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Food Safety from Farm to Fork:
Microbial Attachment and Resilience on Spinach Surfaces

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Abstract of the Dissertation

Food Safety from Farm to Fork:
Microbial Attachment and Resilience on Spinach Surfaces

by

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Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
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Dr. Sharon L. Walker, Chairperson

Foodborne illnesses lead to 400,000 deaths annually worldwide, and microbial contamination of fresh produce continues to pose a risk to human health in even the most developed countries. In the United States, 1 in 6 Americans are impacted by foodborne illness every year as the large number of steps that food goes through from farm to fork makes effectively tracing the source of a foodborne outbreak all the more difficult. Modern food production and distribution systems create complex and dynamic environments in which bacteria may attach and proliferate on produce surfaces, which has created a challenge for developing scientifically rigorous food safety regulations. Therefore, this doctoral research aimed to investigate the effects of various environmental conditions on bacterial adhesion, detachment, and disinfection on leafy greens using colloidal transport theory and fundamental research models.

Overall, the results of this dissertation work indicate that the likelihood of bacterial attachment onto produce surfaces will vary throughout the watering, washing, and rinsing processes that take place between harvest and consumption. Employing a suite of environmentally relevant bacteria, the kinetics of bacterial attachment and removal from

spinach epicuticle surfaces was investigated using a parallel-plate flow cell that allows for simulated gentle rinsing and real-time observation and enumeration of cells. Environmental factors like restricted nutrient availability and complex water chemistry increased attachment rates of foodborne bacteria to the leaf surface, which was attributed to decreases in cell surface charge and changes in extracellular polymers in favor of proteins. The introduction of copper oxide nanoparticles enhanced adhesion and minimized detachment of bacteria from spinach leaf surfaces, while nano-titanium dioxide had the opposite effect, highlighting potential unintended consequences of their application as pesticides or fertilizers in agricultural waters. By modeling standard chlorine disinfection rinses, leaf surface roughness was found to reduce the effectiveness of bleach in removing and inactivating pathogens adhered to spinach leaves. An alternative, enzyme-based disinfectant was produced and shown to increase bacterial detachment from the spinach surface and prevent biofilm formation on polycarbonate. Further, this doctoral research addresses the gap between scientists and policymakers through two projects that identify and explore mechanisms for translating scientific data into public policy change for promoting sustainable diets in the developing world and managing diverse, statewide water data in California. As a whole, this work has elucidated technical and political challenges and opportunities in ensuring food safety and security that warrant further research to inform scientifically rigorous policy and regulations.

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Chapter 1

Introduction

1.1 Background and Motivation

As the global population continues to increase, modern agriculture must adapt to ensure that all communities have access to secure food resources. According to the Food and Agriculture Organization of the United Nations, food security is achieved when sufficient, nutritious, and *safe* food is available (1). However, unsafe food leads to 600 million illnesses and 400,000 deaths annually worldwide (2), and even in the developed world, microbial contamination of fresh produce continues to pose a risk to human health (3). Annually, 1 in 6 Americans will contract foodborne illness (4), while 46% of illnesses that lead to hospitalization or death can be attributed to fresh produce (5). Even with industrialized agricultural systems that regulate and limit direct produce handling, there is a significant need for scientific research to improve our understanding of the complex interactions between microbes and produce to protect human health.

Food safety research often focuses on pre-harvest pathogen contamination while in the field (6-8). Meanwhile, modern food production and processing is increasingly centralized with greater reach and availability of fresh produce nation- and world-wide. Even when low quantities of bacteria are present on produce before or immediately after being harvested, concentrations can significantly increase after being transported, handled, and processed, indicating that contamination may be more likely to occur post-harvest, rather than pre-harvest (9-11). The occurrence of cross contamination during value-added processes like cooling, washing, and packaging has been shown to magnify the risks of foodborne illness outbreaks associated with fresh produce (12, 13). Further, the large number of steps that food goes through from “farm-to-fork” makes effectively

tracing the source of a foodborne outbreak all the more difficult. Thus, washing and packaging plants that are intended to streamline disinfection and improve food safety have the potential to create complex and dynamic environments for bacteria to attach and proliferate on produce surfaces, if they are not well understood and appropriately regulated.

Current food safety policies, such as those of the Leafy Greens Marketing Agreement (LGMA) and newly enacted Food Safety Modernization Act (FSMA), have begun to incorporate strict food safety guidelines for these large-scale processors (14, 15). Unfortunately, the aforementioned standards were hastily developed to assuage consumer perceptions after large foodborne illness outbreaks and are not founded in science. For example, the safety of "triple-washed" spinach lacks scientific evidence and was developed primarily as a marketing tool after the 2006 spinach-associated *E. coli* O157:H7 outbreak that originated from just one farm, but affected products of multiple brands in 26 states, and hundreds of people (16). Since then, leafy greens-associated illness outbreaks have continued to occur, including the 2018 outbreak from romaine lettuce that affected 36 states and was ultimately traced back to contaminated irrigation water in Yuma, Arizona (17).

Challenges in developing scientifically rigorous food safety regulations stem from the fact that modern food systems create complex and dynamic environments in which bacteria may attach and proliferate on produce surfaces. The characteristics and components of water that is used to irrigate, wash, and disinfect produce can vary significantly based on the climate, water source, and crop, while the management

requirements largely remain the same (18). Previous work conducted in our lab, and others, has demonstrated that aqueous bacterial deposition and detachment on surfaces is sensitive to a wide range of parameters, including ionic strength, pH, and valence, as well as the presence of nanomaterials, organic matter, and disinfectants (19-21). Building on said results, the studies presented in this dissertation apply colloidal transport methods and knowledge to agriculturally relevant systems.

This doctoral work aims to expand and elevate our understanding of the role of aqueous environmental conditions in bacterial adhesion, detachment, and disinfection on leafy green surfaces. These factors include cell type (22, 23), water chemistry (21, 24, 25), nutrient availability (26, 27), and the presence of nanoparticles (28, 21) or disinfectants in suspension (29, 30), which contribute to measurable changes in bacteria surface macromolecules (31-33), electrostatic characteristics (34, 35), and hydrophobic interactions (35, 36). The framework of this dissertation considers all of these variables through systematic investigations of how pathogenic and non-pathogenic bacteria interact with model spinach leaf surfaces under dynamic flow conditions. The first factor, cell type, was varied by using 18 different bacteria isolates to identify improved non-pathogen surrogates for two common pathogens through physiochemical characteristics. Second, the effects of water chemistry and nutrient conditions were studied. Results revealed that two pathogens grown in nutrient-restricted environments adhere to spinach leaves at greater rates, with more complex water chemistries magnifying the difference between low- and high-nutrient scenarios. The influence of two agriculturally-relevant nanoparticles in suspension was the third factor considered and resulted in increased

irreversible attachment of the pathogen and minimal effects on a non-pathogen, which could be largely attributed to differences in extracellular polymer production. Lastly, the effects of two disinfectants on bacterial detachment from the leaf surface were studied. In one project, the efficacy of bleach in removing bacteria from spinach leaves was found to be potentially compromised by surface roughness that reduces effective concentration at the surface. In another project, the potential of a novel enzyme-based disinfectant was investigated and shown to increase bacterial detachment rates from leaf surfaces, as well as prevent and remove biofilm growth for several bacteria. As a whole, this dissertation work fills several knowledge gaps in ensuring the food safety of leafy greens from farm-to-fork, including bacterial surrogate optimization, the role of several environmental factors on pathogen transport, and efficacy of both traditional and novel disinfection options.

1.2 Aim and Scope

The overarching objective of this research was to explore the effects of several factors on microbial fate and transport on food surfaces in realistic, dynamic flow conditions. Using a suite of relevant bacteria, the work presented in herein investigates mechanisms of adhesion and detachment in a unique flow cell that allows for simulated gentle rinsing and real-time observation of bacteria. Details of this investigation can be found in five core chapters of this dissertation: selection of better surrogates for foodborne pathogens (Chapter 2), role of water quality and nutrient conditions (Chapter 3), impacts of engineered nanoparticles (Chapter 4), and efficacy of bleach (Chapter 5),

as well as an enzyme disinfectant (Chapter 6). An additional chapter takes a policy perspective on the role of data in managing two important aspects of food safety from the field to our plates: water and sustainable diets (Chapter 7).

1.3 Hypotheses and Objectives

The goals of this doctoral research can be broken down into the following hypotheses and specific objectives, which are addressed in the five technical chapters and one public policy chapter of this dissertation.

Hypothesis 1: Current quality control strains are not representative of the characteristics of real pathogenic strains in ideal and restricted nutrient conditions.

As a part of an ongoing USDA project, the objective of this study was to systematically characterize eighteen environmental *E. coli* isolates from manures and surface waters, to identify physico-chemical properties similar to those of known foodborne pathogens *E. coli* O157:H7 (ATCC 4388) and *Salmonella* enterica serotype Typhimurium (ATCC 13311). Zeta potential, size, relative hydrophobicity, extracellular polymers (EPS) composition, and surface charge density were measured for cells grown in low- and high-nutrient media. Luria-Burtani broth (LB) and a lettuce exudate media (LM) were used to represent standard laboratory and limited-nutrient conditions, respectively. The bacteria varied significantly in zeta potential, hydrophobicity, and surface charge density, while the impact of growth conditions on these characteristics was also strain-dependent. In combination with lettuce leaf adhesion assays performed by

the USDA, these results were used to identify five isolates that may provide improved surrogates for represent the behavior of foodborne pathogens in the environment. This project is detailed in Chapter 2.

Hypothesis 2: Bacteria will attach to spinach leaves at higher rates in nutrient-restricted conditions and simulated aquatic chemistry than in ideal growth conditions and simple solutions, respectively.

The objective of this work was to investigate the effects of solution chemistry and growth conditions on bacterial deposition on spinach leaf surfaces using a microfluidic parallel plate flow cell to simulate gentle produce washing scenarios. Two food safety pathogens of concern (*E. coli* O157:H7 and *Salmonella* enterica serotype Typhimurium), as well as two non-pathogenic potential bacterial surrogates (identified using results from Chapter 1) were grown in LB media or M9 minimal media; then suspended in monovalent, divalent, or complex multivalent salt solutions. Positioned over an inverted light microscope, bacterial attachment to a model spinach leaf surface was directly observed and photographed with a digital camera over 30 minutes. The images generated were then processed to quantify the mass transfer rate coefficients for each bacteria strain in each nutrient and water chemistry condition. Additionally, changes in cell surface charge, hydrophobicity, and EPS composition were measured and used to understand the impact of limited nutrients and complex solution chemistries on the initial stages of biofilm formation. Chapter 3 describes the details of this project.

Hypothesis 3: Presence of metal oxide nanoparticles in suspension with bacteria will increase adhesion and detachment in model 2-D and 3-D systems.

Nanoparticles are increasingly common as fungicides and pesticides in agricultural operations, thus the objective of this project was to study the fate and transport of model bacteria suspended in environmentally relevant solutions with model nanoparticles in 2-D and 3-D systems simulating leafy greens and soils, respectively. The bacteria used in this study were a pathogen, *E. coli* O157:H7 (ATCC 43888) and a non-pathogen, *E. coli* ATCC 25922, while the nanoparticles were CuO and TiO_2. A 2-D parallel-plate flow cell and 3-D saturated sand column were used to systematically examine changes in bacteria deposition and detachment trends as a result of nano-bio interactions under dynamic flow conditions. Surface charge, colloidal size, and bacterial EPS production were analyzed for each bacteria strain and nanoparticle. The aforementioned characterizations were used to explain mechanisms of observed changes in nanoparticle and bacterial transport between the unique scenarios. Experimental results were compared to those predicted by Derjaguin-Landau-Verwey-Overbeek (DLVO) theory to further our understanding of the role of colloidal surface charge in mixed systems. The results of this project are presented in Chapter 4.

Hypothesis 4: Bacterial detachment rates from spinach leaf surfaces will decrease with ionic strength and increase with bleach concentration.

Attachment and detachment kinetics of *E. coli* O157:H7 from spinach leaf epicuticle layers were investigated to determine the impact of water chemistry and

common bleach disinfection rinses on the removal and inactivation of the pathogen. Using multiple ionic strengths and valence conditions, adhesion was observed in the parallel-plate flow cell over a 30 minute period, followed by a 30 minute rinse with either DI water or 1-1000 ppb sodium hypochlorite (bleach) when detachment was observed. Mass transfer rate coefficients were calculated using the cell enumeration of time, and used to quantify the differences in cell attachment and detachment in each scenario. DLVO modeling was once again used to evaluate the contributions of electrostatic and van der Waals forces on the interactions between bacteria and the leaf surface. Additionally, a computational COMSOL model was developed to evaluate fluid flow across the leaf surface and the resulting concentration gradient of bleach during produce rinsing. The project is summarized in Chapter 5.

Hypothesis 5: Rinsing with an engineered enzyme can increase bacterial detachment rates, as well as prevent and remove biofilms.

The objective of this work was to optimize the production of an engineered enzyme and test its usefulness as a food safety disinfectant using biofilm assays on polycarbonate and spinach leaf surfaces in the parallel plate flow cell. A reference *E. coli* strain was used to over-express the enzyme, and mechanical cell lysis followed by protein separation and immobilized metal ion affinity chromatography were used to isolate the enzyme. The enzyme, suspended in 10 mM KCl, was rinsed over the leaf surface with adhered *E. coli* O157:H7 in the parallel plate flow cell at concentrations of 250 and 1000 ppb. Mass transfer rate coefficients were again used to compare the efficacy of the

enzyme rinse with a plain deionized (DI) water rinse. In order to consider enzyme efficacy beyond the initial attachment phase, biofilm formation in 24-well polystyrene plates in the presence of 100 ppm enzyme and after a 10 minute treatment with 100 ppm enzyme was measured by crystal violet staining assays. Biofilm formation of *E. coli* O157:H7, *Salmonella* Typhimurium, and *E. coli* 25922 were measured. Chapter 6 presents the results of this project.

Project 6a: Synthesis of recommendations for improved water data management by a diversity of water industry stakeholders in California.

The objective of this work was to gather and analyze published recommendations for water data management in California. Additionally, informal interviews and ongoing examples of water data management projects were used to provide a preliminary assessment of efforts to improve data collection, organization, and dissemination for water decision-making in California. A literature review and assessment of best practices in California water data management were conducted in parallel. The set of generalized common recommendations, which were identified from recent literature, was used to characterize key features of past water data management strategies. Based on the combination of the most common recommendations from literature and prevalence in observed best practices from past water data management efforts, a subset of key recommendations was produced and used to inform the stakeholder interviews. After aggregating responses to the informal interview questions, the final set of shared key

recommendations for water data management was generated. Chapter 7 includes the details of this process and the resulting recommendations.

Project 6b: Development of a conceptual framework and identification of public policy leverage points for sustainable diets in Vietnam using existing food systems data.

In order to enable Vietnamese decision-makers to identify intervention points that will create positive improvements across multiple sectors of the food system, the objective of this project was to create a conceptual framework and associated metrics for sustainable diets in Vietnam. Through a review of the literature, a framework was developed that includes 8 essential domains of sustainable diets, interactions between them, and over 250 non-overlapping metrics divided according to these domains. In addition to reviewing relevant literature, three distinct but complementary approaches were used: (1) data characterization of existing data sources relevant to sustainable diets, (2) informal interviews with decision-makers, and (3) a stakeholder workshop of national experts representing diverse domains of sustainable diets. The framework and metrics were used alongside stakeholder engagement to identify preliminary leverage points that emerged across multiple domains, which refer to specific policy areas that have the potential to influence multiple aspects of the sustainability of diets in Vietnam. The resulting conceptual framework and policy leverage points for sustainable diets in Vietnam are therefore based on a mix of both data dependent and data independent approaches, which are described in Chapter 7.

1.4 Experimental Approach

In additional to the Introduction (Chapter 1), Conclusion (Chapter 8), and public policy projects (Chapter 7), this dissertation is composed of five experimental chapters. Following the Introduction, Chapter 2 describes the utilization of several key physiochemical characterization methods to highlight the differences and similarities between pathogens and a suite of potential non-pathogen surrogates. Eighteen total cell types were characterized according to their size (using dynamic light scattering (DLS)), surface charge (using electrophoretic mobility or zeta potential), hydrophobicity (using the microbial adhesion to hydrocarbons (MATH) test), and extracellular polymer (EPS) composition (reported as sugar to protein ratio using calorimetric methods). This study revealed that while the current strain of *E. coli* used as a surrogate for quality control protocols poorly matches the surface characteristics of *E. coli* O157:H7 and *Salmonella* Typhimurium, several environmental strains of *E. coli* are promising alternatives. This study is thoroughly described in Chapter 2, entitled "Selection of Better Surrogates for Foodborne Pathogens from the Agricultural Environment."

The crux of this dissertation work begins with Chapter 3, "*Escherichia coli* O157:H7 and *Salmonella* Typhimurium Adhesion to Spinach Leaf Surfaces: Role of Water Chemistry and Nutrient Availability." These two food safety pathogens of concern and two potential non-pathogen bacterial surrogates were grown in ideal (LB media) and environmentally relevant (M9 media) nutrient conditions, then suspended in 10 mM monovalent, divalent, or multivalent salt solutions. Bacteria attachment on a model spinach leaf surface was enumerated in real time over 30 minute experiments using a

parallel plate flow chamber on an inverted fluorescent microscope. Based on images recorded with a digital camera, attachment rate coefficients were calculated using cell attachment rate (cells/min), concentration in suspension (cells/mL), and microscope viewing area (153 × 113 μm). Differences in pathogen attachment were attributed to measurable variability in cell surface charge and hydrophobicity, and both pathogens were significantly more adhesive to spinach surfaces when grown in minimal media. Surrogates, on the other hand, did not follow this trend and showed minimal changes in adhesion kinetics and surface properties between growth conditions. Increased attachment in certain water chemistry and nutrient conditions were attributed to changes in EPS composition in favor of proteins. These results showed the importance of growth conditions and solution complexities in understanding mechanisms of bacterial adhesion at interfaces of water and food surfaces.

The influence of interactions between metal nanoparticles and bacteria on transport was assessed in Chapter 4, "Influence of Nanoparticles on Adhesion and Detachment of *Escherichia coli*." The parallel plate flow cell was again used to quantify attachment and detachment of pathogenic and non-pathogenic stains of *E. coli* (O157:H7 and 25922, respectively) in the presence of either copper oxide (CuO) or anatase titanium dioxide (TiO_2) nanoparticles in 10 mM KCl. Size and surface charge were again utilized to elucidate mechanisms of colloidal interactions, while a scanning electron microscopy was used to visualize the mixtures of nanoparticles and bacteria. Interestingly, the near neutral zeta potential and small size of CuO seemed to promote attachment and reduce detachment of O157:H7 cells on the leaf surface, while TiO_2 had no significant effects. *E.*

coli 25922 transport was unaffected by both nanoparticles, which is attributed to its greater surface charge and EPS production that reduced cellular interactions with nanoparticles.

In order to investigate the effectiveness of rinsing and washing processes after produce leaves the field, Chapters 5 and 6 primarily focuses on removal of bacteria from leaf surfaces. Chapter 5 focuses on traditional disinfection methods and is entitled "Efficacy of Post-Harvest Rinsing and Bleach Disinfection of *E. coli* O157:H7 on Spinach Leaf Surface." Mass transfer rate coefficients and the microfluidic flow cell were again used to quantify the impact of water chemistry and common bleach disinfection rinses on the removal and inactivation of the pathogen. Adhesion was observed for cells suspended in 1-100 mM KCl and 3.3-10 mM artificial groundwater, followed by detachment during a rinse with either DI water or 1 – 1000 ppb bleach solutions. Zeta potential and hydrophobicity were assessed for both the cells and the leaf surface in each solution chemistry and utilized to evaluate the relative contribution of electrostatic and van der Waals interactions with DLVO theory. These results showed the importance of ions present during attachment in cells' ability to remain attached. Additionally, detachment and cell death were dependent on both bleach concentration and exposure time, presenting important challenges to the disinfection of attached cells when compared to the typical industry bleach concentrations and rinsing times.

In Chapter 6, "Disrupting Irreversible Bacterial Adhesion and Biofilm Formation with an Engineered Enzyme," a novel enzyme-based disinfectant is produced and tested. An enzyme with the ability to catalyze degradation of the extracellular matrix was

expressed in a reference strain of *E. coli* and purified via affinity chromatography. Potential of 0.1 mg/mL of the enzyme to both prevent and degrade biofilm formation of *Salmonella* Typhimurium, *E. coli* O157:H7, and *E. coli* 25922 on polystyrene was then investigated by growing bacteria for 48 hours in minimal media and static conditions. Following the removal of planktonic bacteria and rinsing with DI water, crystal violet (CV) solution was used to stain remaining cells. Cell-bound CV was then dissolved in 80% ethanol/20% acetone solution, and absorbance was recorded as an indirect measure of biofilm formation. Additionally, the parallel plate flow cell was employed to compare detachment of *E. coli* O157:H7 when rinsed with 0 ppb, 250 ppb PL, and 1000 ppb enzyme in 10 mM KCl. The results of this project demonstrate a promising disinfection alternative to traditional antimicrobials in the food safety industry.

In order to improve the way that scientific data is managed and ultimately translated to policy change, two projects presented in Chapter 7 (*a* and *b*) utilize a combination of literature review and stakeholder engagements methods, including interviews and structured workshops. Chapter 7*a* addresses data management for water policy in California, and is entitled "Identifying Common Ground for Sustainable Water Data Management: The Case of California." Chapter 7*b* employs these methods to address data-driven policy-making for sustainable food systems in Vietnam and is entitled "Conceptualizing Sustainable Diets in Vietnam: Minimum Metrics and Potential Leverage Points."

1.5 Manuscripts Resulted from Research

Cook, K.L., Givan, E.C., Mayton, H.M., Parekh, R.R., Taylor, R., Walker, S.L. Using the agricultural environment to select better surrogates for foodborne pathogens associated with fresh produce. *International Journal of Food Microbiology*, Vol. 262, 80-88 (2017).

Mayton, H.M., Marcus, I.M., Walker, S.L. *Escherichia coli* O157:H7 and *Salmonella* Typhimurium adhesion to spinach leaf surfaces: Sensitivity to water chemistry and nutrient availability. *Food Microbiology*, Vol. 78, 134-142 (2019).

Mayton, H.M., White, D., Marcus, I.M., Walker, S.L. Influence of engineered nanomaterials on adhesion and detachment of *Escherichia coli* in two model systems. Submitted to *Environmental Science & Technology* (2019).

Kinsinger, N.M., Mayton, H.M., Luth, M.R., Walker, S.L. Efficacy of post-harvest rinsing and bleach disinfection of *E. coli* O157:H7 on spinach leaf surfaces. *Food Microbiology*, Vol. 62, 212-220 (2017).

Mayton, H.M., Walker, S.L., Berger, B.W. Disrupting irreversible bacterial adhesion and biofilm formation with an engineered enzyme. In preparation (2019).

Mayton, H.M. and Story S.D. Identifying common ground for sustainable water data management: the case of California. *Water Policy,* wp2018047 (2018).

Mayton, H.M., Beal, T., Rubin, J., Sanchez, A., Heller, M., Hoey, L., de Haan, S., Doung, T., Dhar Burra, D., Khoury, C.K., Jones, A.D. Conceptualizing sustainability diets in Vietnam: Minimum metrics and potential leverage points. In preparation (2019).

1.6 References

1. Food and Agriculture Organization (FAO). 2003. Trade Reforms and Food Security: Conceptualizing the Linkages. Rome, Italy.
2. World Health Organization (WHO). 2015. WHO Estimates of the Global Burden of Foodborne Diseases: Foodborne Disease Burden Epidemiology Reference Group 2007-2015. Rome, Italy.
3. Callejón RM, Rodríguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and causes. Foodborne Pathogens and Disease 12:32-38.
4. Centers for Disease Control and Prevention (CDC). 2014. CDC and Food Safety. Accessed November 20, 2018 at https://www.cdc.gov/foodsafety/cdc-and-food-safety.html
5. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin, PM. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. Emerging Infectious Diseases, 19(3):407-415.
6. Guo X, Chen J, Brackett RE, Beauchat LR. 2002. Survival of *Salmonella* on tomatoes stored at high relative humidity, in soil, and on tomatoes in contact with soil. Journal of Food Protection, 65(2):274-279.
7. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. 2005. Survival of *Escherichia coli* O157:H7 in soil and on carrots and onions grown in fields treated with

contaminated manure composts or irrigation water. Food Microbiology, 22(1):63-70.

8. Bradford SA, Morales VL, Zhang W, Harvey RW, Packman AI, Mohanram A, Welty C. 2013. Transport and fate of microbial pathogens in agricultural settings. Critical Reviews in Environmental Science and Technology, 43(8):775-893.

9. Gil MI, Selma MV, Suslow T, Jacxsens L, Uyttendaele M, Allende A. 2015. Pre- and postharvest preventive measures and intervention strategies to control microbial food safety hazards of fresh leafy vegetables. Critical Reviews in Food Science and Nutrition, 55(4):453-468.

10. Smolinski HS, Wang S, Ren L, Chen Y, Kowalcyk B, Thomas E, Van Doren J, Ryser ET. 2018. Transfer and redistribution of *Salmonella* Typhimurium LT2 and *Escherichia coli* O157:H7 during pilot-scale processing of baby spinach, cilantro, and romaine lettuce. Journal of Food Protection, 81(6):953-962.

11. Buchholz AL, Davidson GR, Marks BP, Todd ECD, Ryser ET. 2012. Transfer of *Escherichia coli* O157:H7 from equipment surfaces to fresh-cut leafy greens during processing in a model pilot-plant production line with sanitizer-free water. Journal of Food Protection, 75(11):1920-1929.

12. Kuan CH, Lim LWK, Ting TW, Rukayadi Y, Ahmad SH, Wan Mohamed Radzi CWJ, et al. 2017. Simulation of decontamination and transmission of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and Listeria monocytogenes during handling of raw Vegetables in domestic kitchens. Food Control, 80:395-400.

13. Kusumaningrum H. 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. International Journal of Food Microbiology, 85(3):227-236.

14. California Leafy Greens Products Handlers Marketing Agreement (LGMA). 2016. Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens. Sacramento, CA, USA.

15. Food Safety Modernization Act. In Public Law 111-353, Congress, Ed. 2011.

16. Food and Drug Administration (FDA) and California Department of Public Health. 2007. Investigation of an *Escherichia coli* O157:H7 Outbreak Associated with Dole Pre-Packaged Spinach. Sacramento, CA, USA.

17. Centers for Disease Control and Prevention (CDC). 2018. Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Romaine Lettuce (Final Update). Atlanta, GA, USA.

18. Leafy Greens Marketing Agreement (LGMA). 2018. LGMA Accepted Food Safety Practices. Leafy Greens Marketing Agreement, Sacramento, CA, USA.

19. Chen G and Walker SL. 2007. Role of solution chemistry and ion valence on the adhesion kinetics of groundwater and marine bacteria. Langmuir 23:7162-7169.

20. Chowdhury I, Zorlu O, Walker SL, Haznedaroglu BZ. 2014. Impact of growth phase and natural organic matter on the attachment kinetics of *Salmonella* Typhimurium to solid surfaces. Environmental Engineering Science 32:111-120.

21. Chowdhury I, Cwiertny DM, Walker SL. 2012. Combined factors influencing the aggregation and deposition of nano-TiO2 in the presence of humic acid and bacteria. Environmental Science & Technology, 46(13): 6968-6976.

22. Marcus IM, Bolster CH, Cook KL, Opot SR, Walker SL. 2012. Impact of growth conditions on transport behavior of *E. coli*. Journal of Environmental Monitoring, 14(3):984-991.

23. Haznedaroglu BZ, Kim HN, Bradford SA, Walker SL. 2009. Relative transport behavior of *Escherichia coli* O157:H7 and *Salmonella* enterica Serovar Pullorum in packed bed column systems: Influence of solution chemistry and cell concentration. Environmental Science & Technology, 43:1838-1844.

24. Li Q, Logan BE. 1999. Enhancing bacterial transport for bioaugmentation of aquifers using low ionic strength solutions and surfactants. Water Research, 33:1090-1100.

