vigorously shaken to dissolve. Contrary to the EPA protocol, the two water batches were then autoclaved at 121°C for 15 min for sterility, due to issues with TD microbial contamination. Autoclaving was verified to not

change the water turbidity by measuring the water's turbidity before and after autoclaving. After autoclaving, 20 mg and 4 mg of Humic Acid (HA; Sigma Aldrich, Co, Saint Louis, Missouri) (target of 10 mg/L and 2 mg/L TOC in the final turbid water) was added in two sterile 1 L screw cap bottles; 500 ml from the 100 NTU and 2 NTU sterile water batches were added to the bottles, respectively for mixing, where they were vigorously shaken for at least two min to fully hydrate and dissolve the HA and added back to their respective bulk (2 L) solutions. Humic acid sterility was validated on Brain Heart Infusion agar (BHA) prior to use since it was added post water autoclaving. Each of the 2 L water batches were then separated into two equal volume batches and then standardized to achieve a final pH of 6.5 in one batch and 8.4 in the other batch using 1 N HCl and/or 5N NaOH (Thermo Scientific™, Fairlawn, New Jersey) as needed. Each of the four newly made batches (100 NTU,6.5; 100 NTU,8.4, 2 NTU,6.5; 2 NTU, 8.4) were then split into two equal (400 ml) bottles, resulting in a total of eight samples of water. Each set of turbidity-pH combination was calibrated overnight (8-10 hours) at 32°C and the other set at 12°C. Post overnight equilibration, each final water sample was plated on BHI agar and EC Petrifilm during the preliminary work, and only on EC Petrifilm during the inoculation study, to ensure sterility before inoculation. Figure 3.1 shows a summarized flowchart of the inoculated water sample preparation.

3.2.4 Water Inoculation

One ml was removed from each of the prepared 400 ml turbid water samples and used for sterility testing. Then, each 399 ml sample per turbidity and pH of test water samples (e.g., 100 NTU-8.4-32°C and 100 NTU-8.4-12°C) was inoculated with 1 ml of the working inoculum to achieve a target concentration of ca. 5 logs per ml. The concentration of each water sample was enumerated before antimicrobial treatment to document initial generic *E. coli* concentration.

Briefly, dilutions were made in PBDW and plated on EC Petrifilm, which were then incubated at 37°C for 48±4hours. Colonies (blue and purplish with gas bubbles) were counted on EC Petrifilm. Following enumeration, each inoculated sample was then separated into three equal (99 ml) bottles and placed back at their respective temperature for approximately 30 min for temperature equilibration.

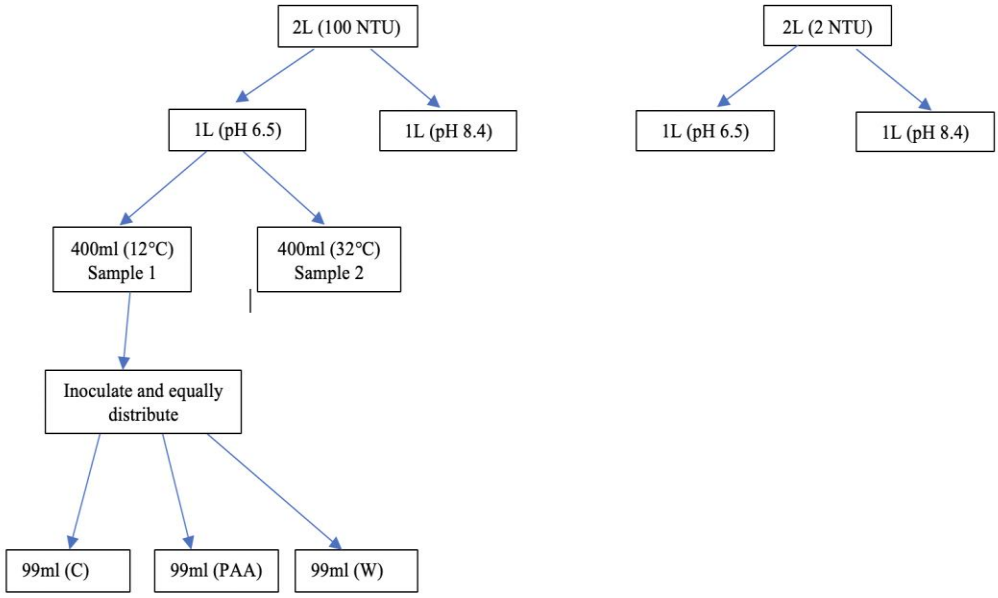

Figure 3.1 Flow chart of the inoculated water sample preparation, the same steps for 2 NTU, 32°C of 6.5 pH, and 8.4 pH of 100NTU samples were followed. Six (99ml) bottles made from sample 1 and 2 (different temperatures of each turbidity and pH combinations) were treated at the same time. Samples were randomly assigned numbers throughout the 3 replicates of the study.

3.2.5 Antimicrobial treatment preparation

The two antimicrobial chemicals used were SaniDate15 (PAA; BioSafe Systems, LLC, East Hartford, Connecticut) and Ultra Clorox germicidal bleach (C; Clorox Professional Products Company, Oakland, California) at 75±5 ppm and 25±3 ppm of PAA and available free chlorine respectively, as a final concentration in the treated water samples. Sterile DI water was used as a

control (W). The concentrations were chosen based on the Produce Safety Alliance (PSA) labeled sanitizers for produce spreadsheet (Cornell CALS, 2020), which summarizes approved sanitizers and their respective concentrations for produce. Stock solutions of PAA and C were prepared and were validated to achieve 75±5 ppm and 25±3 ppm of PAA and free available chlorine, respectively, in the final 100 ml water samples as part of a preliminary study. The treatments were prepared in sterile, aluminum foil-covered flasks to protect against light degradation. The free chlorine concentration was confirmed using a Hanna free and total chlorine high range portable photometer (HI96734; Hanna Instruments, Woonsocket, Rhode Island). The PAA treatment concentration was prepared from SaniDate15 following manufacturer recommendations, and its concentration was confirmed using the Biosafety system peracetic acid test kit (BioSafe Systems, LLC, East Hartford, Connecticut) according to manufacturer instructions. The concentrations of both working solutions were measured at the beginning and the end of the inoculation study to ensure that no change in concentration occurred during the course of use.

3.2.6 Antimicrobial application and microbial analysis

Sample bottles were treated by adding a 1ml aliquot of the appropriate treatment solution (C, PAA, W) into each 99 ml bottle of simulated agricultural water. The bottles were then swirled for 10 sec to evenly distribute the sanitizer and the time 0-min sample was then immediately collected. Each sample bottle was sampled at 0, 5, 10, 60, 1440, and 2880 min and enumerated for generic *E. coli*. Briefly, 1 ml of the treated sample was neutralized in 9 ml of Dey/Engley Neutralizing Buffer (D/E; Difco™, Sparks, Maryland), and 5 ml were neutralized in 45ml of D/E in Whirl-Pak® bags (Nasco, Madison, WI). Subsequent dilutions were made from D/E neutralized tubes using 9 ml PBDW and enumerated on EC Petrifim. The EC Petrifilm®

were then incubated at 37°C for 48±4 hours, and the colonies (blue and purplish with gas bubbles) were enumerated. The EPA protocol calls for the use of a non-selective agar (*e.g.* BHI agar) for enumeration (EPA, 2022). However, preliminary work demonstrated issues with contamination and questionable counts. To overcome this challenge, EC Petrifilm were validated as an effective, more selective alternative as part of a preliminary study (data not shown). To the Whirl-Pak® bags containing 5 ml of sample neutralized in 45 ml of D/E, 50 ml of 2X BHI was added, resulting in 1X BHI upon dilution with the sample and D/E, and incubated at 37°C for 24±2hours for enrichment and recovery of generic *E. coli*. Because the FSMA-PSR requires 'no detectable generic *E. coli*' in agricultural water used for post-harvest washing of produce, the enrichment step was important for detecting generic *E. coli* that might be present, but below the limit of detection for EC Petrifilm. After incubation, the enriched BHI bags were then streaked on MAC and incubated at 37ºC for 18-24 hours to determine the presence/absence of *E. coli* in treated samples. Any MAC plates with pink colonies were interpreted as positive for *E. coli*. Immediately following the 10 min sampling point, sample bottles were returned to their respective temperatures and stored in between treatments, and only briefly removed for each subsequent sampling point.