25. Bradford SA, Kim HN, Haznedaroglu BZ, Torkzaban S, Walker SL. 2009. Coupled factors influencing concentration-dependent colloid transport and retention in saturated porous media. Environmental Science & Technology, 43:6996-7002

26. Hassan AN, Frank JF. 2004. Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. International Journal of Food Microbiology, 96:103-109.

27. Nagar V, Pansare Godambe L, Bandekar JR, Shashidhar R. 2016. Biofilm formation by Aeromonas strains under food-related environmental stress conditions. Journal of Food Processing and Preservation, 41, e13182

28. Khan SS, Mukherjee A, Chandrasekaran H. 2011. Studies on interaction of colloidal silver nanoparticles (SNPs) with five different bacterial species. Colloids and Surfaces B: Biointerfaces, 87(1): 129-138.

29. Calix-Lara TF, Rajendran M, Talcott ST, Smith SB, Miller RK, Castillo A, Sturino JM, Taylor TM. 2014. Inhibition of *Escherichia coli* O157: H7 and *Salmonella* enterica on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. Food Microbiology, 38, 192e200.

30. Han Y, Sherman DM, Linton RH, Nielson SS, Nelson PE, 2000. The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157: H7 to green pepper surfaces. Food Microbiology, 17(5), 521e533.

31. Kuznar ZA, Elimelech M. 2005. Role of surface proteins in the deposition kinetics of Cryptosporidium parvum oocysts. Langmuir, 21:710-716.

32. Kim HN, Hong Y, Lee I, Bradford SA, Walker SL. 2009. Surface characteristics and adhesion behavior of *Escherichia coli* O157:H7: Role of extracellular macromolecules. Biomacromolecules, 10:2556-2564.

33. Kim HN, Walker SL, Bradford SA. 2010. Macromolecule mediated transport and retention of *Escherichia coli* O157:H7 in saturated porous media. Water Research, 44:1082-1093.

34. Gross M, Cramton SE, Gotz F, Peschel A. 2001. Key role of teichoic acid net charge instaphylococcus aureus colonization of artificial surfaces. Infection and Immunity, 69:3423-3426.

35. van Loosdrecht MC, Lyklema J, Norde W, Schraa G, Zehnder AJ. 1987. The role of bacterial cell wall hydrophobicity in adhesion. Applied Environmental Microbiology, 53:1893-1897.

36. Schafer A, Harms H, Zehnder AJ. 1998. Bacterial accumulation at the air− water interface. Environmental Science & Technology, 32:3704-3712.

Chapter 2

Towards Better Surrogates for Foodborne Pathogens from Agricultural Environments

Cook, K.L., Givan, E.C., Mayton, H.M., Parekh, R.R., Taylor, R., Walker, S.L. Using the agricultural environment to select better surrogates for foodborne pathogens associated with fresh produce. *International Journal of Food Microbiology*, Vol. 262, 80-88 (2017).

Abstract

Despite continuing efforts to reduce foodborne pathogen contamination of fresh produce, significant outbreaks continue to occur. Identification of appropriate surrogates for foodborne pathogens facilitates relevant research to identify reservoirs and amplifiers of these contaminants in production and processing environments. Therefore, the objective of this study was to identify environmental *Escherichia coli* isolates from manures (poultry, swine and dairy) and surface water sources with properties similar to those of the produce associated foodborne pathogens *E. coli* O157:H7 and *Salmonella* enterica serotype Typhimurium. The most similar environmental *E. coli* isolates were from poultry (n = 3) and surface water (n = 1) sources. The best environmental *E. coli* surrogates had cell surface characteristics (zeta potential, hydrophobicity and exopolysaccharide composition) that were similar (i.e., within 15%) to those of S. Typhimurium and/or formed biofilms more often when grown in low nutrient media prepared from lettuce lysates (24%) than when grown on high nutrient broth (7%). The rate of attachment of environmental isolates to lettuce leaves was also similar to that of *S.* Typhimurium. In contrast, *E. coli* O157:H7, a commonly used *E. coli* quality control strain and swine isolates behaved similarly; all were in the lowest 10% of isolates for biofilm formation and leaf attachment. These data suggest that the environment may provide a valuable resource for selection of surrogates for foodborne pathogens.

2.1 Introduction

Contamination of food and water by pathogens continues to be a significant public health concern in the United States (U.S.). It is estimated that 48 million foodborne illnesses occur each year and only 9.4 million of those are caused by identified pathogens (1). A substantial percentage of illnesses (around 46%) are thought to be associated with fresh produce (2) and leafy vegetables have been categorized as a food safety priority by the World Health Organization (3). Although the incidence of some important foodborne pathogens, including *Salmonella* sp. and *Escherichia coli* O157:H7, has decreased in recent years, significant outbreaks continue to occur (4-6). This is despite implementation of recommended good agricultural practices (GAPs) targeted to fresh fruits and vegetables (2, 5). Although most of the recommended mitigation strategies have focused on post-harvest processing, environmental monitoring programs for pre-harvest safety are increasingly important. Studies have shown the importance of the environment (irrigation water, soil, wildlife) as the source for contaminants (6, 7, 8, 9) and the FDA Food Safety and Modernization Act (FSMA) has prioritized prevention by strengthening safety on the pre-harvest side of production.

National, international, and industry groups have called for identification of improved surrogates that persist in ways that better mimic the behavior of important foodborne pathogens (9-15). Nonpathogenic bacteria like *E. coli* are often used by industry and regulatory groups as proxies, surrogates and/or indicators of human pathogens (14-18). In production and processing environments, surrogates are used in studies to identify risk and to develop improved management practices to reduce pathogen contamination. Properly quantifying and mimicking the behavior of pathogens in these environmental systems is complicated and many groups have questioned the validity of using generic organisms as indices for evaluating the microbiological quality

of water, soil and produce (16, 19, 20). However, years of precedence, including long-term use in regulated monitoring, as well as the availability of specific, sensitive, user and budget friendly methods make *E. coli* an ideal surrogate for pathogens. The need lies in uncovering strains and properties of this or similar organisms that make them better representatives of the pathogens (11, 18, 20, 21). Having access to a representative selection of well-characterized pathogen surrogates would provide the produce industry with a viable option for evaluating and validating intervention strategies while assuring safety and simplicity of use for research and quality control purposes.

Surrogates are dissimilar to fecal indicator organisms, in that they must behave like the pathogen in a specific environment. In contrast, an indicator simply signifies the potential risk for bacterial contamination in the source environment (14). In general, generic *E. coli* strains such as the American Type Culture Collection (ATCC) strain *E. coli* 25922 have been used as surrogates for pathogenic *E. coli* strains such as O157:H7 and for other research, benchmarking or standard laboratory and industrial testing as recommended by regulatory groups (12, 22-24). These strains are often adapted to laboratory conditions and lack factors that permit persistence under harsher environmental conditions.

Environmental *E. coli* isolates survive extended periods of time in the secondary habitats (soil, manure, or water) where temperatures, moisture levels, UV radiation from the sun, salinity, and other environmental conditions can create a stressful environment (6, 25-27). Strain-level differences in physical, chemical and biological properties of environmental isolates of *E. coli* is extensive and likely serves to improve chances for survival in the host environment and increase fitness in secondary habitats like soil and water sources (28-34). Therefore, selection of more appropriate surrogates may be made possible by using isolates from the same niche as the enteric pathogens (i.e., gastrointestinal bacteria) that are capable of survival in secondary habitats (i.e. following

fecal deposition into the environment). This approach has already been applied in the meat processing industry where biosafety level 1 (BSL-1) isolates of *E. coli*, obtained from beef hides are being used as surrogates for *E. coli* O157:H7 (10, 18, 21). To our knowledge, this approach has not yet been applied for identifying surrogates for foodborne pathogens associated with fresh produce. Therefore, the goal of this research was to identify *E. coli* strains from manure and surface water sources which possess genotypic and phenotypic properties similar to those of foodborne pathogens found in association with fresh produce (6, 29, 35-37).

2.2 Materials & Methods

2.2.1 *E. coli* cultures and bacterial growth conditions.

This work builds on previous research in which environmental *E. coli* isolates (n = 1346) from poultry, swine, dairy and surface water sources were characterized (29). That research was used as the basis for selection of a sub-set of environmental *E. coli* isolates (n = 63) for use in this study. Selected isolates equally represented poultry, swine and dairy manure and surface water sources, included isolates with genes important for survival in secondary habitats and represented each of the *E. coli* phylogroups. These 63 *E. coli* isolates were then characterized to identify surrogates with genotypic and phenotypic properties similar to those reported in the literature to be associated with fitness of human pathogens in environmental sources and on produce (29, 34, 36-41). Biofilm formation, leaf adhesion, growth rates and genes associated with adhesion, biofilm formation and fitness were used as the primary selection criteria for isolates to be used for subsequent studies. Two enteric pathogens, *E. coli* O157:H7 (ATCC 43888) and

S. Typhimurium (ATCC 13311), and a commonly used quality control strain (*E. coli* ATCC 25922) were included for comparative purposes. A flow diagram outlining procedures used for selection of final surrogates is shown in Figure 2.1.

E. coli isolates were recovered from frozen culture by plating onto Luria-Bertani (LB) agar (Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ) and *E. coli* selective agar plates (mTEC; Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ). After incubation overnight at 37°C, a single colony from the plate culture was aseptically selected and used to inoculate growth media for further experiments. Selected *E. coli* strains and pathogens were characterized following growth to stationary phase (18 h to 24 h) on either nutrient rich or nutrient poor medium. Low-nutrient media were derived from lettuce leaf exudates supplemented with minimal salts media (LM) and were prepared as previously described using store bought green leaf lettuce heads (42, 43). Minimal salts medium was added to exudates as previously described, with the addition of 19 mM of ammonium chloride to supplement growth (42). Growth on low-nutrient media was compared to high-nutrient lab growth media LB broth (Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ).

2.2.2 Phenotypic characterization: biofilm formation, curli expression growth rates

Isolates grown on LM or LB media were characterized for growth rate, biofilm formation and curli expression. Briefly, biofilm formation of isolates was determined using the crystal violet stain assay of (44) using a 96 well microtiter culture plate format.

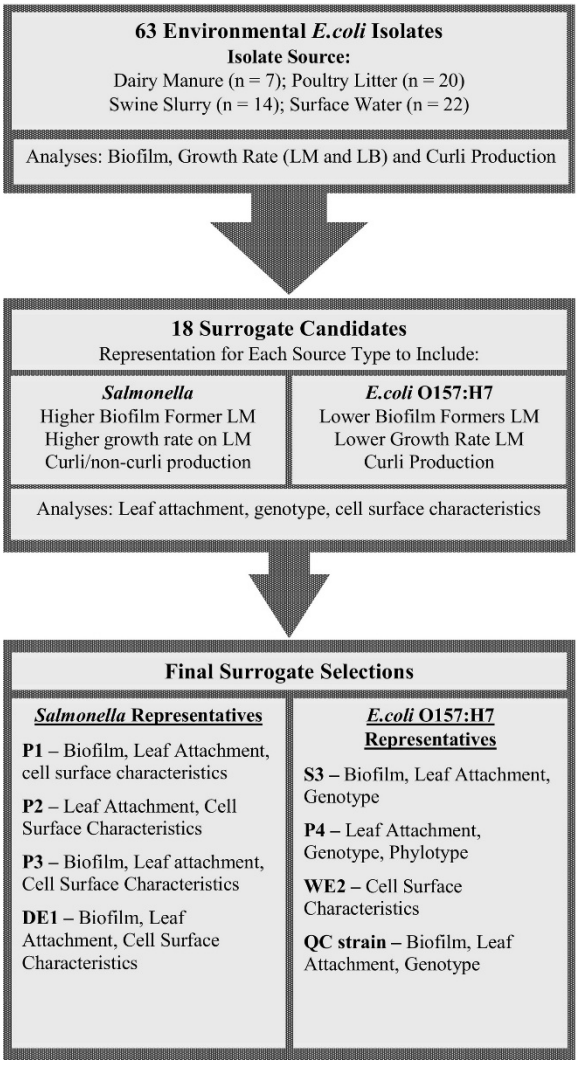

Figure 2.1 Flow diagram illustrating procedures used to select environmental *E. coli* surrogates to represent produce associated foodborne pathogens.

The initial *E. coli* isolates (n = 63) were a subset of 1346 isolates from dairy manure (D), poultry litter (P), swine slurry (S) or surface waters (DE or WE) and also included the common quality control (QC) strain *E. coli* ATCC 25922. Biofilm formation, growth rates and curli production were used as selection criteria to narrow the surrogate pool to 18 isolates (surrogate candidates). Candidates were assessed for lettuce leaf attachment, genotype, and cell surface characteristics. Final *E. coli* surrogate selections included four poultry litter, two surface water and one swine isolate as well as the QC strain.

LM = lettuce lysates with minimal salts media; LB = Luria-Bertani broth. See Table 2.2 for genotype and phylogroup data and Figure 2.4 for lettuce attachment data.

Biofilm readings were completed using a BioTek ELx808 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT) and the optical density (OD) of each well was measured at 546 nm (OD_{546}). Growth rates for isolates grown on LB or LM growth media in 96 well microtiter plates were recorded over a 12-h period using BioTek ELx808 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT) reading at OD_{546}. Growth rates (*K*) were determined using the GrowthRates© software version 1.8 (45). The expression of curli fimbriae was identified by growing all isolates on an agar plate prepared according to methods previously described (46). Red colonies indicated the expression of curli after 48 h of incubation at room temperature, and isolate profiles were categorized by Romling's methods (47).

2.2.3 Cell surface characterization.

Electrophoretic mobility and effective diameter were assessed using a ZetaPALS analyzer (Brookhaven Instruments, NY). The zeta potential, a measure of each cell type's electrokinetic properties and relative charge, was calculated from the electrophoretic mobility using the Smoluchowski equation (48, 49). Relative hydrophobicity was assessed using the semi-quantitative microbial adhesion to hydrocarbons (MATH) test, which involves measuring the percentage of total cells that partition into a hydrocarbon (dodecane) via changes in optical density evaluated with a UV–Vis spectrophotometer (BioSpec-mini, Shimadzu Corp., Kyoto, Japan) at OD_{546}. Potentiometric titration with a microtitrator (798 Titrino, Metroohm) was conducted to determine relative acidity of cell surfaces, from which surface charge density was calculated. Extracellular polymeric

substance (EPS) on the cell surface was characterized for composition of proteins and polysaccharides using a modified EPS extraction technique involving freeze drying and detection via colorimetric methods as previously described (50, 51). All characteristic parameters were measured in triplicate.

2.2.4 Genotypic characterization and serotyping.

All isolates were genetically characterized as previously described for adhesion, capsular synthesis and siderophore formation (29, 52-54). Isolates were placed in one of four phylogenetic groups (A, B1, B2, or D) using the triplex PCR method as previously described (29). Using this method, two genes (*chuA* and *yjaA*) and a non-coding region of the genome (TSPE4.C2) are amplified in one PCR reaction. Isolates were sent to Pennsylvania State University *E. coli* Reference Center (University Park, PA) for serotyping of the somatic (O) and flagellar (H) antigens and virulence gene analysis. O serotyping was conducted using O-antigenic polysaccharides generated against *E. coli* serogroups O1-O187. H typing was performed by PCR-RFLP analysis of the *fliC* gene. Virulence genes were assessed by PCR analysis for LT, STa, STb, stx1, stx2, *eae*, *cnf1* and *cnf2*.

2.2.5 Lettuce adhesion assay.

The attachment of isolates to lettuce leaves was determined using modifications of methods as previously described (42, 55). Green leaf lettuce (Lactuca sativa) seedlings were grown in a commercial potting mixture to the three-leaf stage (approximately 14

days) in a BSL-2 greenhouse located in western Kentucky, U.S.A. Isolates (approximately 1×10^9 cells/ml) were grown in LM media and lettuce leaves were aseptically removed and dipped into inoculum for 30 s. Leaves were immediately washed in sterile 0.1 × PBS buffer for 30 s to remove any loosely adhered cells. DNA was extracted from washed lettuce leaf samples and the concentration of attached surrogate or pathogen cells were quantified.

2.2.6 DNA extractions and quantitative, real-time PCR assays.

Quantitative, real-time PCR (qPCR) was used to evaluate the concentration of *Salmonella*, and *E. coli* cells attached to lettuce leaves. *Salmonella* cell concentrations were targeted using *ttrBCA* gene specific qPCR assay; ttr-F: AGC TCA GAC CAA AAG TGA CCA TC, ttr-R: CTC ACC AGG AGA TTA CAA CAT GG, and ttr-Probe: FAM-CAC CGA CGG CGA GAC CGA CTT T-BHQ as previously described (56). *E. coli* cell concentrations were quantified using primers specific for the *lacY* gene; lacY-F 5'-ACCAGACCCAGCACCAG

ATAAG-3', lacY-R 5'-CTGCTTCTTTAAGCAACTGGCGA-3', and probe; lacY-FAM-5'-CATACATATTGCCCGCCAGTACAGAC-3'-BHQ as previously described (57). DNA from lettuce leaves was extracted by bead-beating and qPCR assays were prepared using Qiagen HotStarTaq Master Mix as previously described (58).

2.2.7 Statistical analysis.

All means and standard deviations were calculated using SigmaPlot 12.5 (Systat Software, 2013) or Excel (Microsoft Office, 2013) using triplicate data points. Significant differences for means were determined using a two sample Student t-tests with differences considered significant if $p < 0.05$.

2.3 Results & Discussion

2.3.1 Isolate selection.

The variability among production and processing environments makes it critical that hazard analysis and controls be site and process specific. On-farm production practices are particularly variable and depend on climate, topography, source materials and management factors. Therefore, research to develop valid mitigation strategies is dependent on the availability of relevant, safe and reliable surrogates that mimic the behavior of important foodborne pathogens in a similar environment. The process of surrogate selection focuses on identifying strains of nonpathogenic bacteria which behave like the relevant pathogen in a particular environment (14). For example, environmental isolates are being used in the meat processing industry as surrogates for *E. coli* O157:H7. These isolates were obtained from beef hides and have been shown to possess similar growth, resistance and attachment properties as the pathogen (10, 18, 21). Surrogates that are non-pathogenic relatives of pathogens (i.e., *E. coli* O157:H7, *Listeria monocytogenes*, and *Clostridium botulinum*) are being used to understand the behavior of pathogens in the

production environment, estimate risk of contamination at harvest, and to develop management practices that reduce risk (59-61). However, the non-pathogenic laboratory strains used in many studies may not be adapted for survival in environmental settings in the same way as the enteric foodborne pathogens that they represent. In fresh produce production, effective surrogates should survive in secondary habitats and adhere to produce surfaces in a manner similar to that of targeted pathogens. Therefore, the goal of this research was to identify a suite of environmental *E. coli* isolates that exhibited phenotypic and genotypic characteristics similar to those of foodborne pathogens found in association with fresh produce (Figure 2.1).

2.3.2 Growth rate, curli and biofilm formation.

To select surrogate candidates from the environmental *E. coli* isolates, isolates were characterized for the ability to grow on either high- or low-nutrient broths, to form curli fibers, and to produce biofilms (Figure 2.1). As expected, the average growth rate of environmental *E. coli* isolates grown in high nutrient LB broth ($0.0132 \pm 0.002\ h^{-1}$) was more than double that of isolates grown in the low nutrient lettuce lysates ($0.0058 \pm 0.0006\ h^{-1}$). Despite lower growth rates in LM media, lettuce lysates were used in all biofilm and lettuce adhesion studies because the nutrient content is similar to levels found under environmental growth conditions. Results from several studies suggest that there is a correlation between produce surface metabolites, exudates and/or damaged plant tissue and the occurrence/growth of enteric pathogens like *Salmonella* and *E. coli* O157:H7 (62-67). In these studies, nutrient analysis of lettuce exudates showed that LB and LM had

similar levels of most cations, but LM was significantly lower in sodium (487 ± 11 mg/L) than was LB (3523 ± 120 mg/L) (Table 2.1). In contrast, concentrations of magnesium and phosphorous were both higher in LM (11.9 ± 0.3 mg/L and 450.9 ± 13.6 mg/L, respectively) than in LB (1.70 ± 0.06 mg/L and 156.7 ± 7.0 mg/L, respectively).

Table 2.1 Comparison of physicochemical properties of inoculating media.

Media[a]	pH	EC[b]	Ca	Fe	K	Mg	Na	P	Zn
		mS/cm	----------mg/L----------						
LL	6.65 ± 0.02	0.56 ± 0.01	8.17 ± 0.15	0.07 ± 0.03	99.90 ± 1.95	5.57 ± 0.06	24.67 ± 0.68	9.50 ± 0.22	0.11 ± 0.00
LM	7.10 ± 0.01	3.21 ± 0.04	18.23 ± 0.37	0.05 ± 0.01	366.26 ± 9.02	11.90 ± 0.30	486.73 ± 11.07	450.85 ± 13.58	0.15 ± 0.01
LB	7.09 ± 0.01	19.15 ± 0.25	5.56 ± 0.18	0.37 ± 0.02	354.33 ± 14.88	1.70 ± 0.06	3522.79 ± 120.14	156.65 ± 7.04	0.91 ± 0.04

[a]LL= lettuce lysate without minimal salts media; LM= lettuce lysate with minimal salts media; LB= Luria-Bertani broth.

[b]EC= electrical conductivity measured in milli-Siemens per cm (mS/cm).

Biofilm formation is crucial to a pathogen's ability to colonize and persist on plant surfaces (36, 37), and has been shown to be an indicator of a pathogen's ability to attach to produce surfaces (44). In this study, biofilm formation by *Salmonella* was more than nine times higher when grown in lettuce lysates than when grown in high nutrient LB broth (Figure 2.2B). In contrast, the QC strain was one of the lowest biofilm formers in lettuce lysates (average OD_{546} 0.122 ± 0.019) and biofilm formation was significantly lower (ten times less) than that of *Salmonella* ($p < 0.05$; Figure 2.2B). Similarly, the *E. coli* O157:H7 did not form significant levels of biofilm when grown on LM compared to

LB and its biofilm formation was similar to the low level of biofilm formed by the QC strain grown in the same media.

As was the case for *Salmonella*, biofilm formation was higher when the environmental *E. coli* isolates (n = 63) were grown on the low nutrient LM media (Figure 2.2A). Mean biofilm formation at OD_{546} of all isolates grown on LB was 0.017 ± 0.066 but was 0.127 ± 0.097 when grown on LM. However biofilm formation was highly variable among *E. coli* isolates from the same source (i.e. poultry) as well as among isolates from different environmental sources (Figure 2.2A). Eleven of the top twenty biofilm formers were from dry event (DE) surface water samples and 24% produced biofilm when grown on LM compared to 7% grown on LB. Isolates DE1 and DE2 had OD_{546} of 1.15 ± 0.20 and 1.28 ± 0.20, respectively (Figure 2.2B), levels similar to or greater than those of *Salmonella* (average OD_{546} of 1.48 ± 0.38).

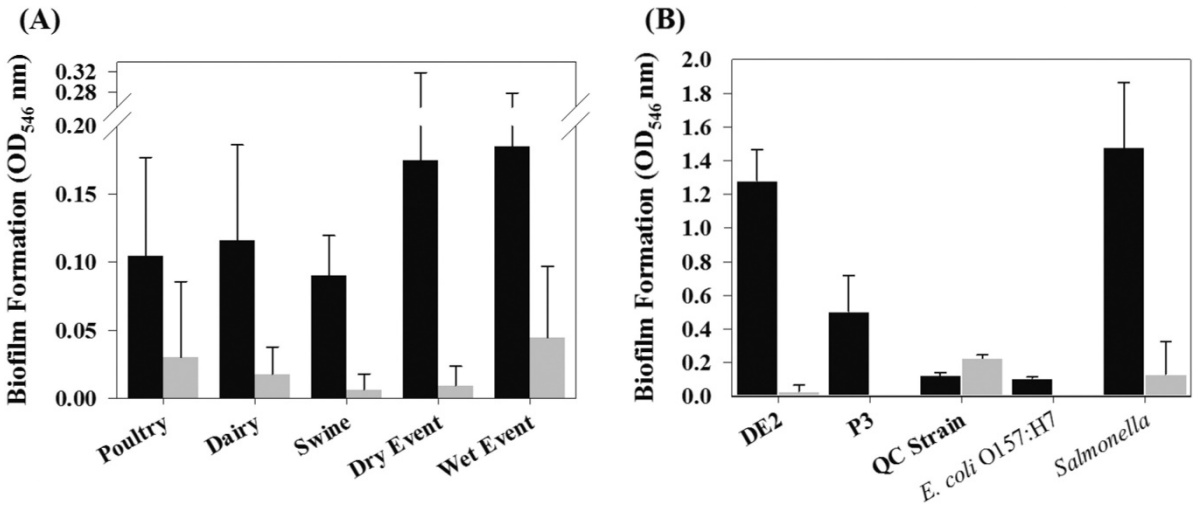

Figure 2.1 Biofilm formation.

Values represent the mean biofilm formation (OD_{546} nm) of (A) environmental *E. coli* isolates (n = 63) from manure and water sources and (B) environmental *E. coli* strains (P3 & DE1), QC strain, *E. coli* O157:H7, and *Salmonella* Typhimurium. Biofilm formation was measured following growth in either lettuce lysates (LM; black) or Luria-Bertani (LB; gray).

Previous studies have shown that biofilm formation increases when bacteria are grown under low nutrient conditions (44, 47, 68). Increased biofilm formation is speculated to be due to changes in cell surface characteristics due to starvation (69). However, there may also be a greater relationship with media composition than with nutrient strength (44, 68). In this study, higher biofilm formation by most isolates grown in lettuce lysates may be due to the especially low sodium in that medium although that was not evaluated.

Most of these environmental *E. coli* isolates produced curli, although not all were positive for cellulose. The high rate of curli positive isolates may be coincidental with these being environmental (manure-associated) isolates. S. Typhimurium were recently shown to lack cellulose and fimbriae when grown at temperatures found in animal hosts (37°C), but the structures were produced when the pathogens were grown at temperatures (28°C) similar to those found in secondary environments 70). Research suggests that curli expression is associated with attachment (38, 72), however, expression is tightly regulated by factors including osmolarity (47). This could explain higher biofilm formation by isolates grown in low sodium LM. In a comparison of *Salmonella* from clinical, meat and produce sources, Solomon et al. (2005) found that 80% of produce-related isolates produced curli and produce-related outbreak strains were more often curli positive than were isolates from food sources (72). Their results emphasize the potential importance of curli in attachment to produce surfaces. However, further research is needed to better understand how media nutrient composition, environmental factors and strain characteristics influence adherence and biofilm formation.

Based on data from these 63 environmental strains, the number of *E. coli* isolates was narrowed (n = 18) to include representatives from each source type. Isolates included those that were either high or low biofilm formers (representing biofilm levels produced by *Salmonella* and *E. coli* O157:H7) and those with different growth rates on nutrient broth and in lettuce lysates. Selected isolates (surrogate candidates) were then characterized for lettuce attachment genotype, serotype and cell surface properties to aid in selection of final surrogates (Figure 2.1).

2.3.3 Leaf attachment.

Leaf attachment is the first step in colonization of produce surfaces and studies have found significant correlations between produce associated pathogens and the ability of the organism to attach to produce surfaces (36, 39, 73, 74). In this study, trends for leaf attachment results were similar to those of biofilm formation. *Salmonella* had the highest attachment to three-leaf stage lettuce plants (1.7×10^8 cells/g; $p < 0.05$). In addition to being strong biofilm formers on LM, environmental *E. coli* from poultry also attached to lettuce leaves at higher rates than other isolates (Figure 2.3). Poultry isolates (P1, P2, P3) and surface water isolate DE1 had the highest leaf attachment and attachment was not significantly different than that of *Salmonella* ($p > 0.05$); Table 2.3). As was the case for biofilm formation, *E. coli* O157:H7 did not attach to lettuce at levels above average and values did not differ significantly from those of the QC strain or the surrogates. *E. coli* surrogates from swine sources were generally below average in leaf attachment (S1 through S5). The swine isolates were also poor biofilm formers when grown in lettuce

lysates (Figure 2.2) characteristics which suggest that they would be suitable environmental surrogates for *E. coli* O157:H7. In a study of *E. coli* surrogates for *E. coli* O157:H7, Kim and Harrison (2009) found that zeta potential and attachment to lettuce was higher for *E. coli* ATCC 25922 than for other surrogates (laboratory stock cultures) and recommend its use as a surrogate for *E. coli* O157:H7 (12). Our results suggest that the QC strain adheres to lettuce and form biofilms in a manner similar to *E. coli* O157:H7, although both were poor performers compared to *Salmonella* and the environmental *E. coli* isolates.

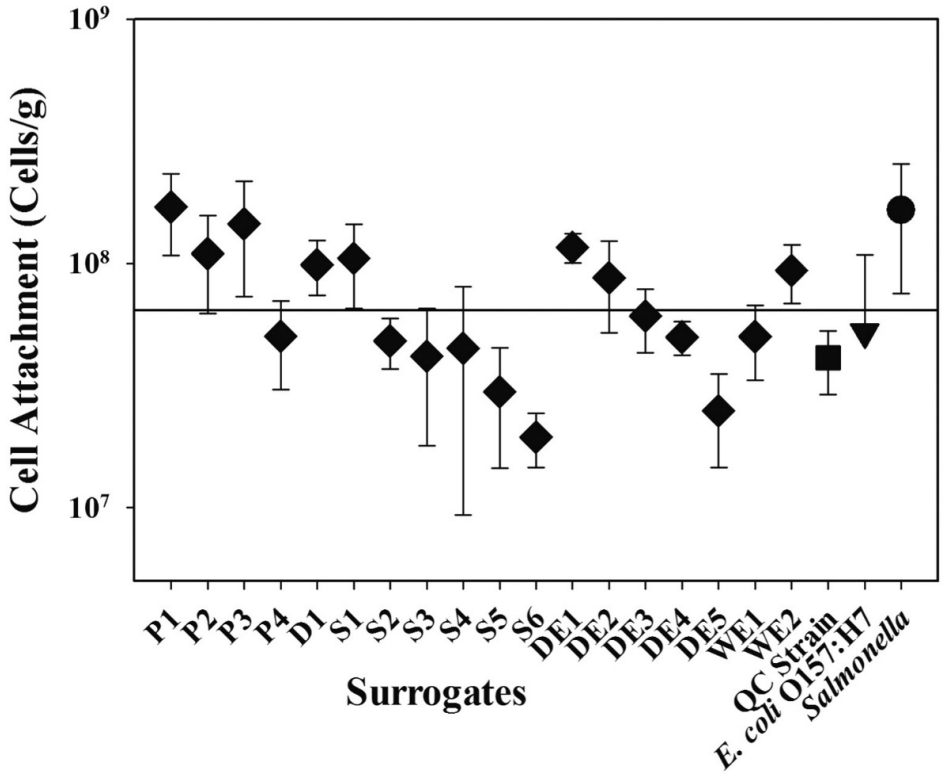

Figure 2.2 Lettuce leaf attachment**.**

E. coli surrogates (⬥); *E. coli* quality control (QC Strain; ■); *E. coli* O157:H7 (O157:H7; ▼); and *Salmonella* Typhimurium (*Salmonella*; •). Values represent average and standard deviation of triplicate experiments.

2.3.4 Phylotyping and serotyping.

All phylogenetic groups of *E. coli* (A, B1, B2, or D) were represented in the 18 surrogate candidates, but most were phylogroup B1 (44%) while groups B2 and D were equally represented (Table 2.2). The surface water isolate and QC strain, both chosen to represent *E. coli* O157:H7, belong to phylogroup B2 and three of the four selected poultry litter isolates were group D. Three of the selected surrogates were group B1, strains which are commonly found in commensals from animals (75).

Serotyping of the somatic (O) and flagellar (H) antigens and PCR analysis of virulence genes (LT, STa, STb, stx1, stx2, *eae*, *cnf1*, and *cnf2*) was carried out on all 18 isolates (Table 2.2 and Table S1, Appendix A). A wide diversity of *E. coli* O:H serotypes and flagellar types existed among the selected isolates, with the only identical types being among isolates from the same source (i.e., O1:H + in two poultry isolates; Table 2.2). Two of the *E. coli*, one from swine and one from a water source had properties similar to those of pathogens (Table S2, Appendix A). All other isolates had serotypes that were not associated with those of common pathogenic strains and were negative for important virulence genes. Lack of O type or H serotypes or virulence genes (LT, STa, STb, stx1, stx2, *eae*, *cnf1*, and *cnf2*) commonly associated with pathogens suggest the selected isolates were nonpathogenic, which is an important criterion for final surrogate selection. However, it may be necessary to obtain genome sequences to confirm isolate safety (10, 14, 18, 76).

Table 2.2 Serotype and presence of gene for adhesion, capsular synthesis, siderophore formation.

Source	Isolate	Serotypes		Phylo-group[c]	Gene Target[d]					
		O[a]	H[b]		*agn*	*ihA*	*paa*	*sfa-foc*	*kpsII*	*ironEC*
Poultry	P1	100	34	B1	+					+
	P2	43	2	D	+					+
	P3	1	+	D	+	+				
	P4	1	+	D	+	+				
Dairy	D1	88	+	B1	+					
Swine	S1	–	+	B1	+					+
	S2	–	–	A						
	S3	111	5	B1	+	+				+
	S4	109	45	A				+		
	S5	86	10	B1	+				+	
	S6	128	–	B1	+		+			
Surface water	DE1	21	–	B1	+					
	DE2	21	–	B1	+					
	DE3	110	+	B2						
	DE4	110	+	B2	+					
	DE5	13	4	B2	+			+	+	+
	WE1	R	18	D	+					
	WE2	19	4	B2	+				+	
Controls	QC Strain	6	1	B2	+	+		+		+
	E. coli O157:H7	157	7	D	+	+	+			

Table 2.3 Statistical comparison of biofilm formation and leaf attachment for top five surrogates and QC strain to pathogens.

		Salmonella		***E. coli* O157:H7**	
Source	**Isolate**	**Biofilm**	**Leaf Attachment**	**Biofilm**	**Leaf Attachment**
		----------t-score[a]----------			
Poultry	P1	-4.17	0.23	1.47	1.80
	P2	-4.36*	-0.95	1.42	1.55
	P3	-2.71	-0.28	3.25	1.67
Surface water	DE1	-0.28	-0.81	9.32*	1.65
	WE2	-3.35	0.25	6.07*	1.76
Control	QC Strain	-4.69*	-3.69*	1.43	1.03

[a]Independent samples t-test comparing each surrogate or QC strains

[b]H serotype shows match to known *fliC* serotypes; (+) indidcates *flicC* present but RFLP pattern does not match known patterns; (–) indicates no *filC* present.

[c]*E. coli* isolates phylogroup determined by PCR

[d](+) indicates positive

*$p < 0.05$

2.3.5 Genotyping.

All isolates were genetically characterized as previously described, targeting genes for adhesion, capsule synthesis and siderophore formation (29, 52-54). Isolates possessed genes conducive to adherence and survival in the environment (Table 2.2). Four of the final selected surrogates (two selected for each pathogen) were positive for the siderophore gene *ironEC* which aids in the capture of iron in low nutrient environments. Surrogates S5, DE5, and WE2 were positive for the capsular synthesis gene *kpsII* which may aid in nutrient scavenging and/or cell-cell communication. Genes for adhesion were common in these isolates. All but three were positive for the adhesion gene *agn* and all but one were positive for the *fimH* gene. Both of these genes have been linked to biofilm formation and adhesion in previous studies (31, 34, 71). Surrogates P3, P4, and S3 were positive for the adhesion gene *ihA*, and two surrogates were positive for adhesion gene *sfa-foc*. These findings taken together with phenotypic analysis results suggest that the environmental isolates have genetic properties conducive to survival in low nutrient environments and attachment to surfaces. However, it is uncertain how these genotypes are expressed in vivo. For example, Yue et al. (2015) showed that *Salmonella* strains that have low-affinity binding in vitro, were able to bind strongly to porcine enterocytes (i.e., in a host-specific) (78). This is similar to our results which show that swine isolates that had high adhesion rates to quartz sand in column studies had very low binding to lettuce leaves.

2.3.6 Cell surface characterization.

Zeta potential, size, relative hydrophobicity, EPS composition, and surface charge density were measured to identify differences in cell surface characteristics between isolates grown in LM or LB and to identify isolates with surface properties most similar to those of the pathogens (Figure 2.4 and Table S3, Appendix A). Cell surface characterization data for isolates with the highest lettuce leaf attachment levels are shown in Figure 2.4.

Mean cell size was greater for cells grown in LB (1482 ± 88 nm) than for those grown in LM (1196 ± 75 nm). The zeta potential for *Salmonella* doubled when grown in LB (-19.7 ± 1.3 mV) versus in LM (-9.9 ± 1.5 mV), but in general isolate zeta potential values were similar (within a 10 mV range) regardless of growth media. Cell surface hydrophobicity varied anywhere from ± 1% to ± 52% relative hydrophobicity between growth in LB or LM. The largest differences were observed in three surface water isolates that had significantly higher cell surface hydrophobicity when grown in lettuce lysates, and in one surface water isolate and two swine isolates which had lower cell surface hydrophobicity. Surface charge density represents the distribution of polar functional groups on the cell surface (79), and decreased in all isolates when grown in LM (data not shown). Levels of total EPS decreased for cells grown in LM, with protein content reduced to less than half of the corresponding value for growth in LB (54.5 ± 9.8 mg protein/10^{10} cells to 28.7 ± 0.65 mg protein/10^{10} cells). Despite the lower overall EPS production for cells grown in LM, there was a general increase in the sugar/protein ratio of EPS composition when cells were grown in LM (averaging 1.5 times higher). These

overall characterization results are similar to trends observed by Marcus et al. (2012) for *E. coli* dairy isolates when grown in manure extract or LB broth (51).

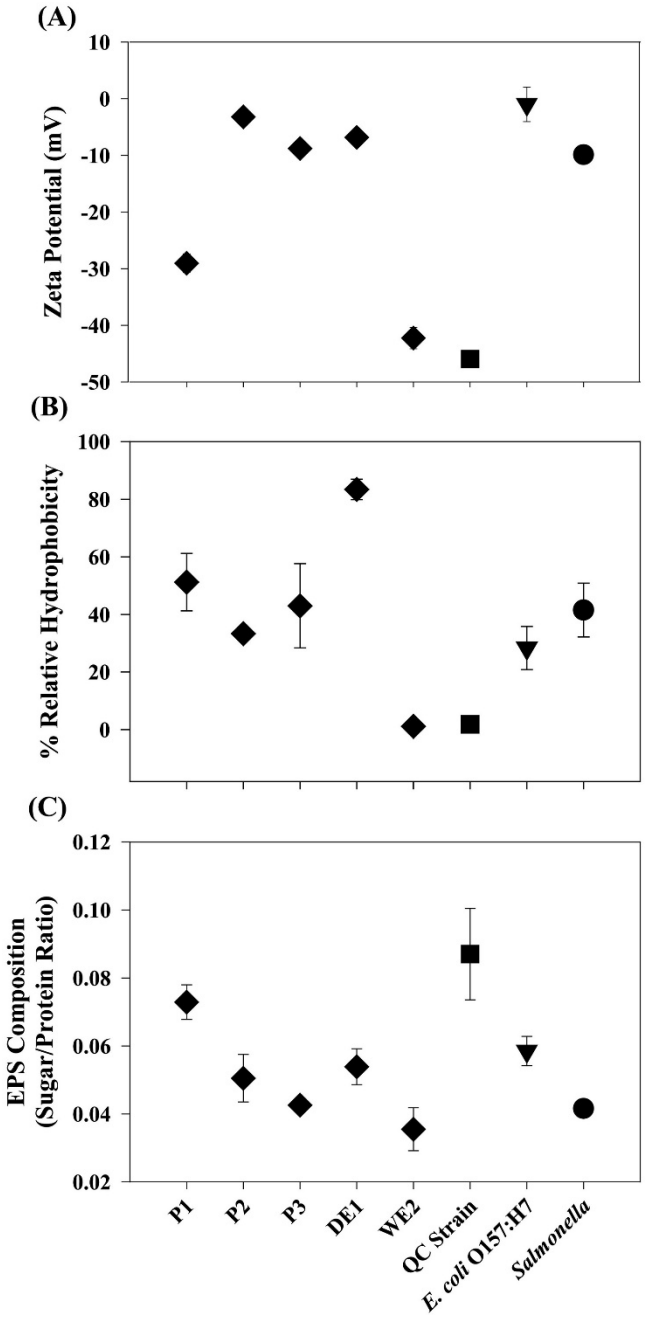

Figure 2.3 Physiochemical cell properties.
Measure of Zeta Potential (A); Relative Hydrophobicity (B); EPS Composition (C) for top five *E. coli* surrogates (◆); *E. coli* quality control (QC Strain; ■); *E. coli* O157:H7 (O157:H7; ▼); and *Salmonella* Typhimurium (*Salmonella*; •). Values represent average and standard deviation of triplicate experiments.

When grown in lettuce lysate media, zeta potential, relative hydrophobicity and EPS composition of the QC strain were all significantly different from those of *E. coli* O157:H7 and *Salmonella* (Figure 2.4; $p < 0.05$). However, there were environmental surrogate representatives with values similar to those of the pathogens for each characteristic (Table S3, Appendix A). For example, zeta potential, relative hydrophobicity, and EPS composition for the poultry isolate P3 were all within 15% of values for *Salmonella*. In general, cell surface characterization showed that the pathogens and environmental *E. coli* isolates that attached at similar rates to lettuce had similar cell surface characteristics (within 15% of each other), including size, zeta potential, relative hydrophobicity and EPS composition (Figure 2.4 and Table S3, Appendix A). These isolates and pathogens all had a negative zeta potential, with three of the five ranging between -1 and -10 mV (Figure 2.4A). One of the five, a surface water isolate (WE2), has significantly lower zeta potential (-42.23 ± 1.89 mV), which is similar to that of the QC strain (-45.93 ± 0.80 mV). This higher magnitude of charge for WE2 and the QC strain causes these cells to be more stable in suspension and potentially capable of further transport when conditions are unfavorable for bacterial attachment (80).

Relative hydrophobicity for four of the five isolates with the greatest adherence to lettuce (52.75 ± 8.96%) was comparable to that of the pathogens (41.54 ± 9.35% and 28.36 ± 7.51% for *Salmonella* and *E. coli*, respectively; Figure 2.4B). Again, the environmental isolate WE2 had values significantly lower ($p < 0.05$) than the others (1.16 ± 0.29%) and similar to the QC strain (1.82 ± 1.04%). These reduced hydrophobic characteristics (nearly hydrophilic) can reduce barriers to attachment on the waxy

hydrophobic epicuticle layer on plant surfaces (44). The EPS composition is defined by its sugar to protein ratio, which ranged from 0.058 ± 0.004 to 0.036 ± 0.006 for four out of five isolates and the pathogens, while the QC strain was highest at 0.087 ± 0.014 (Figure 2.4C). Extracellular substances that make up EPS can influence bacterial attachment and transport mechanisms indirectly by impacting both the cell surface hydrophobicity and surface charge (44). Overall, these results highlight the good comparability of environmental *E. coli* isolates with their pathogen models, and emphasize the need for consideration of growth at environmentally relevant nutrient levels (i.e. those found in lettuce exudates) to accurately characterize bacteria and predict attachment to surfaces.

2.4 Conclusions

Many enteric foodborne pathogens represent a risk to food safety due to their ability to persist in the gastrointestinal tract and also in secondary habitats where they survive until confronted with a suitable host. Similarly, a portion of commensal *E. coli* from the gastrointestinal flora successfully persist in secondary environments. These strains are able to form biofilms and adhere to surfaces quickly and at high levels, increasing the likelihood that they will be maintained and persist in the face of competitive, nutrient-limiting conditions. In these studies we demonstrate the enhanced capability of *Salmonella* and some of the environmental *E. coli* isolates to form biofilms when grown in low nutrient lettuce lysates and to attach to lettuce leaves rapidly and at high rates. The most representative *E. coli* surrogates for *Salmonella* (final surrogate

selections; Figure 2.1) were from surface water (DE1) and poultry litter (P1, P2, P3) sources. Those isolates had genotypic (nutrient scavenging, adhesion and biofilm genes) and phenotypic (biofilm formation, growth rate, lettuce adhesion and cell surface) properties that were similar to those of *Salmonella* and/or were suggestive of enhanced ability to adhere to surfaces and persist for extended periods in the environment. In contrast to *Salmonella*, the *E. coli* O157:H7 produced low levels of biofilm when grown on lettuce lysates and attachment to lettuce leaves was below average. The QC strain and environmental *E. coli* isolates selected as surrogates for *E. coli* O157:H7 were all below average in biofilm formation and/or lettuce leaf attachment. Selected isolates also had similar genotypic and cell surface characteristics as *E. coli* O157:H7. To account for these differences in genotypic and phenotypic properties between both pathogens and surrogates, a diverse suite of environmental *E. coli* were recommended for use as surrogates to represent the two pathogens (Figure 2.1). Future research should verify the behavior of isolates and pathogens under more diverse environmental production (wet/drought conditions, temperature extremes) and processing conditions. However, our results provide strong support for the use of environmental isolates as surrogates for foodborne pathogens.

2.5 References

1. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States-major pathogens. Emerging Infectious Diseases, 17:7-15.
2. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerging Infectious Diseases, 19(3):407-415.
3. Food and Agriculture Organization (FAO) and World Health Organization (WHO). 2008. Microbiological hazards in fresh leafy vegetables and herbs, Microbiological Risk Assessment Series No. 14. Rome, Italy.
4. Crim ST, Griffin PM, Tauxe R, Marder EP, Gilliss D, Cronquist AB, Cartter M, Tobin-D'Angelo M, Blythe D. 2015. Preliminary incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network on 10 U.S. sites, 2006–2014. CDC Morbidity and Mortality Weekly Report, 64(18):495-499.
5. Goodburn C, Wallace CA. 2013. The microbiological efficacy of decontamination methodologies for fresh produce: A review. Food Control, 32:418-427.
6. Olaimat AN, Holley RA. 2012. Factors influencing the microbial safety of fresh produce: A review. Food Microbiology, 32:1-19.

7. Gelting RJ, Baloch MA, Zarate-Bermudez M, Hajmeer MN, Yee JC, Brown T, Yee BJ. 2015. A systems analysis of irrigation water quality in an environmental assessment of an *E. coli* O157: H7 outbreak in the United States linked to iceberg lettuce. Agricultural Water Management, 150:111-118.
8. Jung Y, Jang H, Matthews KR. 2014. Effect of food production chain on outbreak incidence. Microbial Biotechnology, 7(6):517-527.
9. van Boxstael S, Habib I, Jacxsens L, de Vocht M, Baert L, van de Perre E, Rajkovic A, Lopez-Galvez F, Sampers I, Spanoghe P, de Meulenaer B, Uyttendaele M. 2013. Food safety issues in fresh produce: Bacterial pathogens, viruses and pesticide residues indicated as major concerns by stakeholders in the fresh produce chain. Food Control, 32(1):190-197.
10. Cabrera-Diaz E, Moseley TM, Lucia LM, Dickson JS, Castillo A, Acuff GR. 2009. Fluorescent protein-marked *Escherichia coli* biotype i strains as surrogates for enteric pathogens in validation of beef carcass interventions. Journal of Food Protection, 72(2):295-303.
11. Deng K, Wang X, Yen LH, Ding H, Tortorello ML. 2014. Behavior of Shiga Toxigenic *Escherichia coli* relevant to lettuce washing processes and consideration of factors for evaluating washing process surrogates. Journal of Food Protection, 77(11):1860-1867.
12. Kim JK, Harrison MA. 2009. Surrogate selection for *Escherichia coli* O157:H7 based on cryotolerance and attachment to romaine lettuce. Journal of Food Protection, 72(7):1385-1391.

13. Lemarchand K, Lebaron P. 2003. Occurrence of *Salmonella* spp. and Cryptosporidium spp. in a French coastal watershed: relationship with fecal indicators. FEMS Microbiology Letters, 218:203-209.

14. Sinclair RG, Rose JB, Hashsham SA, Gerba CP, Haas CN. 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. Applied Environmental Microbiology, 78(6):1969-1977.

15. Ulbrich CJ, Lucia LM, Arnold AN, Taylor TM, Savell JW, Gehring KB. 2015. Reduction of surrogates for *Escherichia coli* O157:H7 and *Salmonella* during the production of nonintact beef products by chemical antimicrobial interventions. Journal Food Protection, 78(5):881-887.

16. Harwoo VJ, Levine AD, Scott TM, Chivukula V, Lukasik J, Farrah SR, Rose JB. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Applied Environmental Microbiology, 71(6):3163-3170.

17. Jenkins MB, Fisher DS, Endale DM, Adams P. 2011. Comparative die-off of *Escherichia coli* O157:H7 and fecal indicator bacteria in pond water. Environmental Science & Technology, 45(5):1853-1858.

18. Keeling C, Niebuhr SE, Acuff GR, Dickson JS. 2009. Evaluation of *Escherichia coli* biotype I as a surrogate for *Escherichia coli* 0157:H7 for cooking, fermentation, freezing, and refrigerated storage in meat processes. Journal of Food Protection, 72(4):728-732.

19. Field KG, Samadpour M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research, 41(16):3517-3538.

20. Griffith JF, Cao Y, McGee CD, Weisberg SB. 2009. Evaluation of rapid methods and novel indicators for assessing microbiological beach water quality. Water Research, 43:4900-4907.

21. Marshall KM, Niebuhr SE, Acuff GR, Lucia LM, Dickson JS. 2005. Identification of *Escherichia coli* O157:H7 meat processing indicators for fresh meat through comparison of the effects of selected antimicrobial interventions. Journal of Food Protection, 68(12):2580-2586.

22. Cuniff PA. 2002. *Salmonella* in foods: Automated conductance method: Sec. 17.9.20, Method 991.38, Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists International. Gaithersburg, MD.

23. Feng P, Weagant SD, Jinneman K. 2011. Chapter 4A: Diarrheagenic *Escherichia coli*, Bacteriological Analytical Manual. Food and Drug Administration (FDA), Silver Springs, MD, USA.

24. International Organization for Standardization (ISO). 2014. Microbiology of food, animal feed and water -- Preparation, production, storage and performance testing of culture media. ISO 11133:2014, Geneva, Switzerland.

25. Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G. 2006. *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. Journal of Environmental Quality, 35(4):1170-1180.

26. Ishii S, Sadowsky MJ. 2008. *Escherichia coli* in the environment: Implications for water quality and human health. Microbes in the Environment, 23(2):101-108.

27. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. Journal of Food Protection, 67(7):1365-1370.

28. Bolster CH, Cook KL, Marcus IM, Haznedaroglu BZ, Walker SL. 2010. Correlating transport behavior with cell properties for eight porcine *Escherichia coli* isolates. Environmental Science Technology, 44(13):5008-5014.

29. Cook KL, Bolster CH, Ayers KA, Reynolds DN. 2011. *Escherichia coli* diversity in livestock manures and agriculturally impacted stream waters. Current Microbiology, 63(5):439-449.

30. Dixit SM, Gordon DM, Wu XY, Chapman T, Kailasapathy K, Chin JJ. 2004. Diversity analysis of commensal porcine *Escherichia coli* – associations between genotypes and habitat in the porcine gastrointestinal tract. Microbiology, 150:1735-1740.

31. Foppen JW, Lutterodt G, Röling WFM, Uhlenbrook S. 2010. Towards understanding inter-strain attachment variations of *Escherichia coli* during transport in saturated quartz sand. Water Research, 44(4):1202-1212.

32. Méric G, Kemsley EK, Falush D, Saggers EJ, Lucchini S. 2013. Phylogenetic distribution of traits associated with plant colonization in *Escherichia coli*. Environmental Microbiology, 15(2):487-501.

33. Son I, van Kessel JS, Karns JS. 2009. Genotypic diversity of *Escherichia coli* in a dairy farm. Foodborne Pathogens and Disease, 6:837-847.

34. Yang HH, Vinopal RT, Grasso D, Smets BF. 2004. High diversity among environmental *Escherichia coli* isolates from a bovine feedlot. Applied Environmental Microbiology, 70(3):1528-1536.

35. Bolster CH, Haznedaroglu BZ, Walker SL. 2009. Diversity in cell properties and transport behavior among 12 different environmental *Escherichia coli* isolates. Journal of Environmental Quality, 38(2):465-472.

36. Brandl MT. 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annual Reviews in Phytopathology, 367-392.

37. Yaron S, Romling U. 2014. Biofilm formation by enteric pathogens and its role in plant colonization and persistence. Microbial Biotechnology, 7:496-516.

38. Barak JD, Gorsk, L, Naraghi-Arani P, Charkowski AO. 2005. *Salmonella* enterica virulence genes are required for bacterial attachment to plant tissue. Applied Environmental Microbiology, 71(10):5685-5691.

39. Nagy A, Mowery J, Bauchan GR, Wang L, Nichols-Russell L, Nou X. 2015. Role of extracellular structures of *Escherichia coli* O157:H7 in initial attachment to biotic and abiotic surfaces. Applied Environmental Microbiology, 81(14):4720-4727.

40. Tan MSF, White AP, Rahman S, Dykes GA. 2016. Role of fimbriae, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. PLoS ONE, 11(6):e0158311.

41. Wang H, Dong Y, Wang G, Xu X, Zhou G. 2016. Effect of growth media on gene expression levels in *Salmonella* Typhimurium biofilm formed on stainless steel surface. Food Control, 59:546-552.

42. Brandl MT, Amundson R. 2008. Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella* enterica. Applied Environmental Microbiology, 74(8):2298-2306.

43. Kyle JL, Parker CT, Goudeau D, Brandl MT. 2010. Transcriptome analysis of *Escherichia coli* O157:H7 exposed to lysates of lettuce leaves. Applied Environmental Microbiology, 76(5):1375-1387.

44. Patel J, Sharma M. 2010. Differences in attachment of *Salmonella* enterica serovars to cabbage and lettuce leaves. International Journal of Food Microbiology, 139(1-2):41-47.

45. Hall BG, Acar H, Nandipati A, Barlow M. 2014. Growth rates made easy. Molecular Biology and Evolution, 31(1):232-238.

46. Römling U, Bokranz W, Rabsch W, Zogaj X, Nimtz M, Tschäpe H. 2003. Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. International Journal of Medicinal Microbiology, 293(4):273-285.

47. Reisner A, Krogfelt KA, Klein BM, Zechner EL, Molin S. 2006. In vitro biofilm formation of commensal and pathogenic *Escherichia coli* strains: Impact of environmental and genetic factors. Journal of Bacteriology, 188(10):3572-3581.

48. Vigeant MAS, Ford RM, Wagner M, Tamm LK. 2002. Reversible and irreversible adhesion of motile *Escherichia coli* cells analyzed by total internal reflection aqueous fluorescence microscopy. Applied Environmental Microbiology, 68(6):2794-2801.

49. Walker SL, Bhattacharjee S, Hoek EMV, Elimelech M. 2002. A novel asymmetric clamping cell for measuring streaming potential of flat surfaces. Langmuir, 18(6):2193-2198.

50. Chen G, Walker SL. 2007. Role of solution chemistry and ion valence on adhesion kinetics of groundwater and marine bacteria. Langmuir, 23(13):7162-7169.

51. Marcus IM, Bolster CH, Cook KL, Opot SR, Walker S.L. 2012. Impact of growth conditions on transport behavior of *E. coli*. Journal of Environmental Monitoring, 14(3):984-991.

52. Frömmel U, Lehmann W, Rodiger S, Bohm A, Nitschke J, Weinreich J, Grob J, Roggenbuck D, Zinke O, Ansorge H, Vogel S, Klemm P, Wex T, Schröder C, Wieler LH, Schierack P. 2013. Adhesion of human and animal *Escherichia coli* strains in association with their virulence-associated genes and phylogenetic origins. Applied Environmental Microbiology, 79(19):5814-5829.

53. Gomi R, Matsuda T, Matsui Y, Yoneda M. 2014. Fecal source tracking in water by next-generation sequencing technologies using host-specific *Escherichia coli* genetic markers. Environmental Science & Technology, 48(16):9616-9623.