3.2.7 Statistical analysis

All experimental procedures were replicated three times. Statistical analyses were conducted with the Statistical Analysis Software (SAS version 9.4; Cary, NC). All data were analyzed as a repeated measures model using the PROC MIXED linear mixed model and significance level of 0.05. The best covariance structure was determined and used in the model. The Least Squares Means (LSMEANS) were calculated and used to identify statistical significance between individual treatments using the Tukey-Kramer adjustment for multiple

comparisons. The main effects of time, temperature, turbidity, pH and treatment, as well as all two-way interactions, were evaluated for statistical significance. Three- and four-way interactions were not included in the model to avoid potential issues with model nonconvergence. Main effects and interactions not statistically significant were removed from the model using backwards elimination.

3.3Results

All C and PAA samples were below the detection limit (5 CFU/ml, and *E. coli* was not detected on MAC at all time points for all C and PAA treated samples. This indicates that both 25 ppm of free chlorine and 75 ppm PAA were able to achieve approximately a 5-log reduction of generic *E. coli* following a 10 sec mixing period. The main effects of time ($P=0.0004$), temperature ($P<0.0001$), pH ($P=0.0010$), and treatment ($P<0.0001$) were statistically significant. The main effect of turbidity was not statistically significant ($P>0.05$). The following two-way interactions were significant: temperature x time ($P=0.0002$), treatment x time ($P<0.0001$), treatment x pH ($P<0.0001$), and treatment x temperature ($P<0.0001$). Data will be discussed according to these significant interactions.

When comparing *E. coli* populations at each temperature across time, a significant difference was not observed until the 1440- and 2880-min sampling points (Table 3.1), when generic *E. coli* in the 32ºC samples increased to 2.0 log CFU/ml, in comparison to the 1.7 log CFU/ml recovered from samples held at 12ºC ($P\leq0.05$). Samples stored at 32°C increased by 0.2 log CFU/ml from time point 0 to both the 1440 min and 2880 min sampling points ($P<0.0001$). No significant differences in generic *E. coli* concentrations were observed in samples stored at 12°C at any sampling points.

Table 3.1 *E. coli* populations in simulated agricultural surface water analyzed by storage temperature and time. The temperature x time interaction was significant (P=0.0002) and did not include pH, turbidity, or treatment effects. Therefore, data associated with pH, turbidity, or treatment are displayed according to temperature and time.

LS means±SE generic *E. coli* survival (Log CFU/ml)						
Temperature (°C)	Time (min)					
	0	5	10	60	1440	2880
12	1.8 ± 0.024^{Aa}	1.8 ± 0.026^{Aa}	1.7 ± 0.026^{Aa}	1.7 ± 0.031^{Aa}	1.7 ± 0.030^{Aa}	1.7 ± 0.031^{Aa}
32	1.8 ± 0.024^{Aa}	1.7 ± 0.026^{Aa}	1.7 ± 0.026^{Aa}	1.7 ± 0.031^{Aa}	2.0 ± 0.030^{Bb}	2.0 ± 0.031^{Bb}

[ABC] Values with different uppercase superscripts in the same row indicate significant differences between sampling points for a temperature.

[abc] Values with different lowercase superscripts in the same column indicate significant differences between temperature at a sampling point.

Interaction between temperature and time was significant (P=0.0002).

Beginning at sampling point 0, *E. coli* was not detected in the C and PAA samples from both the EC Petrifilm and MAC. When comparing the treatments at each sampling point, the W treatment was significantly different than both the C and PAA treatments (P<0.0001, whereas the C and PAA treatments were not statistically different (P=1.0000). However, the W control samples did change over time, with a significant (P<0.0001) increase of 0.3 to 0.4 log CFU/ml from time points 0, 5, 10, and 60 min in comparison to 1440 min and 2880 min (Table 3.2).

Table 3.2 *E. coli* populations in simulated agricultural surface water analyzed by antimicrobial treatment and time. The treatment x time interaction was significant (P=0.0001) and did not include pH, turbidity, or temperature effects. Therefore, data associated with pH, turbidity, or temperature are displayed according to treatment and time.

	LS means±SE generic *E. coli* survival (Log CFU/ml)					
Treatments	Time (min)					
	0	5	10	60	1440	2880
C	0.0 ± 0.024^{Aa}	0.0 ± 0.026^{Aa}	0.0 ± 0.026^{Aa}	0.0 ± 0.031^{Aa}	0.0 ± 0.030^{Aa}	0.0 ± 0.031^{Aa}
PAA	0.0 ± 0.024^{Aa}	0.0 ± 0.026^{Aa}	0.0 ± 0.026^{Aa}	0.0 ± 0.031^{Aa}	0.0 ± 0.030^{Aa}	0.0 ± 0.031^{Aa}
W	5.3 ± 0.024^{Ab}	5.3 ± 0.026^{Ab}	5.2 ± 0.026^{Ab}	5.2 ± 0.031^{Ab}	5.6 ± 0.030^{Bb}	5.6 ± 0.031^{Bb}

Note: *E. coli* not detected is indicated as 0.0 log CFU/ml

[ABC] Values with different uppercase superscripts in the same row indicate significant differences between sampling points for a treatment.

[abc] Values with different lowercase superscripts in the same column indicate significant differences between treatments at a sampling point.

Interaction between treatment and time was significant (P<0.0001).

The impact of temperature was significant for W control samples (P<0.0001), with an increase of 0.3 logs CFU/ml in samples stored at 32°C compared to samples at 12°C (Table 3.3). Temperature did not significantly change *E. coli* concentrations in samples treated with C (P=0.3468) or PAA (P=0.3468). When comparing each treatment at 12°C, the W treatment was significantly different (P<0.0001) than both the C and PAA treatments, whereas the C and PAA treatments statistically the same (P=1.000). The same was observed at 32°C, where W was significantly different than C and PAA (P<0.0001), but the C and PAA were statistically the same (P=1.0000).

Table 3.3 *E. coli* populations in simulated agricultural surface water analyzed by antimicrobial treatment and temperature. The treatment x temperature interaction was significant (P<0.0001) and did not include pH, turbidity, or time effects. Therefore, data associated with pH, turbidity, or time are displayed according to treatment and temperature.

LS means±SE generic *E. coli* survival (Log CFU/ml)		
Treatments	12ºC	32ºC
C	0.0 ±0.015^{Aa}	0.0±0.015^{Aa}
PAA	0.0±0.015^{Aa}	0.0±0.015^{Aa}
W	5.2±0.015^{Ab}	5.5±0.015 ^{Bb}

Note: *E. coli* not detected is indicated as 0.0 log CFU/ml

^{ABC} Values with different uppercase superscripts in the same row indicate significant differences between temperatures for a treatment.

^{abc} Values with different lowercase superscripts in the same column indicate significant differences between treatments at a temperature.

Interaction between treatment and temperature was significant (P<0.0001).

Although the pH x treatment interaction was significant (P<0.0001), Table 3.4 shows that the C treatment was not impacted by pH (P=1.0000), nor was the PAA treatment (P=1.0000). However, the W treatment was significantly different (P<0.0001) at each pH, with a mere increase of 0.1 log CFU/ml detected in the 6.5 (5.3 log CFU/ml) and 8.4 pH sample bottles (5.4 log CFU/ml). When comparing each treatment at pH 6.5, the C and PAA treatments were statistically similar (P=1.0000), while the W treatment was significantly different (P<0.0001) than both the C and PAA treatments. Similarly, at pH 8.4, the C and PAA treatments were statistically similar (P=1.0000), while the W treatment was significantly different (P<0.0001) than both the C and PAA treatments.

Table 3.4 *E. coli* populations in simulated agricultural surface water analyzed by antimicrobial treatment and pH. The treatment x pH interaction was significant (P<0.0001) and did not include time, turbidity, or temperature effects. Therefore, data associated with time, turbidity, or temperature are displayed according to treatment and pH.

LS means±SE generic *E. coli* survival (Log CFU/ml)		
Treatments	pH	
	6.5	8.4
C	0.0 ± 0.015^{Aa}	0.0 ± 0.015^{Aa}
PAA	0.0 ± 0.015^{Aa}	0.0 ± 0.015^{Aa}
W	5.3 ± 0.015^{Ab}	5.4 ± 0.015^{Bb}

Note: *E. coli* not detected is indicated as 0.0 log CFU/ml

^{ABC} Values with different uppercase superscripts in the same row indicate significant differences between pH for a treatment.

^{abc} Values with different lowercase superscripts in the same column indicate significant differences between treatments with the applied pH.

Interaction between treatment and temperature was significant (P<0.0001).