54. Kaufmann M, Zweifel C, Blanco M, Blanco JE, Beutin L, Stephan R. 2006. *Escherichia coli* O157 and non-O157 shiga toxin–producing *Escherichia coli* in

fecal samples of finished pigs at slaughter in Switzerland. Journal of Food Protection, 69(2):260-266.

55. Shaw RK, Berger CN, Feys B, Knutton S, Pallen MJ. Frankel G. 2008. Enterohemorrhagic *Escherichia coli* exploits EspA filaments for attachment to salad leaves. Applied Environmental Microbiology, 74(9):2908-2914.

56. Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Applied Environmental Microbiology, 70(12):7046-7052.

57. Pavlovic M, Luze A, Konrad R, Berger A, Sing A, Busch U, Huber I. 2011. Development of a duplex real-time PCR for differentiation between *E. coli* and Shigella spp. Journal of Applied Microbiology, 110(5):1245-1251.

58. Cook KL, Netthisinghe AMP, Gilfillen RA. 2014. Detection of pathogens, indicators, and antibiotic resistance genes after land application of poultry litter. Journal of Environmental Quality, 43(5):1546-1558.

59. Girardin H, Morris CE, Albagnac C, Dreux N, Glaux C, Nguyen-The C. 2005. Behavior of the pathogen surrogates Listeria innocua and Clostridium sporogenes during production of parsley in fields fertilized with contaminated amendments. FEMS Microbial Ecology, 54(2):287-295.

60. Wood JD, Bezanson GS, Gordon RJ, Jamieson R. 2010. Population dynamics of *Escherichia coli* inoculated by irrigation into the phyllosphere of spinach grown under commercial production conditions. International Journal of Food Microbiology, 143(3):198-204.

61. Webb CC, Erickson MC, Davey LE, Payton AS, Doyle MP. 2014. Construction and characterization of outbreak *Escherichia coli* O157:H7 surrogate strains for use in field studies. Foodborne Pathogens and Disease, 11(11):893-899.

62. Aruscavage D, Miller SA, Lewis Ivey ML, Lee KEN, LeJeune JT. 2008. Survival and dissemination of *Escherichia coli* O157:H7 on physically and biologically damaged lettuce plants. Journal of Food Protection, 71(12): 2384-2388.

63. Brandl MT. 2008. Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on postharvest lettuce. Applied Environmental Microbiology, 74(17):5285-5289.

64. Goudeau DM, Parker CT, Zhou Y, Sela S, Kroupitski Y, Brandl MT. 2013. The *Salmonella* transcriptome in lettuce and cilantro soft rot reveals a niche overlap with animal host intestine. Applied Environmental Microbiology, 79(1):250-262.

65. Han S, Micallef SA. 2016. Environmental metabolomics of the tomato plant surface provides insights on *Salmonella* enterica colonization. Applied Environmental Microbiology, 82(10):3131-3142.

66. Wells JM, Butterfield JE. 1997. *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. Plant Diseases, 81(8):867-872.

67. Wells JM, Butterfield JE. 1999. Incidence of *Salmonella* on fresh fruits and vegetables affected by fungal rots or physical injury. Plant Diseases, 83(8):722-726.

68. Dewanti R, Wong ACL. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. International Journal of Food Microbiology, 26(2):147-164.

69. Hood SK, Zottola EA. 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. International Journal of Food Microbiology, 37(2–3):145-153.

70. Tan MSF, White AP, Rahman S, Dykes GA. 2016. Role of fimbriae, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. PLoS ONE, 11(6):e0158311

71. Römling U, Rohde M, Olsén A, Normark S, Reinköster J. 2000. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* typhimurium regulates at least two independent pathways. Molecular Microbiology, 36(1):10-23.

72. Solomon EB, Niemira BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli Biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. Journal of Food Protection, 68(5):906-912.

73. Boyer RR, Sumner SS, Williams RC, Kniel KE, McKinney JM. 2011. Role of O-antigen on the *Escherichia coli* O157:H7 cells hydrophobicity, charge and ability to attach to lettuce. International Journal of Food Microbiology, 147(3):228-232.

74. Van Der Linden I, Cottyn B, Uyttendaele M, Vlaemynck G, Heyndrickx M, Maes M. 2013. Survival of enteric pathogens during butterhead lettuce growth: Crop stage, leaf age, and irrigation. Foodborne Pathogens and Disease, 10(6):485-491.

75. Ratajczak M, Laroche E, Berthe T, Clermont O, Pawlak B, Denamur E, Petit F. 2010. Influence of hydrological conditions on the *Escherichia coli* population structure in the water of a creek on a rural watershed. BMC Microbiology, 10:222-222.

76. Kopit LM, Kim EB, Siezen RJ, Harris LJ, Marco ML. 2014. Safety of the surrogate microorganism Enterococcus faecium NRRL B-2354 for use in thermal process validation. Applied Environmental Microbiology, 80(6):1899-1909.

77. Danese PN, Pratt LA, Dove SL, Kolter R. 2000. The outer membrane protein, antigen 43, mediates cell to cell interactions within *Escherichia coli* biofilms. Molecular Microbiology, 37:424-432.

78. Yue M, Han X, Masi LD, Zhu C, Ma X, Zhang J, Wu R, Schmieder R, Kaushik RS, Fraser GP, Zhao S, McDermott PF, Weill FX, Mainil JG, Arze C, Fricke WF, Edwards RA, Brisson D, Zhang NR, Rankin SC, Schifferli DM. 2015. Allelic variation contributes to bacterial host specificity. Nature Communications, 6.

79. Walker SL. 2005. The role of nutrient presence on the adhesion kinetics of Burkholderia cepacia G4g and ENV435g. Colloids and Surfaces B: Biointerfaces, 45(3–4):181-188.

80. Elimelech MG, Jia X, Williams RA. 1995. Particle Deposition and Aggregation - Measurement, Modelling and Simulation. Butterworth-Heinemann, Stoneham, MA.

Chapter 3

Escherichia coli O157:H7 and *Salmonella* Typhimurium Adhesion to Spinach Leaf Surfaces: Role of Water Chemistry and Nutrient Availability

Mayton, H.M., Marcus, I., Walker, S.L. *Escherichia coli* O157:H7 and *Salmonella* Typhimurium adhesion to spinach leaf surfaces: Sensitivity to water chemistry and nutrient availability. *Food Microbiology*, Vol. 78, 134-142 (2019).

Abstract

This study investigated the effects of solution chemistry and growth conditions on bacterial deposition on spinach leaf surfaces using a parallel plate flow cell. Two food safety pathogens of concern and two non-pathogen bacterial surrogates (environmental *E. coli* isolates) were grown in ideal (LB media) and nutrient restricted (M9 media) conditions. Bacterial attachment was quantified as mass transfer rate coefficients for cells suspended in 10 mM KCl, $CaCl_2$ and artificial groundwater, and cell and leaf surfaces were extensively characterized (zeta potential, hydrophobicity, extracellular polymer (EPS) composition). Between the pathogens, *E. coli* O157:H7 attachment was greater than that of *Salmonella* Typhimurium, attributed to measurable variability in cell surface charge and hydrophobicity. When grown in M9 media, both pathogens were significantly more adhesive to spinach surfaces ($p < 0.01$) than when grown in LB media. Surrogates did not follow this trend and showed minimal changes in adhesion kinetics and surface properties between growth conditions. EPS sugar/protein ratios were reduced in some of the highest attachment scenarios, suggesting that changes in EPS composition in favor of proteins may play a role. These results show the importance of growth conditions and solution complexities in understanding mechanisms of aqueous bacterial adhesion to food surfaces.

3.1 Introduction

The World Health Organization estimates that 600 million people fall ill after consuming unsafe food ever year, leading to over 400,000 deaths worldwide (1). While consumption of fruits and vegetables is essential to improving diets and nutrition, prevalence of foodborne illness linked to microbial contamination of fresh produce remains high in both the United States and Europe (2). Contamination has been shown to come from water sources before, during, and after harvest (3), and can be magnified by increasing pressures on water resources and occurrences of extreme weather (4, 5). The potential for cross-contamination during value-added processes like cooling, washing, and packaging have been shown to further magnify the risks of foodborne illness outbreaks associated with several types of fresh produce (6, 7). Leafy greens are considered especially vulnerable to microbial risks due to the common usage of sprinkler irrigation, "triple wash" and other pre-packaging methods, and high likelihood of consumption without a heating or cooking step to kill pathogens (8). In fact, over 600 foodborne illness outbreaks and 20,000 unique illnesses in the U.S. were attributed to leafy greens from 1973 to 2012 (9). Even with industrializing agricultural systems that regulate and limit direct human handling, there is a significant need for scientific research to improve our understanding of the interactions between microbes and produce. This is due to washing and handling processes that create complex and dynamic environments in which bacteria can attach and proliferate on produce surfaces.

The complexities of water chemistry in agricultural systems can vary significantly based on the climate, water source, and crop, while the approach to food safety largely remains the same. For example, nearly all farms in Salinas, California use unregulated groundwater for irrigation, while those in Yuma, Arizona use highly saline Colorado

River water (10, 11). Together, these locations represent 90% of U.S. leafy greens production and operate under one set of food safety standards (12). Previous work has demonstrated that aqueous bacterial deposition on surfaces is sensitive to a wide range of parameters, including ionic strength, pH, and valence (13, 14). However, these trends have not been tested systematically in the context of potential microbial cross contamination and food safety of leafy greens. Similarly, the critical influence of fluid dynamics on cell-surface interactions has been demonstrated in a number of studies and environments, but most research on microbial adhesion and detachment from produce surfaces has been conducted under static conditions that generally involve dipping and rinsing food substrates in inoculated solutions (6, 15, 16), rather than in situ observation methods. This work utilizes a microfluidic flow cell to assess bacterial deposition and rinsing on model spinach leaf surfaces in real time, under well-defined hydrodynamic flow conditions in simple and complex solution chemistries.

Two foodborne pathogens, *Escherichia coli* serotype O157:H7 and *Salmonella enterica* serotype Typhimurium *(Salmonella),* have been implicated in significant foodborne illness outbreaks in recent years (17, 18). Together, they account for nearly one-third of all leafy-vegetable associated outbreaks over the past 30 years (19). Industry, regulatory groups, and researchers often use nonpathogenic bacteria, like other strains of *E. coli,* as surrogates of human pathogens to study risk and improve management techniques for food safety (20). However, the validity and usefulness of generic *E. coli* strains in mimicking pathogen fate and transport in diverse, realistic environments outside of the laboratory has been questioned (21, 22). Additionally, few studies have

considered changes in pathogen and surrogate attachment after growth in nutrient-restricted conditions, which can have a significant influence on cell surface properties (23) and ultimately biofilm formation (24). This study investigates the adhesion behavior of two environmental *E. coli* strains that were previously identified as promising nonpathogenic surrogates (25), as well as that of reference strains the two known pathogens, all grown in nutrient-rich and nutrient-restricted conditions. Characterization of the deposition trends and cell surfaces for *E. coli* O157:H7 and *Salmonella* was conducted in a suite of water quality scenarios, ranging from simple to complex solution chemistries. Following pathogen experiments, adhesion of the two potential surrogates was quantified in one representative complex solution chemistry in order to provide a comparison in the most realistic conditions.

3.2 Materials & Methods

3.2.1 Bacterial growth and preparation.

Escherichia coli O157:H7 (ATCC 43888), *Salmonella* Typhimurium (ATCC 13311), and two non-pathogen environmental *E. coli*, referred to as *E. coli* B01 and B05, were acquired from the USDA (Kimberly Cook, USDA-ARS-FAESR, Bowling Green, KY). *E. coli* O157:H7 and *Salmonella* were chosen as model pathogens for this work because they represent water- and foodborne bacteria that are both rod-shaped and Gram-negative, while they have been shown to have notably different characteristics in hydrophobicity, zeta potential, and attachment (26). *E. coli* B01 and *E. coli* B05 were

chosen based on previous work that identified their potential ability to sufficiently represent these two pathogens in complex systems (25).

In brief, all bacteria were cultured in Luria-Bertani (LB) media at 37°C overnight, before inoculation in either LB or minimal salts (M9) media for 3.5 hours or 6 hours, respectively, at 37 °C and harvested at mid-exponential cell growth phase (see growth curves in Appendix B). LB medium (Fisher Scientific, Fair Lawn, NJ) consists of 0.5% (5 mg/mL) yeast extract, 1% (10 mg/mL) tryptone, and 0.5% (10 mg/mL) NaCl prepared in deionized water. M9 medium was created using 6 mg/mL Na_2HPO_4, 3 mg/mL, KH_2PO_4, 0.5 mg/mL NaCl, and 1 mg/mL NH_4Cl at pH 7.2, supplemented with 1% glucose, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ in deionized water (27). Bacterial cell suspensions were adjusted to a concentration of 5.0×10^7 cells/mL using a counting chamber (Bürker-Türk chamber, Marienfield Laboratory Glassware, Lauda-Konigshofen, Germany) for deposition experiments or OD_{560}=0.2 (approximately 10^8 cells/mL) for bacterial cell characterization.

3.2.2 Quantifying bacterial attachment.

A parallel plate (PP) flow chamber (GlycoTech, Rockville, MA) on an inverted fluorescent microscope (BX-52, Olympus) was used to conduct bacterial adhesion and rinsing experiments. The microfluidic chamber dimensions are 6 cm × 1 cm × 0.0762 cm and is made up of a Plexiglas® block mounted by a flexible silicone elastomer gasket and a polycarbonate microscope slide (supporting isolated spinach epicuticle layer) sealed by vacuum grease. Influent bacteria suspended in one of the solutions (10 mM KCl $CaCl_2$, or

artificial groundwater (AGW)) enters the chamber from a capillary tube connected to a syringe on a syringe pump set to an average flow rate of 0.1 mL/min, which corresponds with a flow velocity of 0.8 m/h and a Péclet number of 6.47×10^{-4} (28). These non-turbulent flow conditions induce a shear rate of 1.7 s^{-1} on the spinach surface and simulating expected conditions in a gentle produce washing process (29). Bacteria are imaged on the surface under 400x total magnification by using a 40× long working distance objective (UPlanFI, Olympus).

After rinsing the epicuticle surface with DI water within the parallel plate flow cell, the kinetics of bacterial attachment was observed over a 30-min. period at ambient temperatures (20-25°C), followed by a 30-min. rinse with an uninoculated (cell-free) solution of the same background solution. Images were recorded with a digital camera (Demo Retiga EXI Monochrome, QImaging) every 30 sec. in order to determine the kinetics of cell attachment to the surface by comparison of successive images. Flow cell experiments were conducted in 10 mM KCl, 10 mM $CaCl_2$, and 10 mM artificial groundwater (AGW) to investigate the impact of solution chemistry on deposition on the leaf surface. AGW was composed of 0.014 g KNO_3, 0.321 g $MgSO_4{\cdot}7H_2O$, 0.112 g $CaSO_4{\cdot}2H_2O$, 0.044 g NaCl, and 0.109 g $NaHCO_3$ per 1 L of deionized water (30). This ionic strength is relatively high compared to that of most surface water, but was chosen as a baseline within the realm of possibility for surface and groundwater to maximize observable attachment, as shown by previously reported trends in microbial adhesion to the epicuticle and other solid surfaces (35).

Images from flow cell experiments were analyzed using the supplied digital camera software, SimplePCI, and Mathworks MATLAB (R2015a) in order to record and enumerate the kinetics of cell attachment. The rate of attachment during the final ten minutes is used to calculate the mass transfer rate coefficients in order to minimize variability in attachment and to maximize the number of cells that can be observed. The number of bacterial cells deposited can be plotted as a function of time, and bacterial flux, J (cells per s × m^2), can be calculated according to:

$$J = m/a$$

where *m* is the linear slope of adhered cells versus time and *A* is microscope viewing area (153 μm × 113 μm). The corresponding bacterial transfer rate coefficient for the bacteria, *k* (m × s^{-1}), can then be calculated:

$$k = J/C$$

where C is the bulk cell concentration (cells per mL).

3.2.3 Surface preparation and characterization.

Baby spinach leaves used in this study were pre-washed and bagged from the same brand and same grocery store, and were used for creating isolated epicuticle layers on the same day as purchase. Otherwise, leaves were stored for a maximum of three days at 4°C. Fully green, healthy-looking, non-damaged leaves were selected for experiments (no visible yellowing, browning, rips, or tears) and were handled aseptically throughout their preparation. A freeze imbedding technique was used to isolate epicuticle layers from the spinach leaf surface, which was developed by Ensikat et al. (2000) (31). The

transparent wax epicuticle layer was immobilized onto a polycarbonate microscope slide in order to view bacterial deposition and release from below using the inverted microscope. The method, in brief, involves placing a dime-sized drop of Triethylene glycol (TEG) on a flat, rigid stainless steel surface. A pre-cut spinach leaf is then placed on top of the drop, with the top side of the leaf in contact with the TEG. The TEG and surfaces are then submerged in liquid nitrogen for approximately 10 sec. Immediately after removal from liquid nitrogen, tweezers are used to remove the leaf sample, leaving the epicuticle layer imbedded in the frozen TEG droplet, and the droplet is allowed to thaw directly on the polycarbonate slide. This allows the epicuticle wax layer to transfer directly to the slide and remnant TEG can be rinsed away with DI water. Immobilized wax layers were stored at 4°C following isolation for up to one week.

The whole spinach leaves were characterized using a streaming potential analyzer (SurPASS, Anton Paar, Graz, Austria) and goniometer to assess surface zeta potential and surface contact angle (SCA), respectively. Whole leaves and isolated epicuticle layers were also visualized using light microscopy and environmental scanning electron microscopy (ESEM) (FEI Quanta 200). Spinach leaves were imaged in the ESEM in low-vacuum mode at 20 kV, without pre-treatment of samples.

3.2.4 Bacterial cell characterization.

Bacteria were characterized following protocols previously developed for relative hydrophobicity, electrophoretic mobility (a surrogate for surface charge), size, surface charge density, and EPS composition. Relative hydrophobicity (%) was measured using a

UV-Vis spectrophotometer (BioSpec-mini, Shimadzu Corp., Kyoto, Japan) and the microbial adhesion to hydrocarbons (MATH) test (32). A ZetaPALS analyzer (Brookhaven Instruments, Holtsville, NY, U.S.A.) was used to measure electrophoretic mobility, which is converted to zeta potential (mV). Extracellular polymeric substances (EPS) on the cell surface were extracted through water bath sonication (at 3.5 Hz for 20 sec. total) and characterized for composition of proteins and polysaccharides using the Lowry and phenol sulfuric acid methods, respectively, as previously described (33).

3.2.5 Statistical analysis.

At least three independent repetitions were performed for characterization and flow cell experiments, including a fresh cell culture for each trial. To test for differences between water chemistry, growth conditions, and bacteria strains in all experiments listed above, a statistical single-factor ANOVA test was conducted for confidence intervals of 95% and 99% ($p < 0.05$ and $p < 0.01$, respectively). For pairs of water chemistry conditions, a t-test was conducted to determine statistically significant differences for confidence intervals of 95% and 99% ($p < 0.05$ and $p < 0.01$, respectively).

3.3 Results & Discussion

3.3.1 Adhesion kinetics of *E. coli* O157:H7 and *Salmonella* Typhimurium.

Systematic flow cell experiments were conducted to evaluate kinetics of cell attachment to spinach surfaces under a range of relevant water chemistries for the two pathogens, including 10 mM monovalent (KCl), divalent ($CaCl_2$), and multivalent

(artificial groundwater (AGW)) salt solutions. The mass transfer rate coefficient (k, m/s) for bacteria was calculated using observed bacterial flux (number of cells per area per time) and bulk cell concentration (number of cells per mL), from which adhesion trends can be evaluated. Deposition behavior of *E. coli* O157:H7 and *Salmonella* Typhimurium on spinach epicuticle wax as a function of nutritional conditions during growth and water chemistry is displayed in Figure 3.1. After growth in nutrient rich conditions (LB), the average mass transfer rate coefficient, k, for cells in the divalent salt solution ($CaCl_2$) ($1.71 \pm 0.64 \times 10^{-7}$ m/s) was more than double that of the monovalent salt (KCl) ($8.14 \pm 1.28 \times 10^{-8}$ m/s) for *E. coli* O157:H7, which was similar to k in AGW ($8.25 \pm 0.70 \times 10^{-8}$ m/s). *Salmonella* Typhimurium followed the same trend when grown in LB, with k values of 6.57 ± 0.34, 7.32 ± 3.39, and $11.7 \pm 2.24 \times 10^{-8}$ m/s in KCl, AGW, and $CaCl_2$, respectively. The differences in average k between mono-, di-, and multi-valent solution chemistries were not highly significantly when grown in LB, with the exception of *Salmonella* in KCl and $CaCl_2$ ($p < 0.01$).

Experiments with pathogens grown in minimal media (M9) indicate notably different deposition behavior for the cells. *Salmonella* (Figure 3.1b) grown in M9 media was significantly more adhesive in $CaCl_2$ ($3.19 \pm 0.30 \times 10^{-7}$ m/s) and AGW ($1.87 \pm 0.13 \times 10^{-7}$ m/s) than cells grown in LB media. *E. coli* O157:H7 was also significantly more adhesive to spinach surfaces in every solution chemistry after growth in minimal media and deviated from the trends expected based on classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of particle stability. DLVO theory can be used to characterize

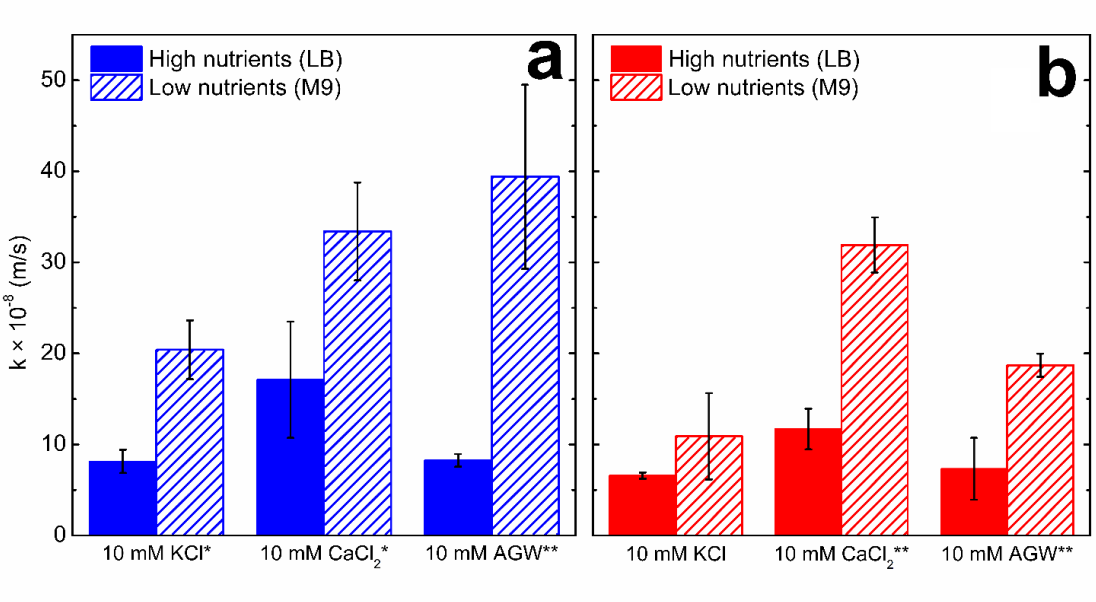

Figure 3.1 Pathogen attachment mass transfer rate coefficients.

Attachment of *E. coli* O157:H7 (a) and *Salmonella* Typhimurium (b) quantified by mass transfer coefficients in monovalent salt solution (10 mM KCl), divalent salt solution (10 mM $CaCl_2$), and a mixture of salts (10 mM AGW) over a 30 min. duration. All experiments were performed in triplicate and error bars indicate one standard deviation. Significant differences between the two growth condition are denoted by *($p < 0.05$) and **($p < 0.01$)

the forces between charged microbial cells and other surfaces interacting in aqueous environments and can therefore provide insight into the favorability of attachment under different conditions (34, 35). For example, the presence of divalent cations in $CaCl_2$ result in charge screening and compression of the electrical double layer between bacteria and the leaf surface, which has been demonstrated to result in more favorable conditions for attachment (36). A more detailed explanation of DLVO theory is available in Appendix B. DLVO calculations revealed that bacteria cells and the spinach leaf surface were only modestly repulsive in all conditions (data not shown). In the absence of

significant electrostatic and van der Waals interactions, hydrodynamic forces within the flow cell are expected to result in cell deposition that generally increases with solution valence (KCl, AGW, and $CaCl_2$, respectively). The attachment mass transfer rate coefficient for undernourished *E. coli* O157:H7 instead reached a maximum of 3.94 ± 1.01 $\times 10^{-7}$ m/s in the mixed valence solution chemistry (AGW).

When grown in LB, no significant differences were observed between attachment rate coefficients in the three different solution chemistries for either bacteria. In contrast, solution chemistry made a significant difference in attachment ($p < 0.05$) after cells were grown in M9 minimal media, with the exception of comparing *E. coli* in $CaCl_2$ and AGW. It is hypothesized that extracellular polymers and other structures on the bacteria surface play a role in magnifying the impacts of undernourishment on bacterial adhesion to the spinach surface in the different solution chemistries. The role of surface macromolecules in pathogen adhesion after growth in minimal media is further explored in the discussion of cell characterization results below.

In comparison to the baseline experiments in KCl and $CaCl_2$, the complex water chemistry of AGW provides more representative conditions in which mechanisms of bacterial attachment in agricultural environments can be considered. Marshall et al. (1971) demonstrated the presence of two distinct stages of aqueous bacterial attachment to surfaces: an instantaneous reversible phase and a time-dependent irreversible phase (37). Therefore, deposition was observed over 30-min. experiments, which would imply that adhesion is occurring within these two early stages of attachment and pre-biofilm development (38, 39). In Figure 3.2a, the average number of *E. coli* O157:H7 and

Salmonella cells adhered to the spinach surface during experiments in AGW is shown as a function of time, separated by M9 and LB growth conditions. For the first 5-10 min., total number of cells remains only slightly above zero due to cells reversibly attaching and then detaching from the surface. Both pathogens grown in each of the nutrient conditions show similar rates of deposition up to this point. The increasing rate of cell attachment is noticeable after the first 10 min. under all conditions. The rate of deposition during the final ten minutes (Figure 3.2b) is where the nuances of cell type and growth condition are most distinct, therefore it is here that the mass transfer rate coefficients are calculated. Cells have likely reached an irreversible attachment phase at this point, as negligible detachment was observed over a 30-min. rinse using the same uninoculated solution following each experiment (data not shown).

When grown in LB media, *E. coli* O157:H7 and *Salmonella* adhere at similar rates in AGW, resulting in mass transfer rate coefficients of $8.25 \pm 0.70 \times 10^{-8}$ and $7.32 \pm 0.34 \times 10^{-8}$ m/s, respectively. This is in contrast with previous work that has shown that these two gram-negative pathogens adhere differently to leafy greens when suspended in simple deionized water and rinsed over the produce surface (40, 41). When grown in M9 minimal media, *Salmonella* cells attached to the spinach epicuticle surface at a significantly higher rate ($1.87 \pm 0.13 \times 10^{-7}$) than in LB media ($p < 0.01$). As noted above, the maximum mass transfer rate coefficient for *E. coli* O157:H7 is observed in AGW after nutrient-restricted growth, which is also significantly higher than that of *Salmonella.* These results are in agreement with previous studies that have shown that bacteria cells

under stress conditions are more likely to adhere to surfaces in order to leave planktonic conditions and begin the process of protective biofilm formation (23, 42).

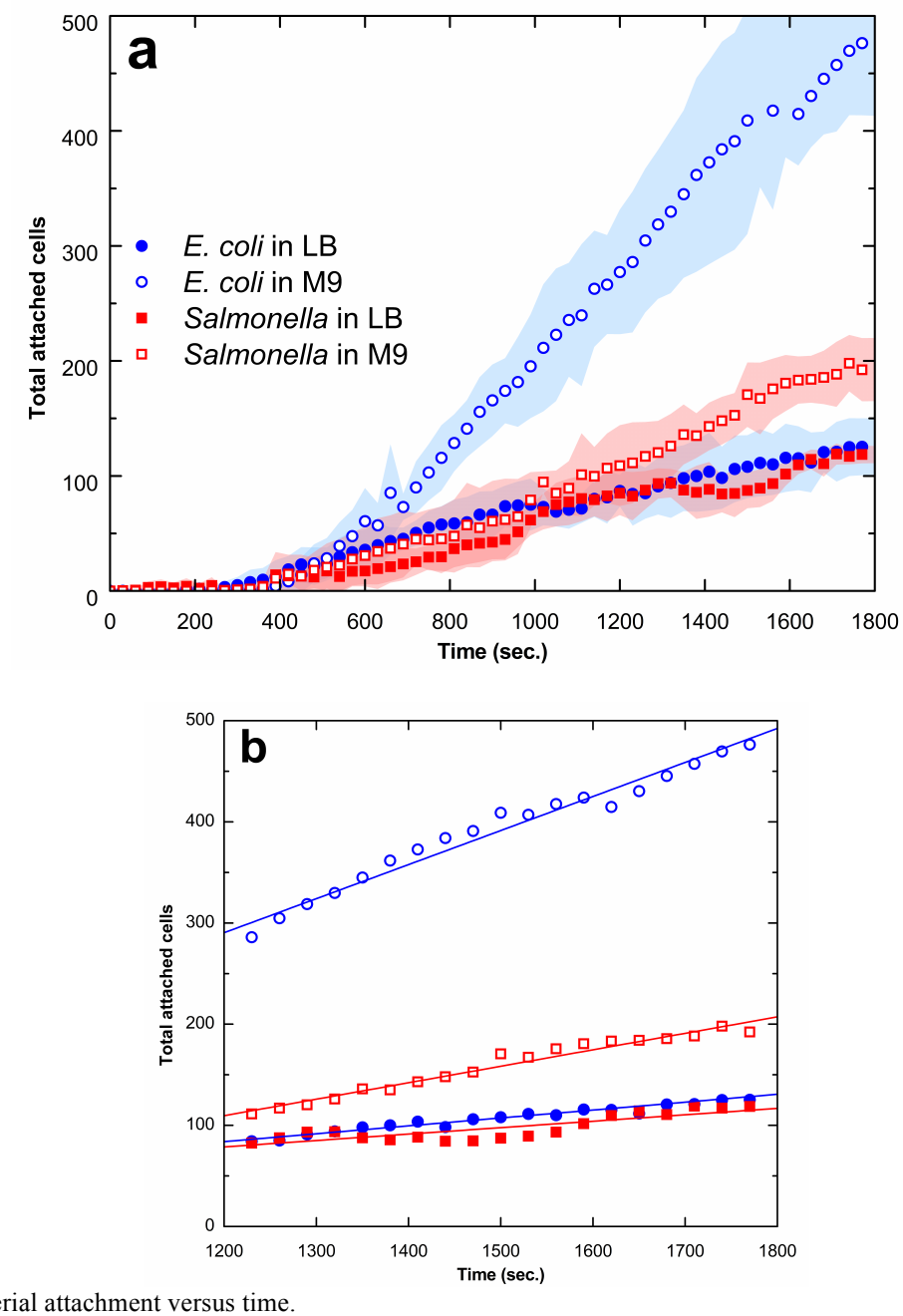

Figure 3.2 Bacterial attachment versus time.

Total *E. coli* O157:H7 and *Salmonella* Typhimurium cells adhered to epicuticle surface in 10 mM AGW over 30 min. experiment (a) and final 10 min. (b). Data points are an average of triplicate trials and standard deviation is represented by the shaded regions surrounding each curve in (a). Adhesion was observed within a 153 µm × 113 µm microscope viewing area.

3.3.2 Epicuticle and cell surface characterization.

To further investigate the physiochemical properties of bacteria and the spinach epicuticle layer that contribute to adhesion, both whole leaves and the immobilized wax layer were characterized by streaming potential, static contact angle, and overall roughness. In addition to these surface properties, Scanning Electron Microscopy (SEM) was utilized to confirm the usefulness of the isolated epicuticle wax as representative of the whole leaf surface for relative comparison of bacterial deposition trends. The leaf surface has a slightly negative surface charge in all three solutions (-12.00 ± 0.43 mV in KCl, -6.81 ± 1.33 mV in $CaCl_2$, and -4.64 ± 1.44 mV in AGW). Static contact angle revealed that the immobilized wax layer was more hydrophobic than the whole leaf, but exhibited less measurement variability, making it more useful for consistent deposition experiments (79.1 ± 2.1° and 69.3 ± 8.5°, respectively). Previous work has also shown that the immobilized epicuticle wax preserves physical morphologies of the leaf surface and minimizes surface roughness (43). This was further confirmed by the observation of preserved leaf surface structures, such as the stomata, within the immobilized epicuticle wax (Figure 3.3).

Bacterial surface properties play a significant role in mechanisms of cell adhesion to solid surfaces. Cells are able to modify the composition and features of their cell wall in response to specific surrounding conditions (44) and surface characteristics have been shown to change in as little as 30 min. of exposure in suspension (13, 43). Electrophoretic mobility, relative hydrophobicity, size, and extracellular polymeric substance (EPS)

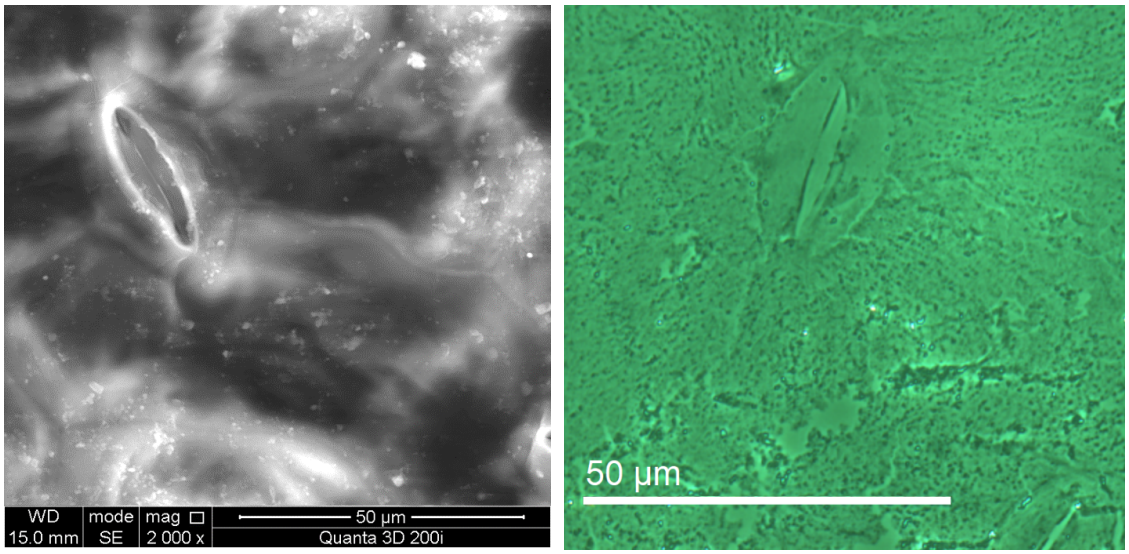

Figure 3.3 Spinach leaf and epicuticle images.

Images of spinach leaf surface (left, SEM image) and isolated epicuticle wax layer on polycarbonate (right, light microscopy image) showing preservation of physical stomata surface structures after physical removal.

composition, were measured at room temperature and unadjusted pH in order to assess the impact of nutrient availability and solution valence on cell surface properties. *E. coli* O157:H7 (ATCC 43888) exhibited a slightly negative charge in all conditions, ranging from -0.63 to -4.53 mV (Table 3.1). *E. coli* cells were also generally hydrophilic, ranging from 12.1% to 41.0% adhesion to dodecane within both growth conditions. *Salmonella* Typhimurium (ATCC 13311) cells had a wider range of surface charge that ranged from 3.75 to -20.65 mV, and were more hydrophobic (40.4% to 83.7% relative hydrophobicity). Zeta potential of cell surfaces did not significantly differ between growth conditions ($p > 0.01$), which is in agreement with previous work that observed differences in transport of *E. coli* in a quartz sand column in high- and low-nutrient scenarios without corresponding changes in cell surface charge (45). However, undernourished *Salmonella* cells are more hydrophilic compared to other conditions,

decreasing to a range of 13.0 % to 48.3% relative hydrophobicity, with the largest reduction (-50% relative hydrophobicity) observed in AGW. This is similar to previous work with various bacteria that have observed lower cell surface hydrophobicity after suppressed growth rates (46) and starvation conditions (47). Alternatively, *E. coli* O157:H7 cells were more hydrophobic after growth in nutrient-restricted conditions and suspended in KCl and $CaCl_2$, but no significant difference was observed when suspended in AGW.

Table 3.1 Bacteria cell surface characteristics and mass transfer rate coefficients (*k*).

Solution [a]	**Growth Media**	**Cell Radius (µm)** [b]	**Zeta Potential (mV)** [c]	**Relative Hydrophobicity (%)** [d]	**$k \times 10^{-8}$ (m/s)**
		E. coli O157:H7			
KCl	LB	0.60 ± 0.006	-4.16 ± 1.38	12.1 ± 4.0	8.14 ± 1.28
	M9	0.54 ± 0.016	-4.53 ± 1.09	25.1 ± 6.4	20.4 ± 3.23
$CaCl_2$	LB	0.58 ± 0.012	-2.22 ± 2.06	30.2 ± 12.0	17.1 ± 6.39
	M9	0.52 ± 0.010	-0.63 ± 2.83	41.0 ± 3.2	33.4 ± 5.38
AGW[e]	LB	0.58 ± 0.011	-3.65 ± 2.12	22.7 ± 2.9	8.25 ± 0.70
	M9	0.54 ± 0.021	-2.47 ± 1.62	19.0 ± 6.6	39.4 ± 10.1
		Salmonella Typhimurium			
KCl	LB	0.69 ± 0.015	-20.6 ± 2.57	40.4 ± 9.8	6.57 ± 0.34
	M9	0.49 ± 0.010	-14.8 ± 2.08	13.0 ± 11.2	10.9 ± 4.74
$CaCl_2$	LB	0.56 ± 0.003	3.75 ± 1.07	72.4 ± 6.7	11.7 ± 2.24
	M9	0.53 ± 0.019	4.61 ± 1.38	48.3 ± 30.8	31.9 ± 3.04
AGW	LB	0.58 ± 0.093	-4.32 ± 1.7	83.7 ± 6.6	7.32 ± 3.39
	M9	0.52 ± 0.003	-3.99 ± 1.28	30.8 ± 3.5	18.7 ± 1.27

[a] Ionic strength of all electrolyte solutions was 10 mM.
[b] Spherical radius calculated from experimentally measured length and width of individual cells
[c] Zeta potential calculated from electrophoretic mobility using ZetaPALS analyzer
[d] Relative hydrophobicity as indicated by microbial adhesion to hydrocarbon (MATH) test
[e] Artificial groundwater

Although both the epicuticle surface and bacteria exhibited a range of negative zeta potentials (Table 3.1), they are relatively small in magnitude and result in DLVO calculations that predict insignificant differences in interactions between cells and epicuticle surfaces for all three combinations of solution valence. Despite similarly repulsive conditions between growth conditions, as suggested by like charges, significant differences in attachment do occur when cells are grown in M9 media, with the most significant differences in AGW. These results indicate that relatively weak electrostatic and van der Waals forces, as indicated by DLVO simulations, are not the dominant mechanism involved in cell interactions with the spinach surface, although they have been shown to be useful in predicting bacterial adhesion trends (46).

3.3.3 Influence of extracellular substances.

Bacterial attachment rate coefficients in the three water chemistries are only significantly different after growth in M9 media, which suggests that environmentally relevant nutrient conditions may magnify changes in cellular surface properties that impact adhesion to produce surfaces. The extracellular matrix has long been reported as a potential means of promoting microbial attachment, but the role of total EPS in attachment to solid surfaces remains uncertain. For example, Oh et al. (2007) found that when *E. coli* O157:H7 was grown in M9 minimal media, cells accumulated more EPS and were more adhesive to glass surfaces than when grown in ideal nutrient conditions (48). In contrast, Ryu et al. (2004) showed that a high EPS-producing mutant of *E. coli* O157:H7 adhered less to a stainless steel surface than the normal strain (49). As shown in

Table 3.2, this study found that the influence of growth in M9 media on total EPS varies by water chemistry and between the strains of these two pathogens. *E. coli* O157:H7 generally produced more EPS (sum of sugar and protein content in mg/cell) after growth in LB media and when suspended in AGW (35.1 ± 4.4 and 11.17 ± 2.3 × 10^{-18} mg/cell after growth in LB and M9 media, respectively). *Salmonella* produced the most EPS in $CaCl_2$ (24.05 ± 1.6 and 38.13 ± 4.4 × 10^{-18} mg/cell for after grown in LB and M9 media, respectively) and did not significantly differ between growth conditions.

Cellular EPS was also characterized by its two major components: carbohydrates and proteins. Carbohydrate content increased from monovalent, to divalent, to multivalent AGW (with the exception of *Salmonella* in M9 and AGW), while trends in protein content are generally inconsistent in relation to water chemistries and growth conditions. Interestingly, the sugar/protein ratio in EPS was significantly higher in cells suspended in complex AGW than those in KCl or $CaCl_2$ (Table 3.2). After growth in LB and M9 media, *E. coli* O157:H7 EPS from AGW treated cultures contained 11.93 ± 1.6 and 8.28 ± 1.6 ratios of sugar to protein, respectively. *Salmonella* cells in AGW likewise decreased from 16.49 ± 4.1 and 7.82 ± 0.8 ratios of sugar to protein in their EPS after growth in LB and M9 media, respectively. For *E. coli,* this change can be attributed to a nearly 70% reduction in carbohydrate content, while protein remains relatively constant. *Salmonella* cells in AGW, on the other hand, show no change in carbohydrate content for cells grown in nutrient-restricted conditions, while protein content doubles. The ratio of sugar/protein was generally higher for cells grown in high-nutrient LB than for those in M9, with the exception of *E. coli* O157:H7 in KCl and *Salmonella* in $CaCl_2$. This

suggests that changes in the makeup of the extracellular matrix in favor of proteins may play a role in promoting bacterial attachment to produce surfaces. Haznedaroglu et al. (2008) also observed that increases in the EPS sugar/protein ratio amongst environmental *E. coli* isolates decreased adhesion to quartz sand (50).

Table 3.2 Extracellular polymeric substance (EPS) composition for *E. coli* O157:H7 and *Salmonella* Typhimurium.

Solution[a]	**Growth Media**	**Sugar $\times 10^{-18}$ (mg/cell)**[b]	**Protein $\times 10^{-18}$ (mg/cell)**[c]	**Sugar/protein ratio**
		Escherichia coli O157:H7		
KCl	LB	0.65 ± 0.2	0.79 ± 0.3	0.94 ± 0.5
	M9	4.74 ± 0.3	0.98 ± 0.1	4.82 ± 0.2
$CaCl_2$	LB	15.92 ± 2.6	2.89 ± 0.3	5.57 ± 1.1
	M9	5.63 ± 1.8	2.56 ± 0.3	2.68 ± 1.7
AGW[d]	LB	32.41 ± 4.2	2.73 ± 0.3	11.93 ± 1.6
	M9	9.96 ± 2.3	1.20 ± 0.1	8.28 ± 1.6
		Salmonella Typhimurium		
KCl	LB	6.51 ± 4.3	4.68 ± 1.2	1.36 ± 0.9
	M9	5.82 ± 4.2	11.23 ± 5.9	0.52 ± 0.2
$CaCl_2$	LB	7.94 ± 2.7	16.11 ± 2.9	0.52 ± 0.3
	M9	27.09 ± 3.5	11.04 ± 1.2	2.45 ± 0.2
AGW	LB	13.25 ± 0.5	0.84 ± 0.2	16.49 ± 4.1
	M9	12.89 ± 0.1	1.66 ± 0.2	7.82 ± 0.8

[a] Ionic strength of all electrolyte solutions was 10 mM
[b] Based on the phenol−sulfuric acid method with xanthan gum as the standard at 10^8 cells/mL
[c] Based on the Lowry method with BSA as the standard at 10^8 cells/mL
[d] Artificial groundwater

Several bacterial strains have been shown to be more adhesive and ultimately more likely to form a biofilm when grown under nutrient-restricted conditions, which is hypothesized to be a protection mechanism under stress conditions (24, 51, 52). Exposure to complex water chemistry may induce similar responses, as evidenced by the higher sugar/protein ratio in EPS of *E. coli* O157:H7 cells in AGW (Table 3.2). Alteration of the combination of carbohydrates and proteins on the cell surface can create a more heterogeneous distribution of surface charge that has been shown to increase bacterial adhesion (52). Junkins and Doyle (1992) have shown that *E. coli* O157:H7 cells are more adhesive when grown under conditions that favor EPS production (53). This is due to the chemical and structural heterogeneity that creates multiple specific interactions with a given substrate surface (54, 55). Hassan and Frank (2004) showed that this trend applies to adhesion of *E. coli* O157:H7 on produce surfaces (23).

Using the same strains of *E. coli* O157:H7 and *Salmonella* Typhimurium employed in this study, Cook et al. (2017) previously demonstrated that growth in low nutrient conditions increases biofilm formation (25). It has been speculated that changes in bacterial attachment and biofilm formation can be attributed to production of specific surface macromolecules. For example, curli and cellulose surface structures have been previously shown to enhance biofilm formation, but not initial attachment, of other *E. coli* O157:H7 strains on stainless steel (56). However, curli expression has been found to be uncommon for pathogenic *E. coli* O157:H7 strain ATCC 43888 on the whole (57). While the literature on curli and cellulose production for *Salmonella* Typhimurium is more limited, some strains have been shown to produce curli and cellulose after

incubation at 30°C in static conditions, and ATCC 13311 was found to produce curli, but not cellulose on LB agar (25, 58). Curli fimbrae and cellulose may aid *Salmonella* in attachment, but flagella have been shown to be the most important factor in adherence to plant surfaces and flagella are still produced when grown at 37°C (59, 60). Although not included within the scope of this study, nutrient deprivation and media composition may induce stress responses in pathogen cells that have been shown to result in curli fimbrae production that can increase bacterial attachment in some cases (61, 62). However, the lack of any clear trends in protein content within cellular EPS implies that production of these surface macromolecules is not the only mechanism of increased attachment to spinach surfaces in this study. Curli are not likely to play a significant role in the attachment of *E. coli* O157:H7 cells grown in ideal nutrient conditions, and this work employs conditions that are not ideal for cellular curli production in either growth scenario, including growth at 37°C and harvest at mid-exponential growth phase (63). Additionally, this work has only employed two reference strains for *E. coli* O157:H7 ATCC 43888 and *Salmonella* Typhimurium, ATCC 13311 and therefore does not account for the variability that has been documented within these serotypes (64, 65). Further research is needed to better understand surface structures of undernourished cells and should employ multiple strains of each pathogen in order to account for variation in bacterial physiology.

3.3.4 Adhesion kinetics of pathogens versus environmental isolates.

Additional experiments were conducted in the parallel plate system to compare the two pathogens to two non-pathogenic environmental isolates in the representative AGW. *E. coli* B01 and *E. coli* B05 have been previously identified as potential non-pathogen surrogates for *E. coli* O157:H7 and *Salmonella* Typhimurium, respectively (25). Attachment trends of these four bacteria on the wax epicuticle layer from the spinach leaf were compared in the complex AGW, after being grown in either LB or M9 media (Figure 3.4). When grown in LB media, the environmental isolates attach similarly to their respective pathogen models, which is promising for their usefulness in predicting pathogen behavior. However, as described above, both pathogens were highly affected by nutrient conditions, becoming significantly more adhesive when grown in M9 minimal media. Environmental isolates did not follow this trend and instead showed insignificant differences in attachment rates between the two growth conditions ($p > 0.05$).

Results of characterization of the surrogates' cell surface properties do not different significantly from pathogenic *E. coli* O157:H7 in zeta potential and relative hydrophobicity (Table S1). None of the cell surface properties measured in this study can wholly describe the differences between the pathogens and non-pathogen isolates. However, the high variability in adhesion trends and EPS composition amongst *E. coli* B05 samples may offer some insight into the unpredictability of bacteria in the natural environment in comparison to pathogen species.

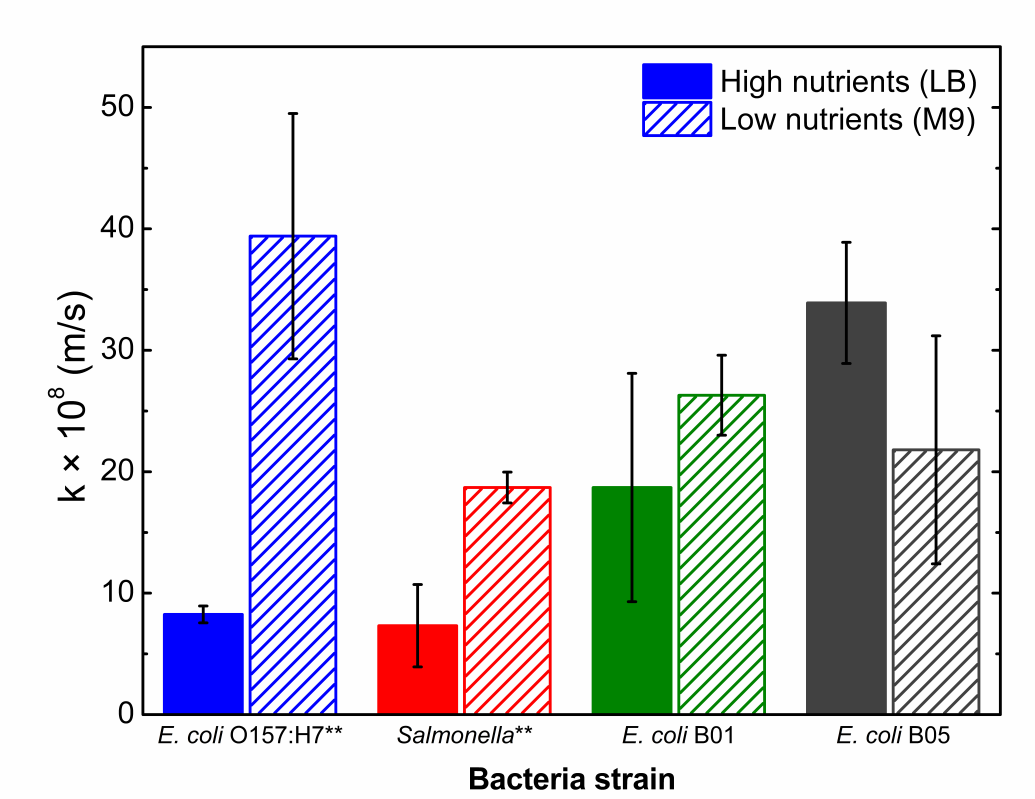

Figure 3.4 Pathogen and surrogates attachment mass transfer coefficients in 10 mM AGW.

Error bars indicate one standard deviation. Significant differences between the two growth condition are denoted **($p<0.01$).

3.4 Conclusions

In summary, the initial phases of biofilm formation were directly observed for two well-known foodborne pathogens and two non-pathogen surrogates in this study. Previous studies on foodborne pathogen attachment to produce surfaces have largely been conducted under simple and static laboratory conditions. Through dynamic flow experiments, this work has shown that growth conditions and solution complexities have significant effects on mechanisms of bacterial adhesion at interfaces of water and spinach

leaf surfaces, and also to present a potential challenge to using stress-tolerant environmental microbes as food safety surrogates. Specifically, these results show that deposition of these two pathogen strains on spinach epicuticle layers significantly increases when cells are grown in nutrient restricted conditions, implying that food safety research that only includes well-nourished cells may underestimate attachment to produce surfaces. This difference in adhesion may be partially attributed to increasing cell surface charge heterogeneity, as characterized by changes in EPS composition and decreasing surface charge density of cell surfaces for both *E.coli O*157:H7 and *Salmonella* Typhimurium. The pathogens followed similar adhesion trends in every tested condition, with the most significant differences observed with suspension in AGW after growth in M9 media ($p < 0.01$), which illustrates the role of complex, environmentally relevant water chemistries in magnifying changes on the cell surface. This ideal water chemistry scenario offers insight into the fundamental mechanisms that result in highly variable transport behavior by *E. coli* and *Salmonella* exposed to realistic aquatic environments, such as wastewater systems (66). Further, these results contribute to potential explanations for previous work that has reported increased bacterial attachment and biofilm formation on solid surfaces after exposure to stress conditions (43, 48, 55, 56). Additionally, we have demonstrated that two environmental isolates that are meant to mimic these pathogens are less impacted by non-ideal nutrient conditions and do not show significant changes in attachment as a result. The minimal media conditions and complex water chemistry employed in this study aim to represent more realistic scenarios in which bacteria pose risks of cross contamination in a food processing plant. The results

contribute to understanding the effects of these environments on bacterial adhesion and ultimately improving removal and inactivation of foodborne pathogens, as well as presenting a potential challenge to using stress-tolerant environmental microbes as food safety surrogates. Understanding the changes in surface properties that pathogens and surrogates undergo when stressed, and the resulting influence on adhesion to organic surfaces, is important for ultimately preventing adhesion and biofilm development that can lead to foodborne illness outbreaks.

3.5 References

1. World Health Organization (WHO). 2015. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015. Rome, Italy.
2. Callejón RM, Rodríguez-Naranjo MI, Ubeda C, Hornedo R, Garcia-Parrilla MC, Troncoso AM. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and causes. Foodborne Pathogens and Disease, 12:32 -38.
3. Olaimat AN, Holley RA. 2012. Factors influencing the microbial safety of fresh produce: A review. Food Microbiology, 32:1-19.
4. Bradford SA, Harvey RW. 2016. Future research needs involving pathogens in groundwater. Hydrogeology Journal, 25:931-938.
5. Shah M, Eppinger M, Ahmed S, Shah A, Hameed A, Hasan F. 2016. Flooding adds pathogenic *Escherichia coli* strains to the water sources in southern Khyber Pakhtunkhwa, Pakistan. Indian Journal of Medical Microbiology, 34:483-488.
6. Kuan CH, Kit Lim LW, Ting TW, Rukayadi Y, Ahmad SH, Jasimah Wan Mohamed Radzi CW, Thung TY, Ramzi OB, San Chang W, Loo YY, Kuan CS, Yeo SK, Radu S. 2017. Simulation of decontamination and transmission of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and Listeria monocytogenes during handling of raw vegetables in domestic kitchens. Food Control, 80:395-400.

7. Kusumaningrum H. 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. International Journal of Food Microbiology, 85:227-236.
8. Food and Drug Administration (FDA). 2008. Guidance for industry: Guide to minimize microbial food safety hazards for fresh-cut fruits and vegetables. Office of Food Safety, Division of Plant and Dairy Food Safety. College Park, MD, USA.
9. Herman KM, Hall AJ, Gould LH. 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. Epidemiology and Infection, 143: 3011-3021.
10. Benjamin L, Atwill ER, Jay-Russell M, Cooley M, Carychao D, Gorski L, Mandrell RE. 2013. Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. International Journal of Food Microbiology, 165:65-76.
11. Fonseca JM, Fallon SD, Sanchez CA, Nolte KD. 2011. *Escherichia coli* survival in lettuce fields following its introduction through different irrigation systems. Journal of Applied Microbiology, 110:893-902.
12. Leafy Greens Marketing Agreement (LGMA). 2016. Annual Report, California Leafy Greens Products Handler Marketing Agreement 2015-16. Leafy Greens Marketing Agreement, Sacramento, CA, USA.
13. Chen G, Walker SL. 2007. Role of solution chemistry and ion valence on the adhesion kinetics of groundwater and marine bacteria. Langmuir, 23:7162-7169.

14. Chowdhury I, Zorlu O, Walker SL, Haznedaroglu BZ. 2014. Impact of growth phase and natural organic matter on the attachment kinetics of *Salmonella* Typhimurium to solid surfaces. Environmental Engineering Science, 32:111-120.

15. Macarisin D, Patel J, Bauchan G, Giron JA, Sharma VK. 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodborne Pathogens and Disease, 9:160-167.

16. Reina LD, Fleming HP, Breidt F. 2002. Bacterial contamination of cucumber fruit through adhesion. Journal of Food Protection, 65:1881-1887.