3.4 Discussion

This study provides initial insights for farmers and the industry regarding efficacy of two chemical interventions that may be efficacious for treating surface water sources for post-harvest use in the produce industry. The objective of this study was to evaluate the efficacy of 75 ppm PAA and 25 ppm of free chlorine at reducing *E. coli* populations in simulated surface water with varying turbidity, pH, and temperatures to satisfy the FSMA-PSR requirement of 'no detectable generic *E. coli*' in agricultural water used for post-harvest use. It should be noted that the FSMA-PSR specifies 'no detectable generic *E. coli*' in a 100 ml sample; however, the EPA protocol followed in this study did not use a 100 ml sample for testing at each time point and only a 5 ml sample was enriched for presence/absence testing. While the data presented herein suggest 'no

detectable *E. coli*' following treatment, they are limited to the 5 ml of water sampled at each time point. Regardless of turbidity, pH, and temperature, both PAA and C were able to achieve 'no detectable generic *E. coli*' in 5 ml of simulated surface water, beginning at the 0 min sampling point, which was collected immediately following a 10 sec mixing period and followed by neutralization in D/E. *E. coli* populations in the water control sample did not decline. These data generally suggest that pH (6.5 & 8.4), temperature (12^{o}C & 32^{o}C), holding time (0 to 2880 min), and turbidity (2 & 100 NTU) had no significant impact on the efficacy of PAA (75 ppm) and C (25 ppm) at reducing generic *E. coli* in simulated surface water.

The lack of influence of pH 6.5 is strongly supported by other publications placing it in the optimal pH ranges of efficiency for both PAA and chlorine sanitizers (McDonnell, 2007; Shaw et al., 2013). A pH of 8.4 was also used because it is slightly basic, which can reportedly affect the efficacy of chlorine sanitizers by converting the most efficient form of chlorine (HOCl) into the less effective form (OCl^{-}) (Fukuzaki, 2006; McDonnell, 2007). However, in this study a simulated surface water of pH 8.4 had no effect on the overall efficacy of either the C or PAA (Table 3.4). Hays et al. (1966) also reported similar log reductions of *E. coli* at a similar pH, with a ~6 log reduction of *E. coli* in hard water of about 500 ppm hardness, at pH of 8.5 using 12 ppm of hypochlorite in 15 sec. The difference in time required between Hays et al. (1966) and the present study may be attributed to the higher dosage of chlorine (25 ppm) that was used in the present study. Also, Fukuzaki (2006) demonstrated that it is only at pH>8.5 that OCl^{-} concentration begins to exceed that of HOCl, which would then shift the equilibrium to the less effective form and, therefore, reduce the efficacy. Conversely, PAA has a broader pH efficiency, which includes the pH of 8.4 tested in this study (Shaw et al., 2013).

Simulated surface water turbidity was not a significant main effect ($P>0.05$), or involved in any significant two-way interactions, which suggests that turbidity values of 100 NTU and 2 NTU do not impact the efficacy of chlorine (25 ppm) and PAA (75 ppm) when used as antimicrobial interventions for reducing generic *E. coli* when all other variables effects are held constant. Total organic content associated with turbidity has been found to be the more likely cause of chlorine demand than turbidity (LeChevallier et al., 1981) where water was filtered losing 99% of its turbidity but kept 90% of its initial unfiltered chlorine demand. The TOC used in this study was very small (10 mg/L and 2 mg/L respective to turbidity), hence didn't affect the efficiency of chlorine or PAA. Also, Banach et al. (2020) observed no significant effect of TOC (500 and 750 mg/L) on the efficacy of PAA at 75 ppm. However, this also contradicts a number of studies that reported that organic matter content and turbidity significantly impact antimicrobial efficacy of both PAA and C (Banach et al., 2015; Gehr et al., 2003; LeChevallier et al., 1981; Van Haute, Sampers, et al., 2013; C. Zhang et al., 2019), with C generally more impacted than PAA (Zhang et al., 2019). Most of the above studies that reported a significant impact of turbidity and organic content on the effectiveness of both chlorine and PAA were either evaluating 1) a very high organic content (e.g.: chemical oxygen demand (COD): 500-1500 mg O_2/L) or 2) a very small concentration of antimicrobial treatment (e.g.: 4.5-6 mg/L PAA). A concentration of 50 ppm PAA or greater is reportedly not affected by organic content (Davidson et al., 2017). The concentrations used in this study (25 ppm of free available chlorine and 75 ppm of PAA) were recommended by the PSA for use in produce handling (Cornell CALS, 2020). The PAA and C concentrations were most likely reduced by organic matter, but if the starting concentration is high enough to quench the organic content demand, leaving enough

residual disinfectant concentration, then overall treatment efficacy is not significantly impacted by turbidity and organic matter (Banach et al., 2015; Gehr et al., 2003; Winward et al., 2008).

Luo et al. (2012) demonstrated that washing produce, such as lettuce, will over time significantly increase the turbidity, as well as the total organic content of the wash water, which will then reduce the available C concentration as well as C efficacy. Hence, even though the total turbidity and organic content (TOC) of the water used in the present study (100 NTU and 2 NTU, and 10 mg/L and 2 mg/L respectively) didn't affect the efficacy of both C and PAA for the purpose of surface water disinfection, if the treated surface water is to be used in produce washing, then the residual concentrations of both treatments should be studied using different sources of water and with varied organic content.

E. coli reductions achieved by both C and PAA were statistically significant at both 12^{o}C and 32^{o}C when compared to the water control samples ($P<0.0001$), and a difference in efficacy was not observed for C or PAA when comparing each individual treatment at 12^{o}C and 32^{o}C ($P=0.3468$) (Table 3.2). This suggests that both 75 ppm PAA and 25 ppm C would be individually effective at a range of temperatures, indicating they are both effective for treating surface water sources during different seasons (*i.e.* water temperatures). The efficacy of antimicrobial interventions generally increases as the temperature increases; however, the present study was effective at achieving no detectable *E. coli* in 5 ml of simulated surface water at the 12^{o}C temperature, indicating efficacy also at a cooler temperature. When comparing chlorine efficacies at 4°C and 50°C, Klaiber et al. (2005) found comparable antimicrobial efficacy of chlorine at both temperatures. Granted, contrary to our study, Klaiber et al., (2005) was looking at the microbial disinfection on carrots instead of water, and the antimicrobial effect on produce is known to be smaller than in the wash water. On the contrary, using smaller

concentrations (2 mg/L), Stampi et al. (2001) observed greater water disinfection when the temperature increased from 12°C to 25°C in secondary effluent water. The difference in other physicochemical characteristics, such as pH and organic content, as well as the higher concentrations of antimicrobial interventions used in the present study, might explain why temperature did not significantly impact treatment efficacy.

The present study resulted in no detectable *E. coli* in 5 ml of simulates surface water at all sampling points, including 0 min, after treatment of simulated surface water by both C and PAA (inoculated with ~5 log CFU/ml). The C and PAA were mixed into each water sample for 10 sec prior to collecting the 0 min sampling point and enumerating generic *E. coli* populations on EC Petrifilm. Because all MAC plates were also negative for *E. coli*, the data presented herein suggest that *E. coli* is rapidly killed by both C and PAA in 5 ml of simulated surface water. It is important to mention that the 1 ml aliquot for D/E neutralization and plating on EC Petrifilm was removed first at each sampling point and the 5 ml was subsequently transferred to a bag containing 45 ml D/E. Therefore, it was likely within 30-60 seconds after each sampling point that the 5 ml aliquot was neutralized. Regardless, *E. coli* was likely rapidly killed within the first minute of exposure. This correlates to a study by Hays et al. (1966), who observed a reduction of 6 logs CFU/ml of *E. coli* from hard water (500 ppm) in 15 sec using 12 ppm sodium hypochlorite. Bester (2015) observed greater than a 4 log reduction of *E. coli* in 15 min using 6 mg/L of PAA, and Koivunen & Heinonen-Tanski (2005) observed over a 3 log reduction in coliforms following a less than 4 min contact time using 15 ppm PAA. This rapid kill reported in the literature and the present study is likely associated with the high starting concentration for both PAA and C. Bester (2015) observed a reduction in the contact time required for increased PAA efficiency as concentration increased, with only 15 min needed by 6 mg/L concentration to

achieve a > 4 log reduction of *E. coli* compared to the 25 min required by 3 mg/L to achieve ~1 log reduction of *E. coli*.

The rapid biocidal activity (~5 log reductions after a 10 sec mixing period) of both the PAA and C treatments is only associated with the ATCC reference strains used in this study. Studies report that the efficacy of chemical interventions may be different depending on the pathogen or microorganisms, including either a phenotypic or genotypic resistance (Banach et al., 2017; De et al., 2008). Similarly, Bester (2015) and Wojcicka et al. (2007) reported that environmental *E. coli* strains demonstrated more resistance when compared to their reference strains. When microorganisms are exposed to harsh environments they develop new mechanisms of defense, which may include genetic alterations, such as mutations, gene transfer, expression of silent genes; or phenotypic alterations, such as biofilm formation, and others that aren't as well understood (Chapman, 2003).