17. Cooley M, Carychao D, Crawford-Miksza L, Jay MT, Myers C, Rose C, Keys C, Farrar J, Mandrell RE. 2007. Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. Plos One, 2:e1159.

18. Ao TT, Feasey NA, Gordon MA, Keddy KH, Angulo FJ, Crump JA. 2015. Global burden of invasive nontyphoidal *Salmonella* disease. Emerging Infectious Diseases, 21:941-949.

19. Herman KM, Hall AJ, Gould LH. 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973–2012. Epidemiology and Infection, 143:3011-3021.

20. Harwood VJ, Levine AD, Scott TM, Chivukula V, Lukasik J, Farrah SR, Rose JB. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Applied and Environmental Microbiology, 71:3163-3170.

21. Deng K, Wang X, Yen L, Ding H, Tortorello ML. 2014. Behavior of shiga toxigenic *Escherichia coli* relevant to lettuce washing processes and consideration of factors for evaluating process surrogates. Journal of Food Protection, 77:1860-1867.

22. Sinclair RG, Rose JB, Hashsham SA, Gerba CP, Haas CN. 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. Applied and Environmental Microbiology, 78:1969-1977.

23. Hassan AN, Frank JF. 2004. Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. International Journal of Food Microbiology, 96:103-109.

24. Teh AHT, Lee SM, Dykes GA. 2016. The influence of prior modes of growth, temperature, medium, and substrate surface on biofilm formation by antibiotic-resistant Campylobacter jejuni. Current Microbiology, 73:859-866.

25. Cook KL, Givan EC, Mayton HM, Parekh RR, Taylor R, Walker SL. 2017. Using the agricultural environment to select better surrogates for foodborne pathogens associated with fresh produce. International Journal of Food Microbiology, 262:80-88.

26. Haznedaroglu BZ, Kim HN, Bradford SA, Walker SL. 2009. Relative transport behavior of *Escherichia coli* O157:H7 and *Salmonella* enterica serovar pullorum in packed bed column systems: Influence of solution chemistry and cell concentration. Environmental Science & Technology, 43:1838-1844.

27. M9 minimal medium (standard). 2010. Cold Spring Harbor Protocols, 8.

28. McClaine JW, Ford RM. 2002. Reversal of flagellar rotation is important in initial attachment of *Escherichia coli* to glass in a dynamic system with high- and low-ionic-strength buffers. Applied and Environmental Microbiology, 68:1280-1289.

29. Kang Huang, Tian Y, Salvi D, Karwe M, Nitin N. 2017. Influence of Exposure Time, Shear Stress, and Surfactants on Detachment of *Escherichia coli* O157:H7 from Fresh Lettuce Leaf Surfaces During Washing Process. Food and Bioprocess Technology, 11:621-633.

30. Bolster CH, Mills AL, Hornberger GM, Herman JS. 1999. Spatial distribution of deposited bacteria following miscible displacement experiments in intact cores. Water Resources Research, 35:1797-1807.

31. Ensikat HJ, Neinhuis C, Barthlott W. 2000. Direct access to plant epicuticular wax crystals by a new mechanical isolation method. International Journal of Plant Sciences 161:143-148.

32. Pembrey RS, Marshall KC, Schneider RP. 1999. Cell surface analysis techniques: What do cell preparation protocols do to cell surface properties? Applied and Environmental Microbiology, 65:2877-2894.

33. Gong AS, Bolster CH, Benavides M, Walker SL. 2009. Extraction and analysis of extracellular polymeric substances: Comparison of methods and extracellular polymeric substance levels in *Salmonella* pullorum. Environmental Engineering Science, 26:1523-1532.

34. Elimelech M, Gregory J, Jia X, Williams RA. 1995. Particle deposition and aggregation: measurement, modelling and simulation. Butterworth-Heinemann, Oxford, England.

35. Rapicavoli JN, Kinsinger N, Perring TM, Backus EA, Shugart HJ, Walker SL, Roper MC. 2015. O antigen modulates insect vector acquisition of the bacterial plant pathogen Xylella fastidiosa. Applied and Environmental Microbiology, 81:8145-8154.

36. Hermansson M. 1999. The DLVO theory in microbial adhesion. Colloids and Surfaces B: Biointerfaces, 14:105-119.

37. 29. Marshall KC, Stout R, Mitchell R. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. Microbiology, 68:337-348.

38. Goulter RM, Gentle IR, Dykes GA. 2009. Issues in determining factors influencing bacterial attachment: a review using the attachment of *Escherichia coli* to abiotic surfaces as an example. Letters in Applied Microbiology, 49:1-7

39. Van Houdt R, Michiels CW. 2005. Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. Research in Microbiology, 156:626-633.

40. Takeuchi K, Matute CM, Hassan AN, Frank JF. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, Listeria monocytogenes, *Salmonella* Typhimurium, and Pseudomonas fluorescens to lettuce leaves. Journal of Food Protection, 63:1433-1437.

41. Barak JD, Whitehand LC, Charkowski AO. 2002. Differences in attachment of *Salmonella* enterica serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. Applied and Environmental Microbiology, 68:4758-4763.

42. Nagar V, Pansare Godambe L, Bandekar JR, Shashidhar R. 2016. Biofilm formation by Aeromonas strains under food-related environmental stress conditions. Journal of Food Processing and Preservation, 41:e13182

43. Kinsinger NM, Mayton HM, Luth MR, Walker SL. 2016. Efficacy of post-harvest rinsing and bleach disinfection of *E. coli* O157:H7 on spinach leaf surfaces. Food Microbiology, 62:212-220.

44. Ploux L, Ponche A, Anselme K. 2010. Bacteria/material interfaces: Role of the material and cell wall properties. Journal of Adhesion Science and Technology, 24:2165-2201.

45. Han P, Shen X, Yang H, Kim H, Tong M. 2013. Influence of nutrient conditions on the transport of bacteria in saturated porous media. Colloids and Surfaces B: Biointerfaces, 102:752-758.

46. van Loosdrecht MC, Lyklema J, Norde W, Schraa G, Zehnder AJ. 1987. Electrophoretic mobility and hydrophobicity as a measured to predict the initial steps of bacterial adhesion. Applied and Environmental Microbiology, 53:1898-1901.

47. Lagha R, Abdallah FB, Masmoudi AS. 2015. Effect of combined long-term starvation and γ-irradiation on membrane fatty acids and cell surface hydrophobicity of

Salmonella enterica serovar Typhimurium. Journal of Dairy Science, 98:8525-8530.

48. Oh YJ, Jo W, Yang Y, Park S. 2007. Influence of culture conditions on *Escherichia coli* O157:H7 biofilm formation by atomic force microscopy. Ultramicroscopy, 107:869-874.

49. Ryu J-H, Kim H, Beuchat LR. 2004. Attachment and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. Journal of Food Protection, 67:2123-2131.

50. Haznedaroglu BZ, Bolster CH, Walker SL. 2008. The role of starvation on *Escherichia coli* adhesion and transport in saturated porous media. Water Research, 42:1547-1554.

51. Reeser RJ, Medler RT, Billington SJ, Jost BH, Joens LA. 2007. Characterization of Campylobacter jejuni biofilms under defined growth conditions. Applied and Environmental Microbiology, 73:1908-1913.

52. Sheng X, Ting YP, Pehkonen SO. 2008. The influence of ionic strength, nutrients and pH on bacterial adhesion to metals. Journal of Colloid and Interface Science 321:256-264.

53. Junkins AD, Doyle MP. 1992. Demonstration of exopolysaccharide production by enterohemorrhagic *Escherichia coli*. Current Microbiology, 25:9-17.

54. Li Q, Wang Q, Zhu J, Zhou S, Gan M, Jiang H, Sand W. 2016. Effect of extracellular polymeric substances on surface properties and attachment behavior of Acidithiobacillus ferrooxidans. Minerals, 6:1-11.

55. Sharma MM, Chang YI, Yen TF. 1985. Reversible and irreversible surface charge modification of bacteria for facilitating transport through porous media. Colloids and Surfaces, 16:193-206.

56. Ryu J-H, Beuchat LR. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. Applied and Environmental Microbiology, 71:247-254.

57. Uhlich GA, Keen JE, Elder RO. 2001. Mutations in the csgD promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. Applied and Environmental Microbiology, 67:2367-2370.

58. Solomon EB, Niemira BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. Journal of Food Protection, 68:906-912.

59. Tan MSF, White AP, Rahman S, Dykes GA. 2016. Role of fimbriae, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. PLoS ONE, 11:e0158311.

60. Wheatley RM, Poole PS. 2018. Mechanisms of bacterial attachment to roots. FEMS Microbiology Reviews, 42:448-461.

61. Olsen A, Arnqvist A, Hammar M, Normark S. 1993. Environmental regulation of curli production in *Escherichia coli*. Infectious Agents and Disease, 2:272-274.

62. Carter MQ, Louie JW, Feng D, Zhong W, Brandl, MT. 2016. Curli fimbriae are conditionally required in *Escherichia coli* O157:H7 for initial attachment and biofilm formation. Food Microbiology, 57:81-89.

63. Hufnagel D, Depas W, Chapman M. 2015. The biology of the *Escherichia coli* extracellular matrix. Microbiology Spectrum, 3.

64. Ratnam S, March SB, Ahmed R, Bezanson GS, Kasatiya S. 1988. Characterization of *Escherichia coli* serotype O157:H7. Journal of Clinical Microbiology, 26:2006-2012.

65. Beltran P, Plock S, Smith N, Whittam T, Old D, Selander R. 1991. Reference collection of strains of the *Salmonella* typhimurium complex from natural populations. Microbiology, 137:601-606.

66. Li J, Zhao X, Tian X, Li J, Sjollema J, Wang A. 2015. Retention in treated wastewater affects survival and deposition of Staphylococcus aureus and *Escherichia coli* in sand columns. Applied and Environmental Microbiology, 81:2199-2205.

Chapter 4

Influence of Nanoparticles on Deposition and Detachment of *Escherichia coli*

Mayton, H.M., White, D., Marcus, I., Walker, S.L. Influence of nano-Cuo and -TiO_2 on deposition and detachment of *Escherichia coli* in two model systems. Submitted to *Environmental Science and Technology*.

Abstract

Increasing evidence suggests that agricultural water quality is closely tied to food safety risks. Therefore, the presence of nanoparticles in environmental waters or utilization as pesticides and fertilizers may have unintended consequences, as the effects of their interactions with foodborne bacteria are not well understood. This project utilizes a 2D parallel-plate flow cell and a 3D saturated sand column to systematically examine changes in bacterial transport trends due to nano-bio interactions under dynamic flow conditions. Two *Escherichia coli* species, O157:H7 and 25922, exposed to nano-CuO (<50 nm) and nano-TiO_2 (<150 nm), were used to mimic agriculturally relevant conditions. In flow cell experiments, the presence of CuO increased deposition and minimized release of pathogenic *E. coli* O157:H7 on a model spinach surface, while TiO_2 had no significant effects. Attachment and detachment – as quantified by mass transfer rate coefficients – of *E. coli* 25922 from the leaf surface were not impacted by the presence of nanoparticles. No breakthrough was observed in the column experiments, with the exception of TiO_2 eluted in the presence of *E. coli* O157:H7. However, column dissection revealed higher proportions of suspended particles retained in the upper portion of the column when either nanoparticle was present. This provides further evidence that nanoparticles can affect bacterial deposition and release, potentially promoting biofilm formation and foodborne illness risks.

4.1 Introduction

Both within the United States and globally, a significant portion of foodborne illness outbreaks are related to microbial contamination of fruits and vegetables (1, 2). This is often caused by irrigation and washing processes, where water potentially harbors harmful bacteria, leading to microbial cross contamination (3-5). *E. coli* O157:H7 is pathogenic bacteria of particular interest due to two 2018 foodborne illness outbreaks associated with romaine lettuce, in which at least 112 people were hospitalized and 5 deaths were reported due to contamination that was found in irrigation water (6). The fate of pathogens within produce irrigation, washing, and processing steps remains of interest for public health, as several recent studies have raised concerns about the efficacy of common rinsing and disinfection procedures (7-9).

In parallel with these challenges, nanoparticles are increasingly common in agricultural waters and are being widely considered for application as pesticides and soil amendments in agricultural operations (10-13). Copper oxide (CuO) and titanium dioxide (TiO_2) nanoparticles are two particular nanoparticles that have demonstrated promise as pesticides or plant growth supplements (14-17). These nanoparticles and others have been shown to induce stress and affect quorum sensing for microbes in environmentally relevant conditions, which can influence cells' likelihood of adhesion and ability to form biofilms (18-20). A review of several copper-based nanomaterials described toxic effects at concentrations as low as 50 ppb in aquatic environments (21). Interactions between TiO_2 and *E. coli* were studied previously in quartz sand column transport experiments, which found that the presence of *E. coli* reduced deposition of industrial grade (P25)

TiO_2 (22). However, few studies have utilized and compared applicable nanoparticles in agriculturally relevant scenarios, which include their interactions with bacteria (22, 23).

The work presented herein contributes to the understanding of aqueous interactions and transport of a specific foodborne pathogen (*E. coli* O157:H7) and metal oxide nanoparticles, specifically nano-CuO and nano-TiO_2 in agricultural systems. Non-pathogenic *E. coli* 25922, which is an industry standard quality control strain, is also used for comparison to the pathogen. A 2D parallel-plate flow cell and 3D saturated sand column were used to systematically examine changes in bacteria deposition and detachment trends as a result of nano-bio interactions under dynamic flow conditions on model leaf and mineral surfaces. By studying these idealized systems in tandem, these results provide unique insights into how physiochemical parameters of colloids affect their interactions with more complex real-life environments (22, 24).

4.2 Materials & Methods

4.2.1 Nanoparticle selection and characterization.

Copper oxide (CuO) was selected as a model nanoparticle for this study because of its use in agriculture as an herbicide (16, 17). Nano-CuO was purchased from Sigma Aldrich (St. Louis, MO) and was reported to have a primary particle size of <50 nm (TEM). Food grade (FG) TiO_2 (anatase, E171, Arizona State University) was selected as the other model nanoparticle with a previously measured primary particle size of 122 ± 48 nm (25). Anatase TiO_2 was chosen due to its promising agricultural applications (26, 27). Stock solutions were prepared using dry nanoparticle powder and were sonicated for

30 min in 10 mM KCl. Then the pH of the solution was adjusted to 7.0 using KOH and HCl, followed by 30 sec of sonication (28). For experiments with *E. coli,* concentrated bacteria stock was then added. With or without bacteria, nanoparticle solutions were gently shaken for 40 min to allow aggregation to occur and stabilize. Both nanoparticles were used at a concentration of 10 mg/mL, corresponding to 10^9 and at least 10^{10} primary particles/mL for TiO_2 and CuO, respectively. This nanoparticle concentration was chosen to mimic environmentally relevant concentrations and stay below previously observed toxic concentrations of nano-TiO_2 and nano-CuO (29, 30).

Zeta potential (electrophoretic mobility) and hydrodynamic diameter were determined for each nanoparticle suspension using a ZetaPALS analyzer and dynamic light scattering (DLS), respectively (Brookhaven Instruments Corp., Holtsville, NY) (31).

4.2.2 Bacteria selection and characterization.

E. coli O157:H7 and 25922 (ATCC 43888 and 25922) were chosen as model bacteria for this study to represent a pathogenic and non-pathogenic strain, respectively, and acquired from the USDA (USDA-ERS-FAESR, Bowling Green, KY). *E. coli* O157:H7 has been recently implicated in several major foodborne illness outbreaks associated with leafy greens (32), while *E. coli* 25922 is a commonly used surrogate for assessing efficacy of food safety processes in the agricultural industry (33). *E. coli* cells were cultured overnight in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37 °C. The overnight culture was then diluted 1:100 in fresh LB, incubated at 37 °C for 3.5 hours, and harvested at the mid-exponential cell growth phase (34). Bacteria were

suspended in 10 mM KCl (with or without nanoparticles) at a concentration of 10^9 cells/mL, and zeta potentials and hydrodynamic diameters were also determined, both separately and in the presence of nanoparticles. For all transport experiments with each of the two strains, scenarios tested were either suspensions of cells only, cells with CuO, or cells with TiO_2.

4.2.3 Parallel-plate experiments.

In a parallel plate flow cell (GlycoTech, Rockville, MA), deposition and release of the model *E. coli* on a spinach leaf surface was directly observed using an inverted microscope (BX-52, Olympus) and digital camera (Demo Retiga EXI Monochrome, QImaging) as previously described (7, 35, 36). In brief, cell suspensions were allowed to attach to the leaf surface at a flow rate of 0.1 mL/min using a syringe pump over 30 min. After a 5 min rinse with sterile 10 mM KCl to remove reversibly attached cells, deionized (DI) water was injected into the flow cell for 25 min to observe detachment. Over the course of the 60 min experiment, photos were taken every 30 sec and a code developed with Matlab (Mathworks, Natwick, MA) was used to quantify the number of cells attached or detached from the surface over time. Enumeration of cells was then used to calculate attachment and detachment mass transfer rate coefficients (k_{att} and k_{det}) as a function of bacteria flux (J, cells s^{-1} m^{-2}) and concentration of cells in suspension (C_0, cells/mL), where

$$k = \frac{J}{C_0}$$

Detachment is also reported as a percentage of cells removed based on the number of counted cells in the last frame of the attachment phase. Additionally, the duration of detachment (before the slope of cells vs. time reaches zero) varied and is therefore also presented in this study. Experimental scenarios with each combination of bacteria and nanoparticle, as well as each individual bacteria strain, were conducted in triplicate and statistical analysis was performed using a statistical single-factor ANOVA test for confidence intervals of 95% and 99% ($p < 0.05$ and $p < 0.01$, respectively).

4.2.4 Saturated sand column experiments.

In the 3D transport experiment through a quartz packed bed, the movement of *E. coli* and metal oxide nanoparticles in saturated soil conditions was observed using an in-line UV-VIS detector, as previously described (37, 38) and documented in the Appendix C. Briefly, the packed columns were primed with 10 mM KCl, before the aforementioned suspensions were pumped into the column at 2 mL/min for approximately 7.5 pore volumes (PV), followed by approximately 7.5 PV of 10 mM KCl, and 5 PV of DI water. The column effluent flowed through a UV-VIS detector (TURNER SP-890) with an in-line cuvette. Measurements were taken every 30 sec at the wavelengths 366 and 600 nm. Measurements taken at 600 nm were used for the generation of breakthrough curves. Scenarios included *E. coli* O157:H7 alone, with CuO, and with TiO_2, and were each conducted in triplicate. One control was conducted with *E. coli* 25922 alone.

Column dissections were performed once for each experimental scenario to elucidate differences in retention that may not be apparent from breakthrough curves(39).

After removing sand from the column in one centimeter increments, the optical density (OD) of the supernatant extracted from each sand segment was measured at 600 nm and normalized based on (1) the weight of the sand in the tube and (2) the proportion to the total absorbance of all five, 1 cm sections.

4.2.5 Scanning electron microscopy.

Scanning electron microscopy (SEM) was utilized to visualize each bacterium and their interactions with ENM suspensions in order to corroborate and provide further insight into the DLS results. A MIRA3 GMU field emission SEM (TESCAN, Brno, Czech Republic) was used to acquire at least 5 images of suspensions from each experimental condition (cells only or cells plus ENM). For imaging by SEM, 15 μL of each sample (10× diluted to 1 mM KCl) was dispensed and dried onto polycarbonate coupons, sputter coated with gold/palladium, and analyzed at 15 kV accelerating voltage, using low vacuum mode at a working distance of 4.80 mm.

4.3 Results & Discussion

4.3.1 Critical observations and implications for pathogen fate.

The results gleaned from this study provide insight into deposition and detachment trends of agriculturally relevant bacteria and nanomaterial mixtures by using fundamental 2D and 3D transport models. The 2D model spinach environment (parallel plate flow cell) provided a physically straightforward, but chemically heterogeneous, environment for direct observation of cell attachment and detachment. Meanwhile, the

3D packed bed provided a physically complex, but chemically simple environment where cell attachment and detachment were indirectly observed. Both systems involved negatively charged collector surfaces, but the hydrodynamics of the two systems have been shown to foster different modes of colloidal deposition (24). However, the particle Peclet numbers in the column and parallel plate chamber are comparable, and are expected to be within the diffusion-limited regime (22, 41). Together, the 2D and 3D systems provide corroborating evidence of the role of irreversible attachment in bacterial fate and transport in the simulated agriculturally relevant scenarios.

In the results presented below, the presence of 10 mg/mL of nanoparticle food grade TiO_2 particles resulted in a steady or slightly increased release of pathogenic *E. coli* O157:H7 from leaf and sand surfaces. This suggests that the application of TiO_2 promotes reversible bacterial attachment and presents a safety consideration due to bacteria release with a change in solution chemistry, such as in a food rinsing process or rain event. Nano-CuO was not shown to lead to a significant increase in detachment, but did cause an increase in irreversible bacterial attachment to both leaf and sand surfaces, possibly fostering increased food illness risk by enhancing irreversible attachment, a critical early stages of the biofilm formation process (42, 43).

The effects of these nanomaterials were observed to be more pronounced on the transport of *E. coli* O157:H7 than the common non-pathogenic quality control strain, *E. coli* 25922, as transport of the more neutrally charged pathogen may be more sensitive to changes in the suspension fluid. This may lead to underestimation of changes in microbial risks through the food system as a result of using nanomaterials in agricultural

operations. Also of environmental relevance is the observed decrease in deposition of TiO_2 in clean bed filtration in the presence of bacteria. This may be due to increased stability associated with extracellular polymeric substances (EPS) on the nanoparticle surface, and may lead to enhanced transport of TiO_2 in soils. The complex physiochemical interactions between nanoparticles and bacteria in can maximize or minimize bacterial transport in agricultural scenarios, based on both cell type and nanoparticle type. The results of this work contribute to greater understanding of the associated food safety and environmental risks.

4.3.2 Nanoparticle and bacteria characterization.

4.3.2a Physiochemical characterization.

The electrophoretic mobility and effective diameter of solutions comprised of each type of nanoparticles, *E. coli*, and the relevant mixtures are displayed in Table 4.1. At pH 7, CuO is near its isoelectric point and therefore under the solution chemistry conditions of this study the particles are close to neutrally charged (-6.11 ± 3.6 mV) (44). Alternatively, TiO_2 is far from its isoelectric point and therefore is more negatively charged at the test pH of 7 in these suspensions (-34.5 ± 9.6 mV) (45). Due to the greater magnitude of charge of TiO_2, these particles are more repulsive and therefore form smaller aggregates relative to their primary particle size (758 ± 111 relative to approximately 120 nm) than CuO (468 ± 28 relative to <50 nm). For the nanoparticles alone, the calculated zeta potential and effective diameter are similar to those of previous work with these metal oxide particles (44-46).

Similar to previous studies, both bacteria cells are negatively charged in these conditions, with *E. coli* O157:H7 close to neutral (-3.4 ± 0.3), while *E. coli* 25922 is highly negatively charged (-44.4 ± 2.1) (33, 47). Comparing the calculated hydrodynamic diameters, *E. coli* 25922 cells are 1821 ± 125 nm while *E. coli* O157:H7 cells are slightly smaller at 1410 ± 161 nm. With the addition of 10 mg/mL of each nanoparticle, the net charge of the particles in suspension, as measured by zeta potential, was not largely affected with CuO in suspension, but measurements showed slightly more negatively charged colloids with TiO_2. The measured effective hydrodynamic diameter of the suspensions was apparently reduced by the presence of nanoparticles, compared to the bacteria alone.

Table 4.1 Characterization of each bacteria, nanoparticle, and combination.

Bacteria	ENM[a]	Effective diameter (nm)[b]	Zeta potential (mV)[c]
E. coli O157:H7	--	1410 ± 161	-3.4 ± 0.3
E. coli O157:H7	CuO	1221 ± 155	-4.0 ± 0.6
E. coli O157:H7	TiO2	608 ± 71	-10.0 ± 0.4
E. coli 29522	--	1821 ± 125	-44.4 ± 2.1
E. coli 29522	CuO	1327 ± 38	-44.9 ± 1.2
E. coli 29522	TiO2	1209 ± 205	-45.1 ± 1.1
--	CuO	468 ± 28	-6.1 ± 3.6
--	TiO2	758 ± 111	-34.5 ± 9.2

[a] ENM = engineered nanomaterial
[b] Measured using dynamic light scattering (DLS)
[c] Measured using ZetaPALS via electrophoretic mobility

4.3.2b SEM images.

To corroborate size and surface charge results, SEM images were taken of the bacteria and nanoparticle mixtures and are displayed in Figure 4.1. Overall, nanoparticles are well-incorporated in bacteria aggregates for both strains of *E. coli*. For *E. coli* O157:H7, images show well-defined cells and nanoparticle aggregates. In contrast, obtaining crisp and clear photos of nanoparticle aggregates in the *E. coli* 25922 mixture was more difficult since 25922 has visibly more extracellular substances than O157:H7, prepared at the same conditions (Figure 4.1). These images provide evidence that extracellular polymers can cover some portion of the nanoparticle surface in suspension.

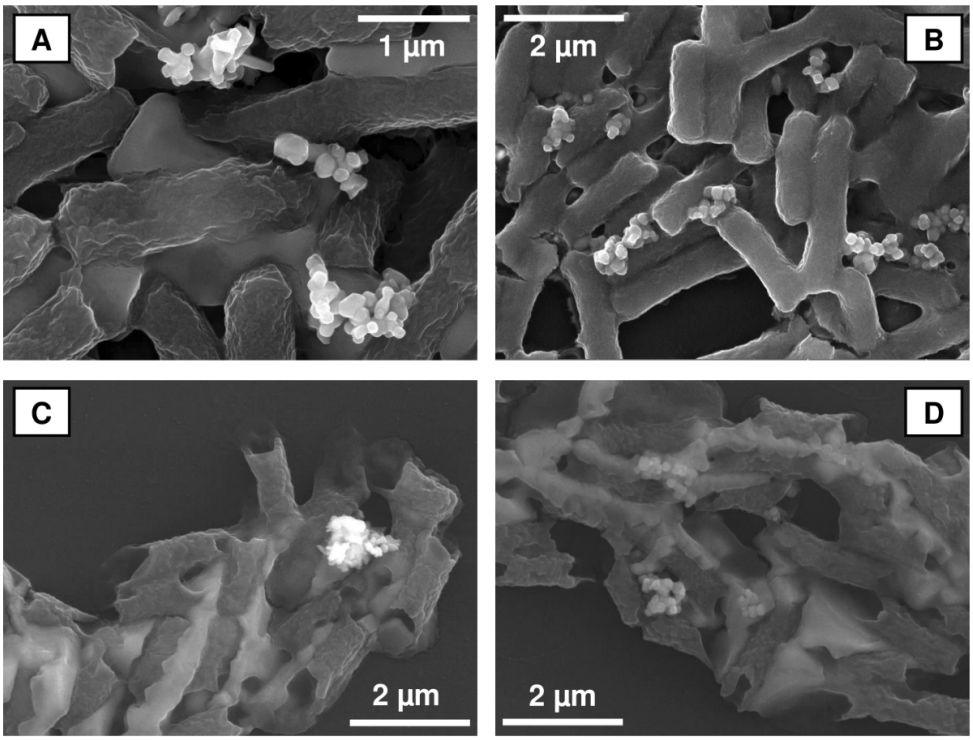

Figure 4.1 SEM images of *E. coli* O157:H7 and *E. coli* 25922 with ENMs.

Images of *E. coli* O157:H7 (A, B) and *E. coli* 25922 (C, D) were captured after suspensions with 10 mg/mL of either CuO (A, C) or TiO_2 (B, D) in 10 mM KCl were deposited and dried on polycarbonate coupons using a modified version of the methods previously described by Chowdhury *et al.* (2012).

Additionally, SEM images show interactions in which nanoparticles are often positioned tightly between two or more bacteria. This is notable because physical interactions have been shown to be one of the primary mechanisms by which nanomaterials induce stress in bacteria cells (18, 48, 49). For example, previous work with antibacterial silver nanoparticles also found that electrostatic forces were a primary mechanism adsorption of nanoparticles to bacteria (50).

4.3.2c DLVO predictions.