3.5 Conclusions

This study found that PAA and C rapidly killed *E. coli* in simulated surface water. The lack of *E. coli* recovery from 5 ml samples enriched and streaked to MAC suggest that *E. coli* were eliminated at the 0 min sampling point, suggesting that both PAA and C were able to achieve reductions of ~5 log CFU/ml of generic *E. coli* within the brief 10 sec mixing period. Efficacy of PAA and C was not impacted by time, turbidity, temperature, and pH, as *E. coli* was not detected in all treated samples at the 0 min sampling point.

Based on the results described herein, this study demonstrates that PAA (75 ppm) and C (25 ppm) are effective antimicrobial interventions for treating surface waters of a variety of physicochemical characteristics. According to the utilized FDA/EPA protocol (with modifications), because these treatments were able to reduce >3 log CFU/ml of generic *E. coli* in

water representing a variety of physicochemical characteristics (temperature, pH and turbidity), these treatments are effective for use as surface water interventions (EPA, 2022). Furthermore, because PAA and C reduced the concentration of generic *E. coli* to the point of not being detected from a 5 ml sample, these data suggest that PAA and C may be effective for treating surface water that will be used for post-harvest uses in the produce industry, as required by the FSMA-PSR.

However, it cannot be overemphasized enough that this study is somewhat limited in scope because only two temperatures, two pH levels, two turbidity levels, and two temperatures were evaluated against three strains of generic *E. coli* inoculated into simulated (laboratory prepared) surface water. Therefore, these data cannot be extrapolated to all surface water conditions and/or microorganisms. As an example, the organic matter in the simulated surface water, which can greatly influence antimicrobial intervention efficacy, was low in the present study. Hence, further research must be completed with the C and PAA treatments in a variety of surface waters that represent different organic matter levels/types to determine if these treatments at the same concentration are effective when used for naturally occurring surface water sources. It is also important to evaluate these chemical interventions at their respective concentrations against wild-type microflora in a variety of surface water sources to ensure efficacy.

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Chapter 4 : Evaluation of Peroxyacetic Acid and Chlorine as Treatments for Agricultural Surface Water Used for Produce Post-Harvest Uses.

Abstract

The adoption of a healthy lifestyle and the awareness of health benefits associated with the consumption of fruits and vegetables has increased produce consumption among consumers. Unfortunately, an increase in food-borne illnesses associated with fruits and vegetables has been observed with several outbreaks linked to the contaminated agricultural water; thus, water type/source, as well as quality, must be considered to improve safety. The effectiveness of peroxyacetic acid and chlorine at reducing *E. coli* in rain barrel and creek water was evaluated in this study to determine if these interventions satisfy the 'no detectable generic *E. coli*' requirement of the Food Safety Modernization Act for water used post-harvest. Rain barrel and creek water were inoculated with ~5 log CFU/ml of *E. coli* cocktail and equilibrated at temperatures 32 and 12°C to simulate different seasons. Samples at each temperature were treated with chlorine (C) 25±2 ppm, peroxyacetic acid (PAA) 75±5 ppm, and ambient sterile distilled water control (W). Populations of *E. coli* were neutralized in Dey-Engley broth and enumerated at time points 0, 5-, 10-, 60,1440-, and 2880-min post-treatment using the FDA-approved Colilert method as well as *E. coli*/coliform (EC) Petrifilm. Neutralized samples were enriched to test for the absence or presence of generic *E. coli*. Using Colilert, *E. coli* was not detected after 60 min post-treatment in samples treated with chlorine (the water source and temperature variables held constant), while *E. coli* was not detected at 0 min in samples treated with PAA. At the 0- and 5-min post-treatment sampling points, PAA was significantly more

effective than C ($P \leq 0.05$). With EC Petrifilm, *E. coli* was not detected in any samples treated with PAA or C, suggesting that both interventions effectively reduce *E. coli* regardless of water source or temperature. This study provides data that allows growers and Extension educators to explore the use of these treatments for use in surface water sources for post-harvest uses in produce.

4.1 Introduction

An estimated 1 in 6 Americans becomes ill with a food-borne illness each year, which leads to 120,000 hospitalizations and 3000 deaths (CDC, 2018b). Produce and nuts cause approximately half of these illnesses (USDA ERS, 2019). Globally, the foodborne illness burden is estimated at 600 million cases with 420,000 deaths (WHO, 2015). Produce contamination can originate 1) in the field following direct contact with animals (their feces) or contaminated water used for irrigation, and 2) during harvest, or 3) from post-harvest handling. Approximately 91% of domestically acquired food-borne illnesses are linked to norovirus, non-typhoidal *Salmonella, Clostridium perfringens, Campylobacter* spp*., and Staphylococcus aureus* (CDC, 2018b). Most of these microorganisms have a fecal-oral route of transmission and cause gastrointestinal (GI) diseases (Gerba, 2009). Generic *E. coli* has a fecal origin, and have readily available detection methods; thus, it is globally adopted by standard setting organizations as a fecal contamination indicator organism (Odonkor & Ampofo, 2013; Pichel et al., 2019). To proactively reduce/prevent the burden caused by produce contamination, the FDA Food Safety Modernization Act (FSMA) includes the Produce Safety Rule (PSR), which establishes required minimum standards for the production and handling of produce to improve the safety of all produce products covered by the rule (FDA, 2022a).

Surface water, such as rivers, rain-harvested, pond, and creek water are utilized sources of water by farmers because they are readily available. However, surface water is open to the environment which makes it susceptible to chemical and microbial contamination from various point and non-point sources. The increase in agricultural activities required to meet the nutritional demand of the estimated world population by 2050, in part, plays a significant role in the contamination of agricultural water (Davis & Bell, 1998; Rodrigues et al., 2020). Other factors, such as rainfall and temperature have been associated with an increasing number of fecal pathogens, such as *Salmonella*, in surface water sources (Haley et al., 2009), as well as the changing physicochemical characteristics of surface water quality (Davis & Bell, 1998). Rain-harvested water provides a great alternative source of water for growers to use post-harvest but their rain catchment method might collect fecal matter deposited on the roof where the rain is collected from, making it susceptible to microbial contamination (Gwenzi et al., 2015; Jordan et al., 2008). The use of untreated, contaminated water during the produce washing step can lead to microbial attachment and internalization on/in the produce and can enhance cross contamination to other produce that weren't initially contaminated (Luo et al., 2014). The FSMA-PSR requires water that is used in post-harvest handling of produce to have 0 CFU generic *E. coli*/100ml (FDA, 2022a).

Chlorine(C) and peroxyacetic acid (PAA) are two FDA-approved chemical interventions most frequently used by farmers and the agriculture industry (21 CFR Part 173.315 ; Northcutt, 2021). The efficacy of these chemical interventions is affected by different physiochemical characteristics of water (e.g. organic content), pathogen target, treatment concentration, and contact time (Goodburn & Wallace, 2013; Van Haute et al. 2013). An increase in organic matter in the water reduces the free available concentration of the antimicrobial interventions, which

would otherwise be available to reduce microbial contamination in wash water (Banach et al., 2015). Biofilm formation, strong attachment of bacteria on the produce surface, as well as the internalization of microorganisms by produce also reduce the effectiveness of chemical disinfection of microorganisms if the water was to be used in treating already contaminated produce (Gil et al., 2009). Since fresh produce is mostly consumed raw, a thermal kill step is lacking, and the interventions used by produce industry are generally inefficient at reducing pathogens on contaminated produce. Thus, it is crucial to use microbial free water in the washing and post-harvest handling of produce to avoid potential microbial contact and cross contamination (Gil et al., 2009). Overall, chemical treatments, such as chlorine and PAA, are most effective at killing microorganisms in wash water and, thus, preventing microbial contact and cross contamination during the washing of produce (Baert et al., 2009). Therefore, demonstrating the efficacy of C and PAA at treating surface water to generate microbially potable water that can be used for post-harvest uses in the produce industry would be significant.

Haley et al. (2022) reported that *E. coli* concentration was statistically greater in surface water sources in comparison to groundwater sources, suggesting the importance of effective treatment of surface water if it is to be safely and effectively used for post-harvest purposes. Not only that, not all growers are able to access and afford ground and municipal water, leading to several growers having asked Dr. Londa Nwadike and other extension educators in Kansas (KS) and Missouri (MO) about the possibility of safely using surface water for post-harvest uses. Therefore, the objective of this study was to evaluate the effectiveness of chlorine and PAA at reducing *E. coli* in rain barrel and creek water to satisfy the 'no detectable generic *E. coli*' requirement outlined by the FSMA-PSR for water to be safely used for post-harvest washing of

produce. This study would then serve as a validation of the two treatments for potential use in surface water to be used post-harvest.

4.2 Materials and Methods

4.2.1 Bacterial strains

Three strains (ATCC 8739, ATCC 13706, and ATCC 23631) of non-pathogenic *E. coli from the* American Type Culture Collection (ATCC) that are recommended for water testing were used in this study (ATCC 8739, 2022; ATCC 13706, 2022; ATCC 23631, 2022). Before each rep, a 10 µl loopful stock culture from each frozen strain was streaked for isolation on Nutrient Agar plates (NA; Difco™, Sparks, MD), and incubated at 37°C for 24±2 hours. A single isolated *E. coli* colony from the NA plates was then used to make the working inoculum immediately (detailed below), however, pure colonies of NA plates remained viable for use if the plates were kept at 4°C for less than a week. Appearance of each strain's pure culture was documented prior to the beginning of the study by plating it on *E. coli*/Coliform Petrifilm (EC Petrifilm™, 3M™, Saint Paul, MN), where the colonies grew blue to purplish with gas bubbles.

4.2.2 Inoculum preparation

An isolated colony of each ATCC strain was added into a 10 ml Brain Heart Infusion broth (BHI broth; Thermo Scientific™ Oxoid, Hants, UK) tube separately. The three tubes were incubated at 37°C for 24±2hours. A pellet of pure *E. coli* with $10^{8\text{-}9}$ CFU/ml was then separated from the broth in each cultured strain's BHI broth tubes by separately centrifuging them at 4,300 x g, for 15 min at 4°C. The supernatant broth was discarded and 10 ml Phosphate Buffered Dilution Water (PBDW; EMD Millipore Corporation, Billerica, Massachusetts) was used to rehydrate each pellet. The concentration of each strain was enumerated. Equal volumes of the three strains were then mixed to form a cocktail solution that was used as the working inoculum

(~27 ml total). The prepared cocktail concentration was also enumerated. The working inoculum was enumerated at the beginning and the end of the trial to ensure that the *E. coli* population was consistent throughout the whole inoculation trial. To enumerate the concentrations of both the strains individually and the utilized cocktail, subsequent dilutions were made in PBDW and plated in duplicate on EC Petrifilms; EC Petrifilms were incubated at 37°C for 48±4hours. Individual strain colonies (blue and purplish with gas bubbles) were counted on EC petrifilm, and they were used as reference for proper enumeration of generic *E. coli* in the inoculated study. The rain barrel and filtered creek water pre-inoculation, native *E. coli* populations were also enumerated using Colilert Quanti-Tray2000 (IDEXX Laboratories, Inc, Westbrook, Maine)

4.2.3 Water sampling, preparation, and inoculation

A 6.5 L batch of rain barrel water was collected from a rain barrel owned by a local KS produce grower. Briefly, a sterilized carboy with a 9 L volume (Thermo Scientific™ Nalgene™) was placed directly under a lid-covered rain barrel's spigot and used to directly collect the rainwater. Before collection, the barrel spigot's external surface was sterilized, and the water was let run for one min before water was collected in the carboy. From a creek in KS used for produce irrigation, 6.5 L of sand-filtered creek water was collected directly into a 10 L sterile carboy. Following each collection, a portion of each water type was enumerated for naturally occurring coliform and *E. coli* populations using Colilert and EC Petrifilm, as described below (results shown in Table 4.6). Both water types were also analyzed for turbidity, pH, electroconductivity, and total dissolved solids (results shown in Table 4.7). Then, each batch of source water was separated into two water samples, each with a volume of 3 L. One 3 L bottle of each water type was stored overnight (8-10 hours) at 32°C and12°C to simulate average spring and summer air temperatures in KS and MO.

Before inoculation the following day, each water sample was plated on EC Petrifilm as described below to ensure that naturally occurring populations did not change during the overnight temperature equilibration. Each 3 L sample bottle per water source and temperature (*e.g.*, rain-32°C and rain-12°C) was inoculated with 1 ml of the working inoculum to a achieve a target concentration of ca. 5 log CFU/ml. The *E. coli* concentration of each 3L bottle of water was confirmed after inoculation by plating on EC Petrifilm as described below. Each inoculated bottle of water was then equally distributed in 3 sub-samples of 990 ml and placed back at their respective temperature for approximately 30 min for temperature equilibration.

4.2.4 Antimicrobial treatment preparation

SaniDate15 (PAA; BioSafe Systems, LLC, East Hartford, Connecticut) and Ultra Clorox germicidal bleach (C; Clorox Professional Products Company, Oakland, California) in concentrations of 75±5 ppm and 25±2 ppm of PAA and free available chlorine, respectively, were the treatments utilized, with sterile DI water used as a control (W). These two treatment concentrations were determined from the Produce Safety Alliance (PSA) recommended sanitizer concentrations for fruits and vegetable washing (Cornell CALS, 2020). Before each replication, titrations were done following manufacturer instructions to confirm stock solution concentrations that achieve the required 75±5 ppm and 25±3 ppm of PAA and free available chlorine respectively in the final 1000 ml treated water samples (treatment details below). Sterile aluminum foil-covered flasks were used to prepare treatments to ensure the treatments were not impacted by light exposure. A Hanna Instrument free and total chlorine high range portable photometer (HI96734; Hannah Instruments, Woonsocket, Rhode Island) was used to validate the free available chlorine concentrations. The PAA treatment concentration was validated using the Biosafety system peracetic acid test kit (BioSafe Systems, LLC, East Hartford, Connecticut)

following the manufacturer instruction. The concentrations of both treatments were confirmed at the beginning and the end of each trial day to ensure the same treatment concentration was used throughout the duration of the trial.

4.2.5 Antimicrobial application and microbial analysis

For time point 0, the sample was collected immediately after a 10 ml aliquot of the appropriate treatment solution (C, PAA and W) was added into each 990 ml sample bottle and swirled to equally distribute the sanitizer for 10 seconds. Water sampling for *E. coli* enumeration from each treated bottle was also performed at time points 5-,10-,60-,1440-, and 2880-min post-treatment. The plate counting and Colilert methods were used to enumerate surviving *E. coli* at each time point. Enumeration using EC Petrifilm was used in objective and thus used in this objective for comparison and validation purposes. Because plating on EC Petrifilm isn't an FDA-approved water testing technique to be used by growers, the IDEXX Colilert Quanti-Tray/2000 was also used to ensure measurable validation. Briefly, for the plate counting method, at each time point, 1 ml of each treated sample was neutralized in 9 ml of Dey/Engley Neutralizing Buffer tubes (D/E; Difco™, Sparks, Maryland) and 5 ml of treated sample was neutralized in 45 ml of D/E in Whirl-Pak® bags (Thermo Scientific™ Nasco, Color Me Whirl-Pak™). Dilutions were made from D/E neutralized tubes using 9 ml PBDW and plated for enumeration on EC Petrifim, and colonies (blue and purplish with gas bubbles) were counted after Petrifilm incubation at 37°C for 48±4hours. For Colilert enumeration, at each sampling point, 100 ml of each treated (C and PAA) sample was neutralized in a transparent, non-fluorescing glass bottle containing 0.2 ml of 10% sodium thiosulfate (Sigma Aldrich, Co, Saint Louis, Missouri), then Colilert reagent was added, and samples were shaken to mix for 30 sec. For the W control samples, 10 microliters from each W treated sample was diluted in 99.99 ml of sterile DI water

containing the Colilert reagent and 0.2 ml of 10% sodium thiosulfate. The use of 0.2 ml of 10% sodium thiosulfate was validated following the neutralizer/control validation procedure of the FDA/EPA protocol (EPA, 2022). Then, all the samples were individually poured in a Colilert Quanti-Tray2000 (IDEXX Laboratories, Inc, Westbrook, Maine), sealed, and incubated at 35°C for 24 hours. For enrichment and recovery of *E. coli*, 50 ml of 2X BHI was added to the 50 ml D/E neutralized sample (5 ml sample+ 45 ml D/E) in Whirl-Pak® bags, resulting in a 1X BHI dilution, and the bags were then incubated at 37°C for 24±2 hours. After incubation, the enriched bags were streaked on MacConkey agar (MAC, Thermo Scientific™ Remel, Lenexa, KS) to determine the presence/absence of *E. coli,* and MAC plates with pink colonies following incubation at 37^{o}C for 18-24 hours were interpreted as positive for *E. coli*. Sample bottles were returned to their respective temperatures after the 10 min time points and were only taken out shortly for the necessary time point sample collection.

4.2.6 Statistical analysis

The experimental procedures were replicated three times; however, only two replications of data are represented for the Colilert method. Statistical analyses were conducted with the Statistical Analysis Software (SAS version 9.4; Cary, NC). Data for the Colilert and EC Petrifilm enumeration methods were analyzed separately. All data were subjected to linear mixed modeling used the PROC MIXED procedure, with a significance level of 0.05. Because repeated measures analysis was used in this study, the best covariance structure for the model was determined. The Least Squares Means (LSMEANS) were calculated and the Tukey-Kramer adjustment for multiple comparisons was used to determine statistical significance between individual treatments. The main effects of time, temperature, treatment, and water source, as well as all two-way interactions, were evaluated for statistical significance. The three- and four-way

interactions were not included in the model to avoid potential issues with model nonconvergence. A backwards elimination strategy was used to remove main effects and interactions not statistically significant from the model.