These size and surface charge results were used to predict the electrostatic and van der Waals forces between particles and cells using Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (51). Traditional electrokinetic characterization and application of DLVO theory provides some insight into the interactions between cells and nanomaterials, as well as with the plant and mineral surfaces. Given the greater magnitude of the measured zeta potential and resulting predicted repulsive forces, it was anticipated that the non-pathogenic *E. coli* 25922 cells would be more stable in the environment than the pathogenic strain (O157:H7), which was expected to be more adhesive, and therefore less mobile, due to its more neutral charge. With addition of nanoparticles in suspension that may associate with the bacteria surface, the overall effective shape can be with a nanoparticle adhered to its surface will still be considered unchanged and used as such in DLVO predictions. Whereas one would expect little observed changes in bacterial transport with the addition of nanoparticles based on DLVO, significant impacts are observed in 2D and 3D transport scenarios, suggesting

that other mechanisms are involved. More details on DLVO calculations are provided in the Appendix C.

4.3.3 Observations and mechanisms of deposition and detachment on spinach leaf surfaces.

Deposition of *E. coli* O157:H7 and *E. coli* 25922 cells was investigated for each strain on its own, and in the presence of each of the two nanoparticles. Presence of nano-CuO increased *E. coli* O157:H7 adhesion to spinach epicuticle surfaces by nearly 50%, from 14.50 ± 2.39 to 28.11 ± 2.77 × 10^{-8} m/s, as shown in Figure 4.2a. There was no significant difference between *E. coli* O157:H7 adhesion alone or in the presence of TiO_2 (16.57 ± 4.72 × 10^{-8} m/s). Adhesion of *E. coli* 25922 to the epicuticle surface was not significantly impacted by the presence of either nanoparticle (15.36 ± 1.43, 12.71 ± 4.29, and 15.55 ± 2.32 × 10^{-8} m/s for *E. coli* alone, with CuO, and with TiO_2, respectively). This may be attributed to its observed high EPS production and agglomeration that screen the impact of nanoparticles on the cell.

Detachment from the epicuticle surface is presented in detachment rate coefficients, in Figures 4.2a and 4.2b. Detachments rates of *E. coli* O157:H7 in every scenario were similar, and less than half of the magnitude of the mass transfer rate coefficients, ranging from -6.05 ± 2.62 to -7.46 ± 0.89 × 10^{-8} m/s. Essentially no detachment of *E. coli* 25922 was observed over the period of time tested. Additionally, this outcome was not impacted by the presence of either nanoparticle. However, when

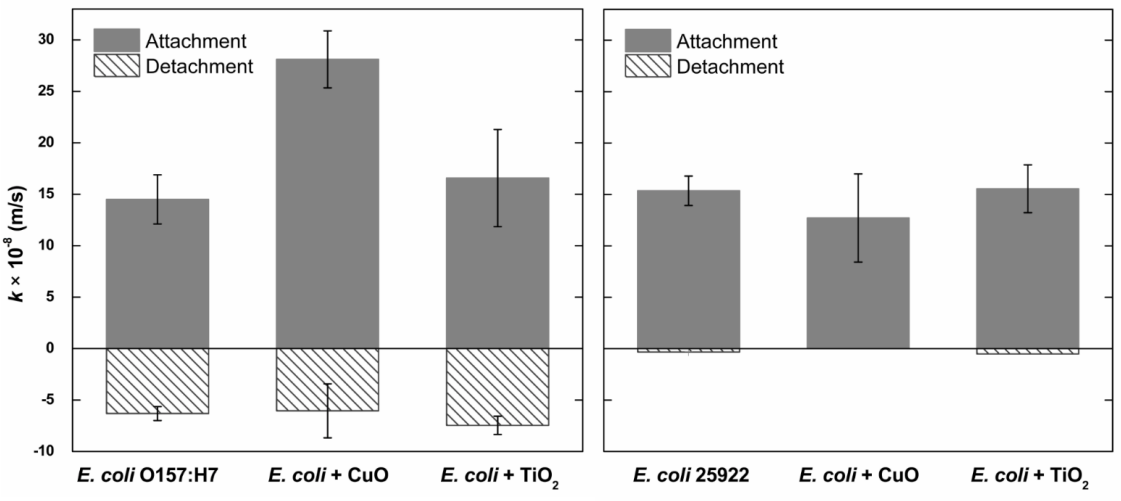

Figure 4.2 Bacterial adhesion and detachment on spinach surface.

Attachment (top) and detachment (bottom) mass transfer rate coefficients for *E. coli* O157:H7 (left) and *E. coli* 25922 (right) in 10 mM KCl on spinach leaf surfaces. Error bars represent on standard deviation from 3 replicates.

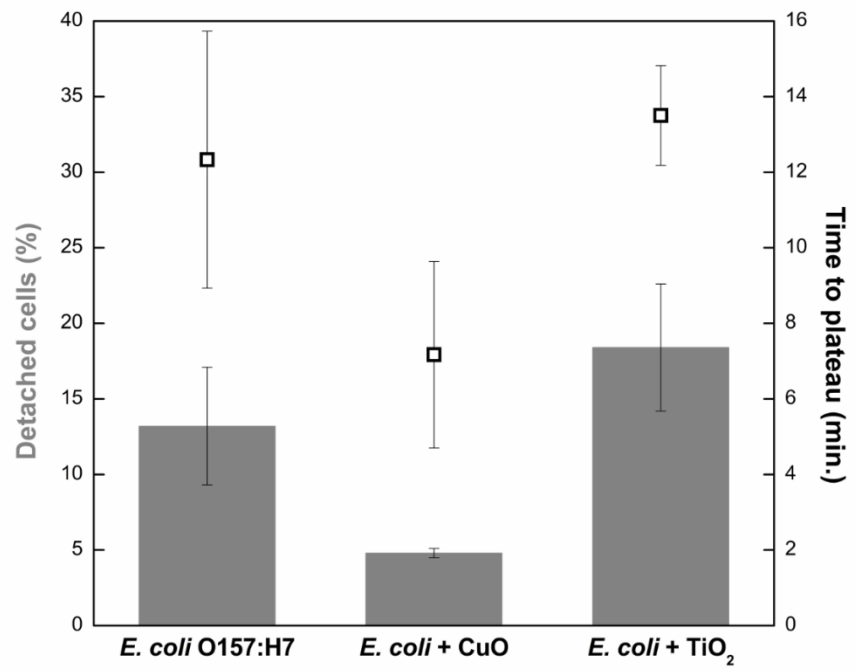

Figure 4.3 Pathogen detachment from spinach leaf surface.

Percentage of *E. coli* O157:H7 cells detached over 30 min with DI water rinse (gray bars, left axis) and time over which the rate of detachment is greater than zero (□, right axis). Error bars represent the standard deviation from 3 replicates.

detachment was normalized by the total cells observed at the end of the attachment phase, nuances in the trends became apparent (Figure 4.3). Only 5% of *E. coli* O157:H7 cells in the presence of CuO detached, while 14% and 18% of cells were removed when *E. coli* O157:H7 attached alone and in the presence of TiO_2, respectively. The amount of time over which detachment was observed is referred to as "Time to plateau" in Figure 4.3. This refers to the point in which no additional cells are being removed from the epicuticle surface during the 30 min rinse with DI water. This length of time varied between scenarios, with *E. coli* O157:H7 alone detaching over 13.5 min, O157:H7 with TiO_2 detaching over 14.5 min, and O157:H7 with CuO detaching over just 7.0 min. These results imply the CuO not only increases deposition rates of *E. coli* O157:H7 but increases the extent of irreversibly attached of cells.

4.3.4 Observations and mechanisms of deposition and release in the packed bed column.

For column experiments, breakthrough curves were generated to represent elution of the *E. coli* strains and nanoparticles from the packed bed. Removal of *E. coli* O157:H7 alone and in the presence of nano-CuO was essentially complete (100.0 ± 0.4% and 100.0 ± 0.6%, respectively). These scenarios showed minimal release in the phase of the experiment in which the column is flushed (rinsed) with DI H_2O (1.2 ± 0.4% and 1.5 ± 0.6%), as shown in Figure 4.4. Conversely, significantly higher release of 7.5 ± 2.6% was observed with *E. coli* O157:H7 in the presence of TiO_2 ($p < 0.01$).

In addition to the observed reduction in removal of *E. coli* O157:H7 in the column with TiO_2, breakthrough of TiO_2 was also observed in this scenario, as shown in the

breakthrough curves in Figure 4.5. This is in contrast with control experiments with each nanoparticle alone, in which complete removal was observed for both nanoparticles in the simple electrolyte background (data not shown), despite significant repulsive forces between TiO_2 and quartz predicted by particle-plate DLVO modeling. It is hypothesized that interactions between *E. coli* O157:H7 cells and TiO_2 in suspension results in increased stability for TiO_2 particles in suspension due to extracellular polymers that have been shown to increase steric hindrance (22, 52). Specifically, Chowdhury *et al.* (2012) utilized TiO_2 in a similar column apparatus under similar conditions (pH 7, 10 mg/mL TiO_2, 10 mM KCl) and also found that particle transport increased in the presence of *E. coli* due to increased electrosteric repulsion.

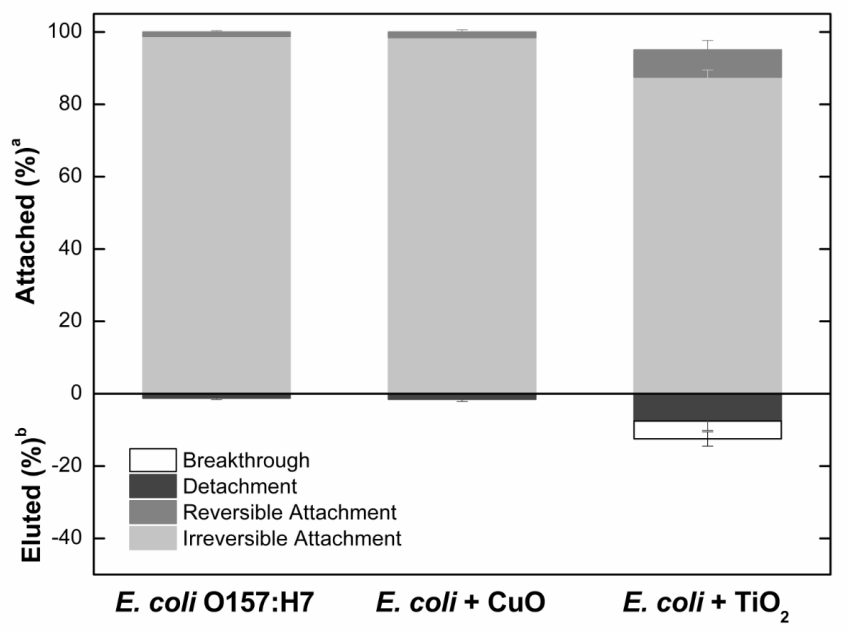

Figure 4.4 *E. coli* O157:H7 removal and release in the saturated sand column.
Breakthrough and release values were calculated by integrating under the breakthrough and DI rinse curves, respectively. Deposition values were calculated using the breakthrough and release values and mass balances. Error bars represent one standard deviation from three replicates.
[a] Calculated based on mass balances.
[b] Calculated based on UV-VIS absorbance.

Column retention profiles were created to further elucidate retention trends between these three scenarios (Figure 4.6). The trend for the cell-CuO suspension is expected to be a good indicator of bacterial retention, since cells have much higher absorbance than CuO at the concentrations used in this study (approximately 26 times as much), based on suspension C_0 absorbance values at 600 nm. The retention curves for the pathogen with nanoparticles show exponential decay in concentration of retained particles (bacteria cells and nanoparticles) with increasing depth of the column, which is expected based on clean bed filtration theory, as governed by first-order attachment (53). The shape of the retention curve in for the *E. coli* O157:H7 and TiO_2 suspension suggests that the first 2 cm of the column became saturated with retained particles. In the absence of nanoparticles, nearly linear decay of pathogen retention is observed. This trend is unexpected because it implies zeroth order deposition kinetics, which are not characteristic of deposition driven by DLVO forces.

The presence of nanoparticles appears to increase the retention of suspension species in the porous media. This is suggested by a greater fraction of retention in the upper portion of the column (C_C/C_N = 0.3, 0.4, and 0.5 at the inlet for *E. coli* O157:H7 alone, with TiO_2, and with CuO, respectively). Specifically, the presence of CuO has a pronounced effect on the retention profile of the *E. coli* O157:H7-nanoparticle suspension. This mirrors the observed increase in bacterial attachment rates to spinach leaf surfaces in the presence of CuO.

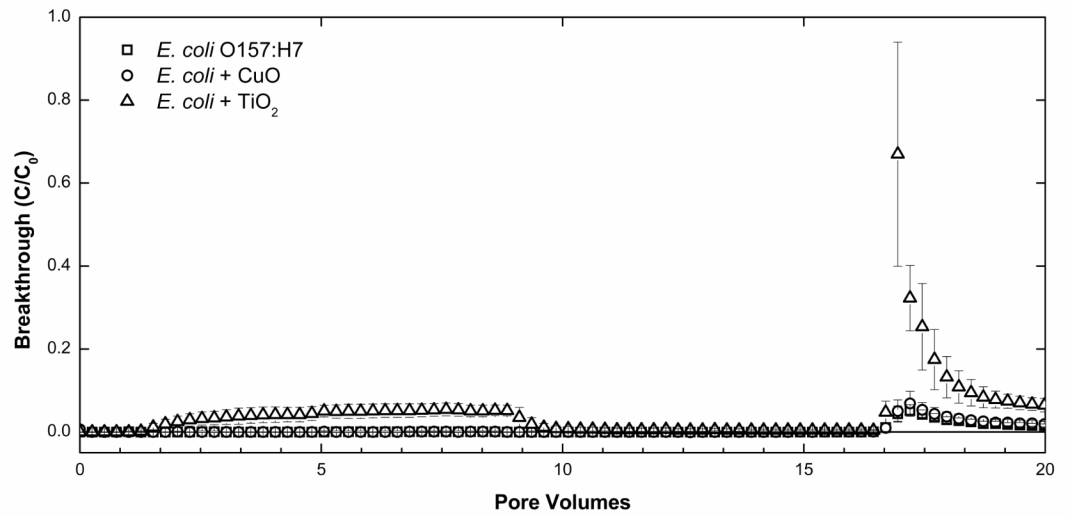

Figure 4.5 Breakthrough curves for saturated sand column transport experiments with *E. coli* O157:H7.

Columns were injected with 10^9 cells/mL in 10 mM KCl electrolyte at pH 7, with or without 10 mg/mL of ENM. Error bars represent one standard deviation from three replicates. Experimental conditions: Darcy velocity = 1 cm/min, Reynolds = 0.1, bed length = 5 cm, bed diameter = 1.5 cm, porosity = 0.45, average grain diameter = 275μm.

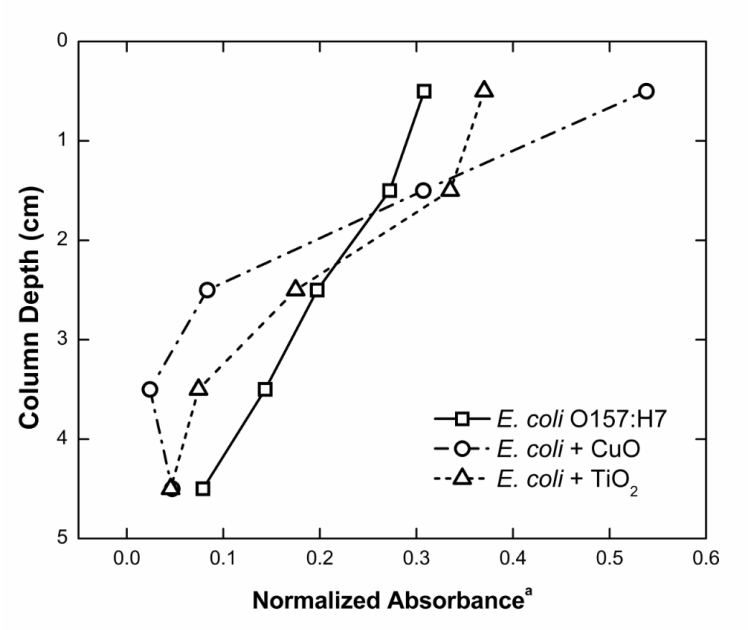

Figure 4.6 Column retention profiles.

Column retention profiles of *E. coli* O157:H7 alone and with each ENM. C_C and C_N are defined as concentration of recovered particles and the sum of concentrations of recovered particles for a given suspension, respectively. A depth of 0 cm corresponds to the entrance of the column.

4.3.5 Nanoparticle impacts on bacteria fate.

By using and comparing 2D and 3D transport models, the impacts of relatively low concentrations of nano-CuO and -TiO_2 on bacterial fate in relevant agricultural environments has been demonstrated. Overall, the impact of nanoparticles on the fate and transport of *E. coli* O157:H7 were greater than that of non-pathogen *E. coli* 25922. This may be attributed to the near neutral surface charge and lesser EPS production of the pathogen, which is supported by previous studies that have demonstrated enhanced resistance to nanomaterial toxicity by cells that overproduce EPS (54, 55). These results highlight the potential for emergent microbial contamination risks and poor representativeness of non-pathogen food safety surrogates from the increased utilization of nanomaterials in agriculture.

The aforementioned results have shown that even at low concentrations, the presence of nano-CuO can increase bacterial attachment on both leaf and sand surfaces. Previous studies have shown that copper-based nanomaterials can induce stress responses in bacterial cells (18, 56, 57). The small primary particle size and near neutral zeta potential may allow nano-CuO to interact more strongly with the cell surface, or even enter the cell (18). Additionally, CuO may dissolve into copper ions in suspension, which are highly toxic to bacteria (18, 58). At these experimental conditions, CuO has been shown to be relatively stable in solution, assuming KCl and NaCl contribute to stability similarly (59). Further, the presence of EPS in suspension has been demonstrated to increase stability leading to an increase dissolution of CuO nanoparticles over long term studies in 10 mM NaCl at pH 7 (59), causing more oxidative stress. One common stress

response in bacteria is the overproduction of EPS and increased adhesion to surfaces, in order to begin the process of forming a protective biofilm (60). Once formed, mature biofilms have been shown to protect *E. coli* O157:H7 cells from several common disinfectants used in the food industry (61, 62).

In many ways, nano-TiO_2 is similar to nano-CuO. Indeed, TiO_2 is stable in the presence of natural organic matter (22). It has also been shown to induce stress in several types of bacteria (63-65). However, TiO_2 had no significant impact on the deposition and detachment of *E. coli* on the spinach surface, and even reduced irreversible deposition on quartz collectors. Jomini *et al.*, (2015) observed an increase in planktonic, versus adhered, environmental bacteria over long term exposure to nano-TiO_2 (66). One important difference between the two studied nanoparticles is that of size: smaller particles have been shown to be more toxic than larger counterparts (67, 68), potentially making the antibacterial influence of primary TiO_2 nanoparticles (~122 nm) less than that of CuO nanoparticles (<50 nm). At these solution conditions, TiO_2 is also considerably more negatively charged than CuO (-34.5 versus -6.11 mV, respectively). While this does not make it significantly less likely to interact with the *E. coli* O157:H7 cells, it does result in significant energy barriers between TiO_2 and the spinach and quartz surfaces, which may reduce opportunities for particles to interact with adherent bacteria.

The results of this work elucidate the impacts of complex physiochemical interactions between nanoparticles and bacteria by using model systems that simulate relevant agricultural environments. Low concentrations of nanoparticles are likely to be present in these settings as they are adopted as efficient pesticide and fertilizers, and the

impacts on pathogen fate can be significant and may vary by cell type and nanoparticle type. Specifically, nano-CuO caused an increase in irreversible pathogen attachment to both leaf and sand surfaces, potentially fostering increased food illness risk by enhancing the early stages of the biofilm formation process. In contrast, the application of nano-TiO_2 may promote reversible bacterial attachment and present a safety consideration due to bacteria release with a change in solution chemistry, such as in a food rinsing process or rain event. The effects of these nanomaterials are more pronounced on the transport of pathogenic *E. coli* O157:H7 than the common non-pathogenic quality control strain, *E. coli* 25922, presenting a challenge for predicting the efficacy of washing and disinfection processes throughout the food chain. It is essential that further research is conducted to inform decision-making that aims to manage microbial risks throughout the food system that may result from increased use of nanomaterials in agricultural operations.

4.4 References

1. Interagency Food Safety Analytics Collaboration (IFSAC). 2015. Foodborne illness source attribution estimates for *Salmonella*, *Escherichia coli* O157, Listeria monocytogenes (Lm), and Campylobacter using outbreak surveillance data, December 2016.
2. World Health Organization (WHO). 2015. WHO estimates of the global burden of foodborne diseases: Foodborne disease burden epidemiology reference group 2007-2015.
3. Olaimat AN and Holley RA. 2012. Factors influencing the microbial safety of fresh produce: A review. Food Microbiology, 32(1):1-19.
4. Bradford SA and Harvey RW. 2017. Future research needs involving pathogens in groundwater. Hydrogeology Journal, 25(4): 931-938.
5. Jongman M and Korsten L. 2018. Irrigation water quality and microbial safety of leafy greens in different vegetable production systems: A review. Food Reviews International, 34(4):308-328.
6. Center for Disease Control and Prevention (CDC). 2018. Multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce (Final Update). Atlanta, GA, USA.
7. Kinsinger NM, Mayton HM, Luth MR, and Walker SL. 2017. Efficacy of post-harvest rinsing and bleach disinfection of *E. coli* O157: H7 on spinach leaf surfaces. Food Microbiology, 62:212-220.
8. Luo Y, Nou X, Yang Y, Alegre I, Turner E, Feng H, Abadias M, and Conway W. 2011. Determination of free chlorine concentrations needed to prevent

Escherichia coli O157: H7 cross-contamination during fresh-cut produce wash. Journal of Food Protection, 74(3):352-358.

9. Goodburn C and Wallace CA. 2013. The microbiological efficacy of decontamination methodologies for fresh produce: A review. Food Control, 32(2):418-427.
10. Keller AA and Lazareva A. 2014. Predicted releases of engineered nanomaterials: From global to regional to local. Environmental Science & Technology Letters, 1(1):65-70.
11. Kah M and Hofmann T. 2014. Nanopesticide research: Current trends and future priorities. Environment International, 63: 224-235.
12. Asadishad B, Chahal S, Akbari A, Cianciarelli V, Azodi M, Ghoshal S, and Tufenkji N. 2018. Amendment of agricultural soil with metal nanoparticles: Effects on soil enzyme activity and microbial community composition. Environmental Science & Technology, 52(4):1908-1918.
13. Dimkpa CO and Bindraban PS. 2017. Nanofertilizers: New products for the industry? Journal of Agricultural and Food Chemistry.
14. Yang F, Liu C, Gao F, Su M, Wu X, Zheng L, Hong F, and Yang P. 2007. The improvement of spinach growth by nano-anatase TiO_2 treatment is related to nitrogen photoreduction. Biological Trace Element Research, 119(1):77-88.
15. Feizi H, Moghaddam PR, Shahtahmassebi N, and Fotovat A. 2012. Impact of bulk and nanosized titanium dioxide (TiO_2) on wheat seed germination and seedling growth. Biological Trace Element Research, 146(1):101-106.

16. Kiaune L and Singhasemanon N. 2011 Pesticidal copper (I) oxide: Environmental fate and aquatic toxicity. Reviews of Environmental Contamination and Toxicology, 213:1-26.

17. Servin A, Elmer W, Mukherjee A, De la Torre-Roche R, Hamdi H, White JC, Bindraban P, and Dimkpa C. 2015. A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. Journal of Nanoparticle Research, 17(2):92.

18. Applerot G, Lellouche J, Lipovsky A, Nitzan Y, Lubart R, Gedanken A, and Banin E. 2012. Understanding the antibacterial mechanism of CuO nanoparticles: Revealing the route of induced oxidative stress. Small, 8(21):3326-3337.

19. Naik K and Kowshik M. 2014. Anti□quorum sensing activity of AgCl□ TiO_2 nanoparticles with potential use for food packaging. Journal of Applied Microbiology, 117(4):972-983.

20. Singh BR, Singh BN, Singh A, Khan W, Naqvi AH, and Singh HB. 2015. Mycofabricated biosilver nanoparticles interrupt pseudomonas aeruginosa quorum sensing systems. Scientific Reports, 5:13719.

21. Keller AA, Adeleye AS, Conway JR, Garner KL, Zhao L, Cherr GN, Hong J, Gardea-Torresdey JL, Godwin HA, Hanna S, Ji Z, Kaweeteerawat C, Lin S, Lenihan HS, Miller RJ, Nel AE, Peralta-Videa JR, Walker SL, Taylor AA, Torres-Duarte C, Zink JI, and Zuverza-Mena N. 2017. Comparative environmental fate and toxicity of copper nanomaterials. NanoImpact, 7:28-40.

22. Chowdhury I, Cwiertny DM, and Walker SL. 2012. Combined factors influencing the aggregation and deposition of nano- TiO_2 in the presence of humic acid and bacteria. Environmental Science & Technology, 46(13):6968-6976.

23. Battin TJ, Kammer Fv, Weilhartner A, Ottofuelling S, and Hofmann T. 2009. Nanostructured TiO_2: Transport behavior and effects on aquatic microbial communities under environmental conditions. Environmental Science & Technology, 43(21):8098-8104.

24. Redman JA, Walker SL, and Elimelech M. 2004. Bacterial adhesion and transport in porous media: Role of the secondary energy minimum. Environmental Science & Technology, 38(6):1777-1785.

25. Yang Y, Doudrick K, Bi X, Hristovski K, Herckes P, Westerhoff P, and Kaegi R. 2014. Characterization of food-grade titanium dioxide: The presence of nanosized particles. Environmental Science & Technology, 48(11):6391-6400.

26. Hong F, Zhou J, Liu C, Yang F, Wu C, Zheng L, and Yang P. 2005. Effect of nano-TiO_2 on photochemical reaction of chloroplasts of spinach. Biological Trace Element Research, 105(1):269-279.

27. Paret ML, Vallad GE, Averett DR, Jones JB, and Olson SM. 2013. Photocatalysis: Effect of light-activated nanoscale formulations of TiO_2 on *Xanthomonas perforans* and control of bacterial spot of tomato. Phytopathology, 103(3):228-236.

28. Chowdhury I, Hong Y, and Walker SL. 2010. Container to characterization: Impacts of metal oxide handling, preparation, and solution chemistry on particle stability.

Colloids and Surfaces A: Physicochemical and Engineering Aspects, 368(1):91-95.

29. Keller AA and Lazareva A. 2013. Predicted releases of engineered nanomaterials: From global to regional to local. Environmental Science & Technology Letters, 1(1):65-70.

30. Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, and Kahru A. 2013. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: A critical review. Archives of toxicology, 87(7):1181-1200.

31. Waller T, Marcus IM, and Walker SL. 2018. Influence of septic system wastewater treatment on titanium dioxide nanoparticle subsurface transport mechanisms. Analytical and Bioanalytical Chemistry:1-8.

32. Center for Disease Control and Prevention (CDC). 2018. Reports of selected *E. coli* outbreak investigations. Atlanta, GA, USA.

33. Cook KL, Givan EC, Mayton HM, Parekh RR, Taylor R, and Walker SL. 2017. Using the agricultural environment to select better surrogates for foodborne pathogens associated with fresh produce. International Journal of Food Microbiology, 262:80-88.

34. Haznedaroglu B, Kim H, Bradford S, and Walker S. 2009. Relative transport behavior of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar pullorum in packed bed column systems: Influence of solution chemistry and cell concentration. Environmental Science & Technology, 43(6):1838-1844.

35. McClaine JW and Ford RM. 2002. Characterizing the adhesion of motile and nonmotile *Escherichia coli* to a glass surface using a parallel‐plate flow chamber. Biotechnology and Bioengineering, 78(2):179-189.

36. Chen G, Beving DE, Bedi RS, Yan YS, and Walker SL. 2009. Initial bacterial deposition on bare and zeolite-coated aluminum alloy and stainless steel. Langmuir, 25(3):1620-1626.

37. Kim HN, Walker SL, and Bradford SA. 2010. Macromolecule mediated transport and retention of escherichia coli o157: H7 in saturated porous media. Water Research, 44(4):1082-1093.