4.3 Results

Petrifilm Method

Only the main effect of treatment was statistically significant (P<0.0001); however, because the temperature x treatment interaction was also significant (P<0.0001), the results will only be discussed according to temperature and treatment. At each temperature, *E. coli* was not detected in samples treated by both C and PAA, and both were significantly different than the W control (P<0.0001). *E. coli* in the W control sample stored at 32°C was significantly different than W samples stored at 12°C (P<0.0001), though the difference was 0.3 log CFU/ml (Table 4.1). *E. coli* populations in samples treated by PAA and C were statistically the same at 32°C and 12°C (P=1.000).

Table 4.1 *E. coli* survival in surface water using *E. coli*/Coliform Petrifilm and analyzed by antimicrobial treatment and temperature. The treatment x temperature interaction was significant (P<0.0001) and did not include source or time effects. Therefore, data associated with source, or time are displayed according to treatment and temperature.

	LS means±SE *E. coli* survival (Log CFU/ml)	
Treatments	12ºC	32ºC
C	0.0±0.012^{Aa}	0.0±0.012^{Aa}
PAA	0.0±0.012^{Aa}	0.0±0.012^{Aa}
W	5.3±0.012^{Ab}	5.6±0.012 ^{Bb}

Note: *E. coli* not detected is indicated as 0.0 log CFU/ml

^{ABC} Values with different uppercase superscripts in the same row indicate significant differences between temperatures for a treatment.

^{abc} Values with different lowercase superscripts in the same column indicate significant differences between treatments at a temperature.

Interaction between treatment and temperature was significant (P<0.0001).

Colilert Method

Using the Colilert detection method, the main effects of time (P=0.0027), treatment (P<0.0001), temperature (P<0.0001) and source (P=0.0065) were statistically significant. However, all main effects were involved in statistically significant two-way interactions as follows: time x source (P=0.0479), time x treatment (P<0.0001), source x treatment (P=0.0293), and temperature x treatment (P<0.0001). Hence, the results will only be discussed according to the two-way interactions.

Table 4.2 illustrates that *E. coli* populations did vary between rain barrel and creek water at 0 min (P=0.0024) but were statistically the same at each sampling point thereafter (P>0.05). A smaller standard error of the mean (SEM) was observed for both sources at time 60 and 1440 minutes, which impacted statistical differences when other time points were compared within a water source. For example, within creek water, the 10 min time point (2.1 log MPN/ml) was

statistically the same as all other time points, including 2880 min (0.8 log MPN/ml), except for the 1440 min time point (1.5 log MPN/ml). This is likely because the 0.8 log MPN/ml reported at 2880 min has an SEM of 0.26, whereas the 1.5 log MPN/ml reported at 1440 min has a smaller SEM of 0.09. In general, *E. coli* populations declined over time in both rain barrel and creek water sources.

Table 4.2 *E. coli* survival populations in surface water using the Colilert method and analyzed by water source and time. The source x time interaction was significant (P=0.0479) and did not include treatment or temperature effects Therefore, data associated with treatment or temperature are displayed according to source and time.

	LS means±SE *E. coli* survival (Log MPN/ml)					
Source	Time (min)					
	0	5	10	60	1440	2880
Rain Barrel	$1.6^{ABCa} \pm 0.21$	$1.8^{ABa} \pm 0.18$	$1.6^{ABCa} \pm 0.17$	$1.6^{Aa} \pm 0.03$	$1.3^{Ba} \pm 0.09$	$1.2^{Ca} \pm 0.26$
Creek	$2.6^{Ab} \pm 0.21$	$2.0^{ABCDa} \pm 0.18$	$2.1^{ABDa} \pm 0.17$	$1.7^{BCa} \pm 0.03$	$1.5^{Ca} \pm 0.09$	$0.8^{CDa} \pm 0.26$

[ABC] Values with different uppercase superscripts in the same row indicate significant differences of time points for a source.

[abc] Values with different lowercase superscripts in the same column indicate significant differences between sources at a sampling point.

Interaction between source and time was significant (P=0.0479).

At each sampling point, PAA and C were significantly different from the control (W) (P<0.0001), achieving a reduction of ~5 logs MPN/ml of *E. coli* at 0 min and 60 min for PAA and C, respectively (Table 4.3). At the 0- and 5-min sampling points, PAA was significantly more effective than C at reducing *E. coli* populations (P<0.05). However, PAA and C were statistically the same (P>0.05) from the 10 min through the 2880 min sampling points.

Table 4.3 *E. coli* survival in surface water using the Colilert method and analyzed by antimicrobial treatment and time. The treatment x time interaction was significant (P<0.0001) and did not include source or temperature effects. Therefore, data associated with source or temperature are displayed according to treatment and time.

LS means±SE generic *E. coli* survival (Log MPN/ml)

Treatments	Time (min)					
	0	5	10	60	1440	2880
PAA	$0.0^{Aa} \pm 0.26$	$0.0^{Aa} \pm 0.22$	$0.0^{Aa} \pm 0.21$	$0.0^{Aa} \pm 0.03$	$0.0^{Aa} \pm 0.12$	$0.0^{Aa} \pm 0.32$
C	$1.3^{Ab} \pm 0.26$	$0.8^{Aba} \pm 0.22$	$0.5^{BCa} \pm 0.21$	$0.0^{Ca} \pm 0.03$	$0.0^{BCa} \pm 0.12$	$0.0^{BCa} \pm 0.32$
W	$4.9^{ABc} \pm 0.26$	$5.0^{ABb} \pm 0.22$	$5.0^{Ab} \pm 0.21$	$5.0^{Ab} \pm 0.03$	$4.2^{Bb} \pm 0.12$	$2.9^{Cb} \pm 0.32$

Note: Generic *E. coli* not detected is indicated as 0.0 MPN/ml.

[ABC] Values with different uppercase superscripts in the same row indicate significant differences of time points for a treatment.

[abc] Values with different lowercase superscripts in the same column indicate significant differences between treatments at a sampling point.

Interaction between treatment and time was significant (P<0.0001).

The efficacy of PAA at reducing *E. coli* populations was significantly better ($P \leq 0.05$) than that of C and W when used to treat both rain barrel and creek water sources. All treatments achieved significantly different ($P \leq 0.05$) reductions in creek water in comparison to rain barrel water, with larger *E. coli* populations recovered from creek water (Table 4.4). The largest observed difference (~0.5 log MPN/ml) between the two water sources was observed for W (P=0.0005).

Table 4.4 *E. coli* survival in surface water using the Colilert method and analyzed by antimicrobial treatment and water source. The treatment x source interaction was significant (P<0.0293) and did not include time, or temperature effects. Therefore, data associated with time and temperature are displayed according to treatment and water source.

LS means±SE *E. coli* survival (Log MPN/ml)		
Treatments	source	
	Rain Barrel	Creek
PAA	0.0 ± 0.13^{Aa}	0.2 ± 0.13^{Ba}
C	0.3 ± 0.13^{Ab}	0.6 ± 13^{Bb}
W	4.3 ± 0.13^{Ac}	4.7 ± 0.13^{Bc}

Note: Generic *E. coli* not detected is indicated as 0.0 MPN/ml.

[ABC] Values with different uppercase superscripts in the same row indicate significant differences between sources for a specific treatment

[abc] Values with different lowercase superscripts in the same column indicate significant differences between treatments for a specific source.

Interaction between treatment and source was significant (P<0.0293).

The efficacy of PAA at reducing *E. coli* populations was significantly better (P≤0.05) than that of C and W at 12°C and 32°C (Table 4.5). The W treatment was the only treatment that varied across temperatures (P<0.0001), with larger *E. coli* populations recovered from 12°C water (4.8 log MPN/ml) in comparison to 32°C (4.2 log MPN/ml). A difference of ~0.2 log MPN/ml between each temperature was observed for C and PAA (P>0.05).

Table 4.5 *E. coli* survival in surface water using the Colilert method and analyzed by antimicrobial treatment and temperature. The treatment x temperature interaction was significant (P<0.0001) and did not include source or time effects. Therefore, data associated with source and time are displayed according to treatment and temperature.

	LS means±SE generic *E. coli* survival (Log MPN/ml)	
Treatments	32ºC	12ºC
PAA	0.0± 0.13^{Aa}	0.1± 0.13^{Aa}
C	0.3± 0.13^{Ab}	0.5± 0.13^{Ab}
W	4.2± 0.13^{Ac}	4.8± 0.13^{Bc}

Note: Generic *E. coli* not detected is indicated as 0.0 MPN/ml.

^{ABC} Values with different uppercase superscripts in the same row indicate significant differences between temperatures for a specific treatment

^{abc} Values with different lowercase superscripts in the same column indicate significant differences between treatments at a temperature.

Interaction between treatment and temperature was significant (P<0.0001).

Table 4.6 Physical parameter analysis of the collected surface water samples

Replications	Water Source	Sub-Sample Replicate	pH	Electrical Conductivity (µS/cm)	Turbidity (NTU)	Total Dissolved Solids (mg/L)
Rep 1	Filtered creek	1	8.30	702	2.47	432
		2	8.31	719	2.22	365
		3	8.31	727	2.27	395
	Rain barrel	1	7.04	126	0.53	49
		2	6.94	118.5	0.52	55
		3	6.98	118.2	0.54	53
Rep 2	Filtered creek	1	8.1	460	5.05	465
		2	8.08	460	4.79	340
		3	8.11	473	5.24	462
	Rain barrel	1	7.04	554	0.82	453
		2	6.91	89.2	0.74	33
		3	6.88	80.5	0.91	56
Rep 3	Filtered creek	1	8.16	500	4.45	490
		2	8.14	519	4.56	486
		3	8.18	515	4.57	489
	Rain barrel	1	6.91	69.8	1.02	52
		2	6.91	70.3	1.12	52
		3	6.87	58.3	1.27	57

Table 4.7 Microbial analysis of the collected surface water before inoculation

Replications	Sources	Sub- Sample Replicate	*E. coli*
			Total (MPN/100ml)
Rep 1	Filter creek	1	20.1
		2	14.6
		3	14.6
	Rain barrel	1	0
		2	0
		3	0
Rep 2	Filter creek	1	178.5
		2	143.9
		3	193.5
	Rain barrel	1	0
		2	0
		3	0
Rep 3	Filter creek	1	68.3
		2	57.3
		3	105.4
	Rain barrel	1	0
		2	0
		3	0

4.4Discussion

The objective of this study was to evaluate the effectiveness of 25 ppm C and 75 ppm PAA at reducing the *E. coli* in rain barrel and creek water to a level of 'no detectable generic *E. coli*' to determine if surface waters can be effectively treated for post-harvest use in produce according to the FSMA-PSR (FDA, 2022a). Using the EC Petrifilm plate counting method followed by enrichment in 2X BHI streaked on MAC, neither the source nor the temperature generally had an impact on the efficacy or PAA and C. The application of PAA and C resulted in no detectable *E. coli* in any samples of both water sources at the 0 min sampling point, which

suggests that the 10 sec mixing period was sufficient for killing ~5 log CFU/ml of *E. coli*. As a general comparison, the Colilert method recovered generic *E. coli* from samples treated with C through the 60 min sampling point. Like the Petrifilm method, *E. coli* were also not detected from PAA samples at the 0 min sampling point using Colilert. This discrepancy is likely because Petrifilm plates hold 1 ml of sample, whereas the Colilert method is based on a 100 ml sample, and the larger sample size improves the likelihood of recovering generic *E. coli* when microbial populations are low.

While *E. coli* was detected until the 60 min sampling point with the Colilert method in samples treated with C, *E. coli* was not recovered at the 0 min sampling point in samples treated with PAA when using Colilert. This suggests that PAA is more efficient at killing *E. coli* in surface water sources. The efficacy of PAA has been reported by a number of other studies as being more stable in the presence of organic matter and dissolved substances in comparison to C (Fatica & Schneider, 2009; Petri et al., 2021). When used in produce wash water sources with varied levels of organic matter, most of the chlorine is reportedly associated with small molecular substances, and 50% of the chlorine is utilized in the first 5 min (Weng et al., 2016). Winward et al. (2008) also reported that the protection provided to microorganisms by water particles would significantly decrease as initial chlorine concentration increases. These two studies would suggest a rapid killing of suspended microorganisms in the water if the concentration of chlorine is high enough to withstand chlorine demand. McFadden et al. (2017) also demonstrated that PAA required a shorter lag time to inactivate *E. coli* than chlorine, so if the *E. coli* population in the water source was resistant or attached to particles, then it might explain why PAA achieved inactivation of *E. coli* more rapidly than chlorine. The present study

also shows that 25 ppm provided enough residual C treatment to continually decrease *E. coli* populations until they were not detected.

The impact of the source of water on the treatment's efficacy either individually or comparatively was also studied, and the results showed that PAA's efficacy was statistically better than C in both sources of water ($P<0.05$). Also, more *E. coli* was recovered from creek water than rain barrel water. The difference in microbial presence and survival of these two sources of water could be hypothesized to be associated with the physicochemical characteristics of each water source. For instance, the average turbidity and total dissolved solids (TDS) of the creek water were higher (3.96 NTU, 436 ppm) than those of the used rain barrel water (0.83 NTU, 51.1 ppm; Table 4.6) respectively. The higher turbidity and TDS of creek water compared to rain barrel water could have influenced the bacterial protection by particles which then made *E. coli* more recoverable in creek water compared to rain barrel water. Turbidity is directly correlated to the increase of total suspended solids, and they have both been shown by other researchers to have a significant effect on the efficacy of treatment by protecting the microorganisms from treatments and harsh environmental conditions (Falsanisi et al., 2008; Fondriest environmental Inc, 2014; LeChevallier et al., 1981; McFadden et al., 2017). The creek water initially had on average a greater *E. coli* population (88.44 MPN/100ml) compared to the rain barrel water (0 MPN/100ml) (Table 4.7), however, this population difference was very small compared to the added inoculum (~5 log CFU/ml). It is important to also note that the microbial recovery difference in creek water compared to rain barrel water in all treatments is <0.4 MPN/ml, which is likely too small to be of biological relevance. Peroxyacetic acid has been shown to be more effective, even when in the presence of higher organic matter content, and is not greatly affected by TDS compared to C treatments, which have been reported to be greatly

impacted by physicochemical characteristics of water (McFadden et al., 2017; Petri et al., 2021). The latter could explain the overall better efficacy of PAA compared to C in both the rain barrel and creek water sources.

This study also evaluated the effect of water temperature on the efficacy of PAA and C at reducing generic *E. coli* populations. At both temperatures (32°C and 12°C), PAA was statistically more effective than C when using the Colilert method; however, the largest difference between PAA and C was 0.4 log MPN/ml (P=0.0212) in the 32ºC water. This small difference may be statistically significant, but the biological significance of such a marginal difference must also be considered. The difference in temperature didn't significantly affect the efficacy of PAA, with statistically similar (P>0.05) surviving *E. coli* populations recovered at 12°C and 32°C. This is in agreement with the findings of Hassaballah et al. (2020), who reported no significant impact of 4°C compared to room temperature on the efficacy of PAA and C in waste water treatment. This allows farmers to use these two validated treatment concentrations for post-harvest in both cool and warm temperature seasons.

Another important finding of study was the reduction (~1 log CFU/100 ml *E. coli*) of *E. coli* in water control samples over time. This reduction was statistically similar in both rain barrel and creek water sources; however, rain barrel water was consistently associated with a greater microbial die-off of raw *E. coli* (before statistical analysis) populations than those found in than creek water. The roof material from which the rainwater is collected is known to influence the chemical properties of the final collected water (Uba & Aghogho, 2000). One could hypothesize that this chemical catchment might include toxic chemicals to the microbial load of the water which manifests in increased die-off overtime. More research studies would be necessary to prove this hypothesis.

Finally, the microbial reductions achieved by this study may have been influenced by the *E. coli* ATCC strains used in the inoculum, as well as the naturally occurring *E. coli* populations (~2 logs CFU/100 ml) present in the water sources, which is not necessarily a reflection of the entire bacterial load in the water source. For instance, Evans et al. (2006) found negligible coliform counts compared to the total bacterial population of 77 roof-collected rainwater run off samples, which indicates that other bacterial types including pathogens maybe present in different and more significant amounts than coliforms. Different microorganisms or strains of the same microorganisms may also have different resistance to the PAA and C treatments due to different defense mechanisms genotypically, as well as developed phenotypic resistance induced by environmental exposure (Banach et al., 2017; Chapman, 2003; Sahulka et al., 2021).

Haley et al. (2022) tested 247 surface water samples from Kansas and Missouri, and reported the average generic *E. coli* levels to be 158.7 MPN/100 ml, which calculates to 2.2 log MPN/ml. Thus, if these surface water sources were to be treated and used for post-harvest purposes in the product industry, the potential for both PAA and C to reduce >3 logs of *E. coli* in rain barrel and creek water sources in the first 10 sec at both 12ºC and 32ºC is promising with the use of 75 ppm PAA and 25 ppm C. This suggests that 75 ppm PAA and 25 ppm C may be effective chemical interventions for a variety of surface water sources during different seasons.

4.5 Conclusions

The results presented herein suggest that, at the tested doses, both PAA and C are effective at eliminating *E. coli* within 60 min of treating rain barrel and creek water at temperatures of 32ºC and 12ºC. Although the EC Petrifilm method indicated that C achieved no detectable generic *E. coli* at the 0 min sampling point, the Colilert method suggests that complete elimination of generic *E. coli* was more rapid with PAA. Overall, both treatments were effective

at satisfying the FSMA-PSR requirement for ‘no detectable generic *E. coli*’ in agricultural water used post-harvest. However, the EC Petrifilm method was based upon a 1 ml sample per plate, followed by a 5 ml enrichment method, in comparison to the 100 ml sample required by the FSMA-PSR and used by the Colilert method. This study provides evidence that PAA and C can be used as effective interventions for treating rain, creek, and other surface water to provide a microbially potable water source that can be safely used post-harvest in produce.

While these data provide promising results, it must be emphasized that the data are limited to *E. coli*, two temperatures, and two water sources. It must be considered that a large amount of variation likely exists within a single type of water source, and the water recovered from one creek to the next, or even from the same creek throughout different times of the year, will likely vary greatly. Similarly, the data presented herein cannot be extrapolated to pathogens or other microorganisms. Additional research is necessary to fully understand the efficacy of PAA and C at treating surface water for post-harvest use. These findings will help the farmers growing covered produce in abiding by the FSMA-PSR requirements for agricultural water, thereby allowing them to supply to bigger markets which will increase profits while also playing an important role reducing produce associated food-borne disease.

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Chapter 5 : Summary and Conclusions

5.1 Comparison of treatment efficacy in simulated surface water, rain barrel water, and filtered creek water.

When tested in laboratory-prepared, simulated surface water for objective 1, both 75 ppm PAA and 25 ppm C were effective at achieving no detectable generic *E. coli* (~5 log CFU/ml reduction) when using EC Petrifilm and a 5 ml sample enrichment, at 0 min, which was immediately following a 10 sec mixing period. While PAA and C were not significantly different from one another ($P=1.0000$), they were each significantly different from the W control ($P \leq 0.05$). The temperature, time, turbidity, and pH did not impact the efficacy of the PAA and C treatments. Similar results were observed in objective 2 when rain barrel and creek water collected from Kansas farmers were treated with W, PAA, and C, and the *E. coli* enumeration was conducted using EC Petrifilm. Conversely, when rain barrel and creek water treated with PAA and C were enumerated for generic *E. coli* with the Colilert method, *E. coli* were not detected at the 0 min sampling point in samples treated with PAA, whereas *E. coli* was detected in samples treated with C until the 60 min post-treatment sampling point. The efficacy of PAA was also significantly better ($P<0.05$) than C and W control for both creek and rain barrel water sources, and at both temperatures (12°C, 32°C), when enumerated using the Colilert method; however, the largest observed difference between reductions achieved by PAA and C were ~0.4 log MPN/ml or less, which is likely too small of a difference to be practically relevant.

The difference in the results obtained with the two enumeration methods (EC Petrifilm and Colilert) are likely the result of differences in limit of detection, where that of EC Petrifilm (<5 CFU/ml) is higher than that of Colilert (1 MPN/100 ml). Even though enrichment in BHI followed by streaking on MAC was included to ensure the absence of generic *E. coli*, only 5 ml

of each sample was used; thus, the detection limit of EC Petrifilm combined with this enrichment method would still be higher than that of Colilert. Considering the consistency in PAA and C results measured by EC Petrifilm in both objectives, it can be hypothesized that the two studies are comparable in regard to EC Petrifilm enumeration data. Conversely, a general comparison of the EC Petrifilm results with the Colilert results in objective 2 suggests that enumeration using EC Petrifilm may have exaggerated the generic *E. coli* reductions, particularly as they pertain to the C treatment.

Regardless of enumeration method, the PAA treatment reduced *E. coli* to undetectable levels at the 0 min sampling point, suggesting that the 10 second mixing period was sufficient for eliminating the *E. coli* population. Because the Colilert method did not report no detectable *E. coli* levels until the 60 min sampling point for C, a conservative recommendation would be to allow a minimum post treatment hold time of 60 min, particularly if using 25 ppm of C and treating surface water for post-harvest use, since FSMA-PSR requires no detectable generic *E. coli* for post-harvest use. Additionally, recognizing that environmental strains may be different from laboratory-grown reference strains, a more conservative contact time of the antimicrobial treatment with the surface water of at least 60 minutes should be utilized for both PAA and C. Regardless, additional research is necessary to further support these findings before specific recommendations can be made regarding the use of surface water for post-harvest purposes.

Most treatments studied effectively reduced cross contamination of produce during the post-harvest wash step and are effective at completely killing the microorganisms in the wash water; however, depending on their concentration, research suggests that microbial reduction is minimal during washing when produce is already contaminated (Barrera et al., 2012; Luo et al., 2011). Therefore, additional efforts are as necessary regarding the prevention of produce

contamination from planting, harvesting, handling to consumption. The use of microbially potable water (0 CFU/100 ml *E. coli*) during post-harvest washing of produce to avoid cross contamination is another important intervention. The results of this study provide preliminary evidence that surface water, which is more abundantly available, may be safely treated and used post-harvest for produce. However, additional research is necessary before this recommendation can be made.

5.2 Gaps and suggested further research

Generic *E. coli* is globally recognized as a fecal contamination indicator and used to determine the cleanliness of water; however, the presence of generic *E. coli* doesn't guarantee pathogen presence and, likewise, the absence of generic *E. coli* does not always correlate to the absence of pathogens. Research has also shown that different strains and microbial species can have varying virulence and resistance to interventions, with viruses and protozoa demonstrating an even higher resistance (Hassaballah et al., 2020). Therefore, more studies evaluating efficacy of chemical interventions at reducing other major microorganisms of concern in surface water, such as *Giardia*, *Cryptosporidium*, *Salmonella*, and *Campylobacter*. (Pandey et al., 2014) would provide the industry with a better understanding of the ability of these interventions to effectively protect the produce from cross contamination with a variety of microorganisms.

These concentrations of C and PAA studied in this work are to determine efficacy of both C and PAA in disinfecting surface water to be microbially potable for post-harvest use. If the water is used for produce washing, additional understanding of the residual concentration of the chemical interventions (C and PAA) in the wash water and how their reaction with organic content might affect the sensory, and physical characteristics of the produce should be evaluated. Residual concentration upkeep is important to avoid cross contamination. It is important to keep

in mind that 80 ppm of PAA is the maximum EPA acceptable concentration for use in treating wash water to be used in produce washing (21CFR173.315). The production of DBPs, especially with C treatment, is another consideration, especially as the organic content increase. However, Klaiber et al. (2005) reported no significant by-product release when washing carrots with 200 ppm of chlorine, which is a concentration that is substantially higher than the 25 ppm used in objectives 1 and 2. Van Haute, et al. (2013) also reported that no DBP was found on the lettuce washed in chlorine wash water (35 ppm), even in the water with COD as high as 1000 mg O_2 /L, after produce were rinsed with tap water. Furthermore, since results from objectives 1 and 2 suggest a rapid elimination of *E. coli*, additional studies could aim to determine the minimum concentration that is equally efficacious, but also cost effective and reasonably rapid to enhance benefit to farmers. When lower chemical intervention concentrations are evaluated, however, the potential regrowth associated with PAA, and C treatments must be considered to understand the overall fate of the microflora and avoid more public health problems. For instance, another study showed that the application of 1.55 mg/L of chlorine in harvested rain barrel water, with about 12 mg/L TOC, led to the complete inactivation of ~3 logs of coliform, but the regrowth led to a microbial population about the same initial coliform levels in 4 weeks when the water wasn't used (Richards et al., 2021). This study was explained with complete reduction of free available residue chlorine to <0.2 mg/L residue in the 37 days.

In conclusion, comparison of disinfectant efficacy is still a challenging concept considering that the type of water, sanitizer, concentration, contact time, target microorganism, application process, microbial enumeration methods, and many more factors that influence the results of one study to another (Gil et al., 2009). The FDA/EPA established a standard protocol to evaluate the efficacy of disinfectants for treating surface water, which can serve as a general

guide for determining if a sanitizer is efficacious; however, the results from a single study or trial conducted using this protocol will not translate to all water types. Thus, more studies like the two described in this thesis are necessary for validating antimicrobial interventions in different turbidities, concentrations, contact time, sources of water, etc. The results provide important educational information for farmers and the industry to better understand/compare/contrast different treatments for use with a variety of water sources.

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