38. Hong Y, Honda RJ, Myung NV, and Walker SL. 2009. Transport of iron-based nanoparticles: Role of magnetic properties. Environmental Science & Technology, 43(23):8834-8839.

39. Lanphere JD, Luth CJ, and Walker SL. 2013. Effects of solution chemistry on the transport of graphene oxide in saturated porous media. Environmental Science & Technology, 47(9):4255-4261.

40. Weiss L. 1968. Studies on cellular adhesion in tissue-culture: X. An experimental and theoretical approach to interaction forces between cells and glass. Experimental Cell Research, 53(2-3):603-614.

41. Kim HN, Bradford SA, and Walker SL. 2009. *Escherichia coli* O157:H7 transport in saturated porous media: Role of solution chemistry and surface macromolecules. Environmental Science & Technology, 43(12):4340-4347.

42. Palmer J, Flint S, and Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol, 34(9):577-588.

43. Stanley PM. 1983. Factors affecting the irreversible attachment of pseudomonas aeruginosa to stainless steel. Canadian Journal of Microbiology, 29(11):1493-1499.

44. Conway JR, Adeleye AS, Gardea-Torresdey J, and Keller AA. 2015. Aggregation, dissolution, and transformation of copper nanoparticles in natural waters. Environmental Science & Technology, 49(5):2749-2756.

45. Chen C, Marcus IM, Waller T, and Walker SL. 2018. Comparison of filtration mechanisms of food and industrial grade TiO_2 nanoparticles. Analytical and bioanalytical chemistry:1-8.

46. Keller AA, Adeleye AS, Conway JR, Garner KL, Zhao L, Cherr GN, Hong J, Gardea-Torresdey JL, Godwin HA, and Hanna S. 2017. Comparative environmental fate and toxicity of copper nanomaterials. NanoImpact, 7:28-40.

47. Bradford SA, Simunek J, and Walker SL. 2006. Transport and straining of *E. coli* O157: H7 in saturated porous media. Water Resources Research, 42(12).

48. Thill A, Zeyons O, Spalla O, Chauvat F, Rose J, Auffan M, and Flank AM. 2006. Cytotoxicity of CeO_2 nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism. Environmental Science & Technology, 40(19):6151-6156.

49. Hajipour MJ, Fromm KM, Ashkarran AA, de Aberasturi DJ, de Larramendi IR, Rojo T, Serpooshan V, Parak WJ, and Mahmoudi M. 2012. Antibacterial properties of nanoparticles. Trends in biotechnology, 30(10):499-511.

50. Khan SS, Mukherjee A, and Chandrasekaran N. 2011. Studies on interaction of colloidal silver nanoparticles (SNPs) with five different bacterial species. Colloids and Surfaces B: Biointerfaces, 87(1):129-138.

51. Derjaguin B and Landau L. 1993. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. Progress in Surface Science, 43(1):30-59.

52. Lin D, Story SD, Walker SL, Huang Q, Liang W, and Cai P. 2017. Role of pH and ionic strength in the aggregation of tio2 nanoparticles in the presence of extracellular polymeric substances from *Bacillus subtilis*. Environmental Pollution, 228:35-42.

53. Bradford SA, Simunek J, and Walker SL. 2006. Transport and straining of *E. coli* O157:H7 in saturated porous media. 42(12).

54. Liu Y, Li J, Qiu X, and Burda C. 2007. Bactericidal activity of nitrogen-doped metal oxide nanocatalysts and the influence of bacterial extracellular polymeric substances (eps). Journal of Photochemistry and Photobiology A: Chemistry, 190(1):94-100.

55. Joshi N, Ngwenya BT, and French CE. 2012. Enhanced resistance to nanoparticle toxicity is conferred by overproduction of extracellular polymeric substances. Journal of Hazardous Materials, 241-242:363-370.

56. Gaetke LM and Chow CK. 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology, 189(1-2):147-163.

57. Bondarenko O, Ivask A, Käkinen A, and Kahru A. 2012. Sub-toxic effects of CuO nanoparticles on bacteria: Kinetics, role of cu ions and possible mechanisms of action. Environmental pollution, 169:81-89.

58. Baek Y-W and An Y-J. 2011. Microbial toxicity of metal oxide nanoparticles (CuO, NiO, ZnO, and Sb_2O_3) to escherichia coli, bacillus subtilis, and streptococcus aureus. Science of The Total Environment, 409(8):1603-1608.

59. Adeleye AS, Conway JR, Perez T, Rutten P, and Keller AA. 2014. Influence of extracellular polymeric substances on the long-term fate, dissolution, and speciation of copper-based nanoparticles. Environmental Science & Technology, 48(21):12561-12568.

60. Landini P. 2009. Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. Research in Microbiology, 160(4):259-266.

61. Niemira BA and Cooke PH. 2010. *Escherichia coli* O157: H7 biofilm formation on romaine lettuce and spinach leaf surfaces reduces efficacy of irradiation and sodium hypochlorite washes. Journal of food science, 75(5):M270-M277.

62. Somers EB, Schoeni JL, and Wong AC. 1994. Effect of trisodium phosphate on biofilm and planktonic cells of C*ampylobacter jejuni*, *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella* typhimurium. International Journal of Food Microbiology, 22(4):269-276.

63. Kumar A, Pandey AK, Singh SS, Shanker R, and Dhawan A. 2011. Engineered ZnO and TiO_2 nanoparticles induce oxidative stress and DNA damage leading to reduced viability of escherichia coli. Free Radical Biology and Medicine, 51(10):1872-1881.

64. Zavilgelsky G, Kotova VY, and Manukhov I. 2011. Titanium dioxide (TiO_2) nanoparticles induce bacterial stress response detectable by specific lux biosensors. Nanotechnologies in Russia, 6(5-6):401-406.

65. von Moos N and Slaveykova VI. 2014. Oxidative stress induced by inorganic nanoparticles in bacteria and aquatic microalgae–state of the art and knowledge gaps. Nanotoxicology, 8(6):605-630.

66. Jomini S, Clivot H, Bauda P, and Pagnout C. 2015. Impact of manufactured tio2 nanoparticles on planktonic and sessile bacterial communities. Environmental Pollution, 202:196-204.

67. Ivask A, Kurvet I, Kasemets K, Blinova I, Aruoja V, Suppi S, Vija H, Käkinen A, Titma T, and Heinlaan M. 2014. Size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells in vitro. PLoS One, 9(7):e102108.

68. Nair S, Sasidharan A, Rani VD, Menon D, Nair S, Manzoor K, and Raina S. 2009. Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. Journal of Materials Science: Materials in Medicine, 20(1):235.

Chapter 5

Efficacy of Post-Harvest Rinsing and Bleach Disinfection of *E. coli* O157:H7

Kinsinger, N.M., Mayton, H.M., Luth, M.R., Walker, S.L. Efficacy of post-harvest rinsing and bleach disinfection of *E. coli* O157:H7 on spinach leaf surfaces. *Food Microbiology*, Vol. 62, 212-220 (2017).

Abstract

Attachment and detachment kinetics of *Escherichia coli* O157:H7 from baby spinach leaf epicuticle layers were investigated using a parallel plate flow chamber. Mass transfer rate coefficients were used to determine the impact of water chemistry and common bleach disinfection rinses on the removal and inactivation of the pathogen. Attachment mass transfer rate coefficients generally increased with ionic strength. Detachment mass transfer rate coefficients were nearly the same in KCl and AGW rinses; however, the detachment phase lasted longer in KCl than AGW (18 ± 4 minutes and 4 ± 2 minutes, respectively), indicating that the ions present during attachment play a significant role in the cells' ability to remain attached. Specifically, increasing bleach rinse concentration by two orders of magnitude was found to increase the detachment mass transfer rate coefficient by 20 times (from $5.7 \pm 0.7 \times 10^{-11}$ m/s to $112.1 \pm 26.8 \times 10^{-11}$ m/s for 10 ppb and 1000 ppb, respectively), and up to 88 ± 4% of attached cells remained alive. The spinach leaf texture was incorporated within a COMSOL model of disinfectant concentration gradients, which revealed nearly 15% of the leaf surface is exposed to almost 1000 times lower concentration than the bulk rinse solution.

5.1 Introduction

Within the United States, food safety is frequently compromised via microbial contamination (1, 2) and sickens one in six Americans annually (3). Additionally, foodborne illness results in significant economic losses within the agricultural industry, estimated to be over $75 billion per year (4). Approximately 20% of single food commodity outbreaks from 2003-2008 were attributed to leafy green produce alone (3). Contamination of such minimally processed and ready-to-eat produce is of specific concern since it is frequently consumed uncooked or raw (5).

The bacterial pathogen *Escherichia coli* serotype O157:H7 (*E. coli* O157:H7) was associated with 350 outbreaks over a twenty year period, with 52% of those being foodborne (6). Several farms identified in an *E. coli*-associated spinach outbreak from the Salinas Valley had water contaminated with this pathogen onsite, specifically associated with cattle farms and wild pigs in close proximity (7). *E. coli* O157:H7, a gram-negative rod-shaped bacterium (8), is an enterohemorrhagic strain of the bacterium that causes acute diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (9).

It is important to note that contamination by pathogens may occur at almost any step from farm to table, and they can further survive during cleaning, packaging, and storage (1, 2). However, all of the transmission modes intersect at one critical step – the initial stage of cell adhesion and contamination. Also understanding potential detachment and/or disinfection by various cleaning methods is critical. To the authors' knowledge, most produce rinsing and disinfection studies (10-17) have been conducted without *in situ* observation methods. Instead, the methods used have generally involved removal of substrate and rinsing to remove loosely adhering organisms. This exposes adhering particles (bacteria) to extreme and inconsistent forces, specifically by crossing the liquid-air interface. Thus, previously published results reflect not only initially adhered bacteria

on the substrate, but also those that are retained on the surface by external forces (19). Therefore, the attachment and detachment of *E. coli* O157:H7 from isolated epicuticle layers of spinach leaves was investigated using a parallel plate flow chamber (Figure S2, Appendix D). With this experimental design, bacterial attachment can be imaged onto a variety of surfaces with real-time *in situ* analysis (20-24) and track individual cells within field of view during rinsing and disinfection experiments.

E. coli O157:H7 is also known to seek stomatas and damaged regions of leaves for protection, nutrients, and moisture, which may extend its survival within the field (25, 26). Therefore, it is crucial to understand the initial attachment of the bacteria in various source waters that may be used for irrigation in order to prevent initial contamination. Hence, mass transfer rate coefficients for both attachment to and detachment from spinach leaf epicuticle layers are determined as a function of a range of relevant environmental parameters. The impact of ionic strength, water chemistry, and typical bleach disinfection rinse concentrations on the removal and inactivation of *E. coli* O157:H7 was investigated within this flow chamber.

5.2 Materials & Methods

5.2.1 Bacterial cell preparation and characterization.

E. coli O157:H7/pGFP was used as a model pathogen in this study (6). *E. coli* cells were cultured in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37°C overnight and harvested at the mid-exponential cell growth phase (27). Cells were characterized for relative hydrophobicity using microbial adhesion to hydrocarbons (MATH) test and zeta potential (28). A detailed description of bacterial growth, preparation, and characterization techniques is provided in SI. Bacteria were

fluorescently labelled with enhanced green fluorescent protein (*E. coli* O157:H7/pGFP) to characterize attachment and detachment (29, 30). In the presence of UV light, the persistence of the cells' fluorescence could be assessed visually using the inverted fluorescent microscope.

5.2.2 Surface preparation and characterization.

All of the baby spinach leaves used in this study were pre-washed, bagged spinach from the same brand and purchased from the same local grocery store. Spinach leaves were stored in sealed containers at 4°C for a maximum of 5 days. For isolated epicuticle layers, spinach was purchased on the day of preparation. Only green, healthy, non-damaged leaves were selected to be used in these experiments (no visible yellowing, browning, rips, or tears) and were handled aseptically throughout their preparation.

A freeze imbedding technique was used to isolate epicuticle films from the spinach leaf surface and immobilize onto a synthetic substrate (31). The method, in brief, involves placing a drop of Triethylene glycol (TEG) on a flat, rigid stainless steel surface. A spinach leaf is then pressed against the metal surface (with the top side of the leaf in contact with the TEG) (31, 32). Next, the surfaces are slowly submerged into liquid nitrogen for approximately 10 seconds and then removed. The leaf is then removed from the frozen droplet in which the epicuticle was imbedded. The droplet is allowed to thaw on a polycarbonate coupon, at which point the epicuticle wax is transferred to the coupon and the remnant TEG is washed away with DI water. Immobilized waxes were stored at 4°C following isolation for up to one week.

Spinach leaves were characterized by streaming potential, Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), and Static Contact Angle (SCA). The zeta potential of spinach leaves was calculated from the streaming potential measured by a streaming potential analyzer (SurPASS, Anton Paar, Graz, Austria) with an adjustable gap cell (33).

5.2.3 Bacterial attachment and rinsing.

Bacterial attachment and detachment experiments were conducted in a parallel plate flow chamber (20, 22, 34-36) (GlycoTech, Rockville, MA) positioned on an inverted fluorescent microscope (BX-52, Olympus). The inner dimension of the chamber is 6 cm × 1 cm × 0.08 cm and is composed of a Plexiglas® block that is mounted by a flexible silicone elastomer gasket and a microscope slide sealed by vacuum grease. The fluid stream enters the chamber from a capillary tube that is connected to a syringe, which is controlled by a syringe pump at a flow rate of 0.1 mL/min, corresponding to an average flow velocity of 0.79 m/h, and a Péclet number of 6.47×10^{-4}. The fluorescently labeled bacteria were imaged by a 40× long working distance objective (UPlanFl, Olympus) using a filter at excitation and emission wavelengths of 480 nm and 510 nm, respectively (Chroma Technology Corp., Brattleboro, VT).

Attachment was observed over a 30-minute period (Stage 1, Figure 5.1) with images recorded with a digital camera (Demo Retiga EXI Monochrome, QImaging) every minute to determine the kinetics of cell adhesion. Enumeration of cells was

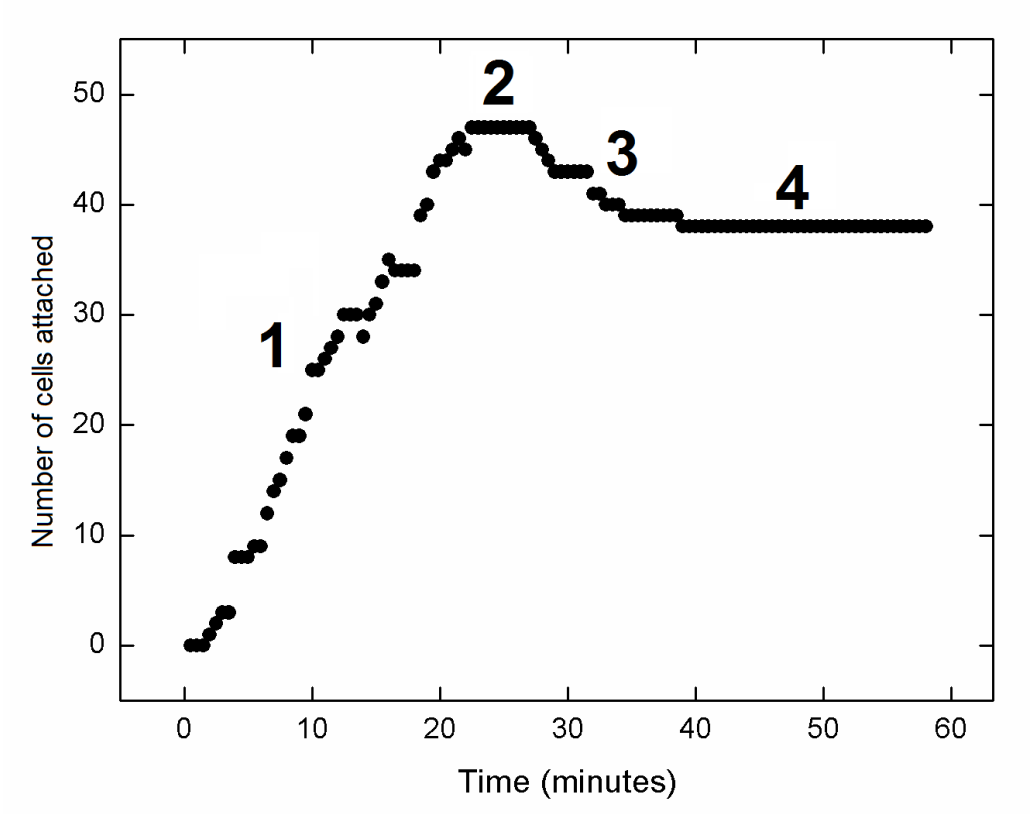

Figure 5.1 Representative experiment profile**.**
Profile showing number of attached cells over time in 10 mM KCl and rinsed with 20 ppb bleach. (1) Attachment, (2) background salt solution rinsing, (3) DI water and bleach rinsing, and (4) detachment plateau stages.

determined by comparison of successive images. A suspension of cells (5×10^7 cells/mL) was utilized and the concentration was determined with a counting chamber (Bürker-Türk chamber, Marienfield Laboratory Glassware, Lauda-Konigshofen, Germany). Adhesion experiments were conducted in 1 to 100 mM KCl and 3.3 to 10 mM artificial groundwater (AGW) (37) at ambient temperature (22 - 25 °C) to investigate the impact of ionic strength and solution chemistry on attachment to the leaf surface. Surfaces were rinsed with DI water within the parallel plate flow cell prior to cell adhesion experiments.

Following deposition, background salt solution used during attachment (KCl or AGW) was rinsed through the flow cell (Stage 2, Figure 5.1) to remove unattached

bacteria from the surface. After the background salt rinse, the rinsing experiment began in Stage 3 by introducing either deionized water (DI water) or sodium hypochlorite (bleach). Bleach rinsing experiments were only conducted on bacteria attached using 10 mM KCl in Stage 1. Although bleach is commonly used in the 50 – 200 ppm range in industry (38), a lower range (1 ppb to 1000 ppb) was used in these experiments to assess the impact of reduced bleach concentration in certain parts of the leaf surface. During all rinsing experiments, the bacterial detachment was observed to cease beyond a certain point, resulting in a plateau in the number of remaining, attached bacteria (Stage 4, Figure 5.1).

5.2.4 Mass transfer rate coefficients.

Enumeration of cells was used to calculate attachment and detachment mass transfer rate coefficients (k_{att} and k_{det}). The number of bacterial cells deposited was plotted versus time, and calculation of bacterial flux, J, was achieved by dividing the initial slope of the line by the microscope viewing area (230 μm × 170 μm). Representative attachment and detachment curves are shown in Figure 5.1 in Stages 1 and 3, respectively. The mass transfer rate coefficient for the bacteria, k_{pp}, is calculated using the bacterial flux (number of cells per area per time), and the bulk cell concentration (number of cells/mL), C_0, via (20 21, 39:

$$k = \frac{J}{C_0}$$

The mass transfer rate coefficients for these experiments are identified as k_{att} or k_{det}. Each experiment was performed in triplicate on each batch of generated surfaces.

Statistical analysis was performed using the student t-test to identify significant differences between data sets where a 95% confidence level was confirmed (when $p < 0.05$).

The detachment $k_{det,DI}$ due to rinsing with DI water will be discussed with respect to both the ionic strength and solution chemistry (KCl vs AGW) used during Stage 1. The detachment $k_{det,bleach}$ due to bleach rinsing will be discussed with respect to bleach concentration used during Stage 3. The duration of detachment, or duration of Stage 3, will be discussed with respect to solution chemistry (KCl vs AGW) for DI rinsing experiments, and with respect to bleach concentration for bleach rinsing experiments.

5.2.5 Modeling: DLVO model formulation.

In order to further evaluate the relative contribution of electrostatic and van der Waals interactions between the bacteria cells and the spinach leaf surface, Derjaguin-Landau-Verwey-Overbook (DLVO) theory was applied (40). Zeta potentials for *E. coli* O157:H7 and spinach leaf surface were used to calculate interaction energy profiles based on DLVO theory, assuming a sphere-plate geometry. *E. coli* zeta potential was measured using a ZetaPALS analyzer (Brookhaven Instruments Corporation, Holtsville, NY), and spinach leaf zeta potential was measured using a streaming potential analyzer (SurPASS, Anton Paar, Graz, Australia). Measurements and calculations were done using ionic strengths of 1 to 100 mM. Further information and equations used in the DLVO model formulation can be found in Appendix D.

5.2.6 Modeling: COMSOL model formation.

Using a commercial finite element package (COMSOL Multiphysics v4.3, COMSOL, Inc., Palo Alto, CA), a computational model was developed to evaluate fluid flow across the surface of a spinach leaf and the concentration gradient of bleach during rinsing. The velocity field is described by the equations of motion for an incompressible fluid and the continuity equation. The following boundary conditions were imposed: 1) at the inlet, a fully developed laminar velocity profile was specified with a cross flow velocity of 0.79 m/h to simulate the parallel plate conditions, while the out flow boundary was set to constant (atmospheric) pressure, 2) at the leaf surface, the tangential velocity was set to zero (no-slip condition).

Once the velocity field was computed, the bleach concentration profile was able to be determined. The bleach concentration field is described by advection–diffusion equation. The following boundary conditions were imposed: 1) a constant concentration at the inlet, ranging between 4 ppm bleach to 200 ppm bleach, and 2) an initial zero concentration at the outlet and at the wall, consequently the lowest bleach concentration. The computational domain was meshed using a structured, boundary-layer type mesh, with an increasing mesh density near the leaf surface features. This enabled efficient computation while retaining accuracy where the largest concentration variations were expected in the system. Mesh refinement was carried out to ensure the independence of solved bleach concentration profile on the solved mesh. In all simulations, the solution was assumed to be water with fluid density and dynamic viscosity taken as 1000 kg/m^{-3} and 10^{-3} Pa•s, respectively.

5.3 Results & Discussion

5.3.1 Characterization of spinach leaves and epicuticle wax film.

In order to differentiate the effects of large-scale roughness associated with leaf topography versus chemistry of the surface epicuticle wax layer on the attachment of cells, experiments were conducted on an immobilized wax film in the absence of bulk roughness. However, to compare the surface of the leaf to the extracted wax layer, Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) were utilized in evaluating surface morphologies and roughness. The spinach leaf exhibited epicuticle waxes with irregular and undefined morphologies (Figure 5.2A). Waxes with a well-defined prism structure, approximately 500 nm, were also consistently observed but irregularly dispersed across the leaf surface (Figure 5.2A inset). The same epicuticle wax morphologies were observed for the immobilized epicuticle waxes on polycarbonate, indicating little or no damage was inflicted on the waxes during the embedding, removal, and immobilization process (Figure 5.2B).

High resolution AFM images (Figure 5.2C) reveal nanoscale roughness, 129.4 ± 26.2 nm, on the surface of the leaf. This roughness is associated with the epicuticle wax morphologies observed via SEM (Figure 5.2A). The measured roughness of the immobilized epicuticle waxes was 23.3 ± 5.4 nm. The immobilized epicuticle wax is less rough than the leaf, due its immobilization on a flat, smooth substrate, which eliminates underlying texture of the leaf itself. Thus, we would expect to observe increased microbial attachment, and increased resistance to detachment and disinfection on a real

leaf surface versus the isolated epicuticle layer (20, 41). On both surfaces, the prismatic structures observed in SEM were measured in AFM to be 482 ± 155 nm high (protruding from surface).

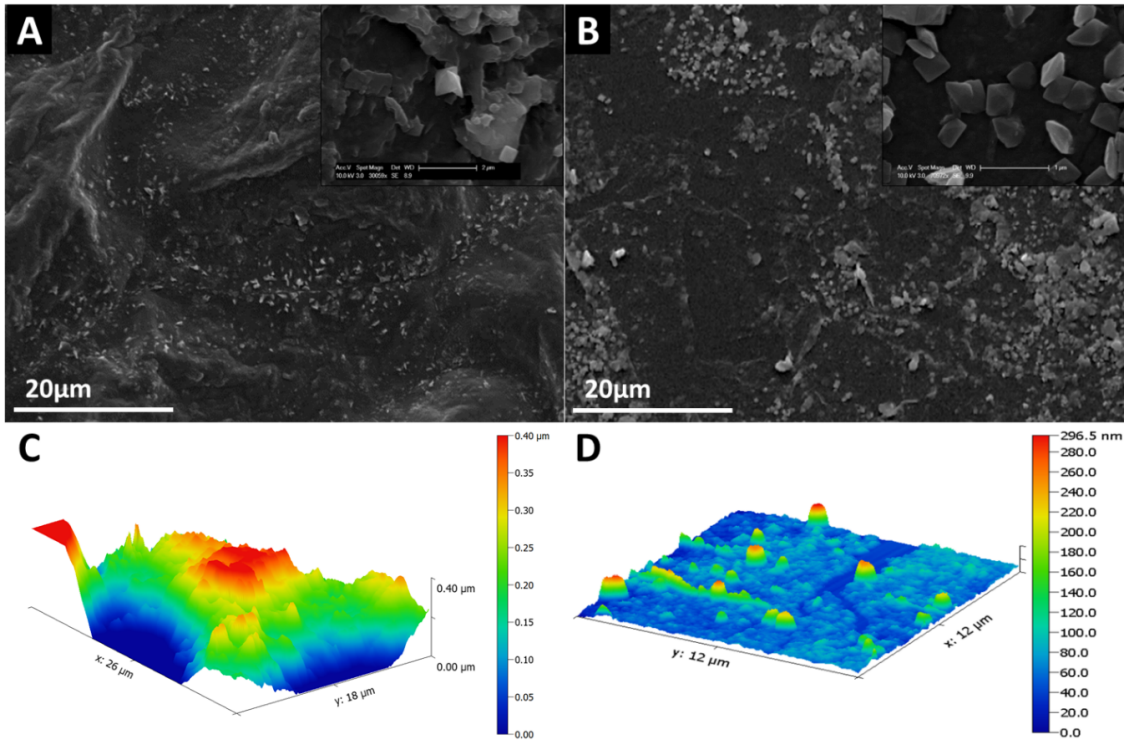

Figure 5.2 SEM and AFM images of leaf surface.

SEM images of dried spinach leaf (A) and immobilized epicuticle layer on polycarbonate (B). Inset scale bar: 1 µm. AFM images of spinach leaf immersed in water (C) and immobilized epicuticle layer on polycarbonate in air (D).

5.3.2 Bacteria characterization.

E. coli O157:H7/pGFP cells exhibited a slight net negative charge (averaging -1.5 ± 2.7 mV) in both KCl and AGW systems and did not change significantly with ionic strength, as determined by zeta potential measurements listed in Table 5.1. Using streaming potential, negative surface charges were measured on leaves (averaging -5.7 ± 2.0 mV) at all ionic strengths and solution chemistries investigated.

Table 5.1 Spinach leaf and *E. coli* O157:H7/pGFP properties.

Salt	Ionic Strength (mM)	Spinach Leaf ζ Potential (mV)	*E. coli* O157:H7/pGFP ζ Potential (mV)	Spinach Leaf Contact Angle[b] (°)	*E. coli* O157:H7 MATH[c] (%)
KCl	1	-8.98 ± 1.27	-2.71 ± 4.11		28.4 ± 3.9
KCl	3.3	ND	-3.95 ± 1.67		26.1 ± 10
KCl	10	-4.78 ± 0.97	-3.84 ± 2.03		34.9 ± 10.8
KCl	100	ND	2.70 ± 6.80	56 ± 10	ND
AGW[a]	3.3	-5.65 ± 1.15	-2.93 ± 2.51		25.3 ± 5.0
AGW[a]	5.7	-5.38 ± 0.72	-1.54 ± 3.71		35.5 ± 8.7
AGW[a]	10	-3.77 ± 0.26	1.81 ± 3.41		40.0 ± 2.6

ND: values not determined
[a] Artificial Ground Water
[b] Spinach leaf contact angle was measured using DI water
[c] Microbial adhesion to hydrocarbons (relative hydrophobicity)

5.3.3 Bacterial adhesion to spinach leaf and epicuticle surface.

The adhesion trends of *E. coli* O157:H7/pGFP on epicuticle layers as a function of ionic strength and source water chemistries (simple KCl solutions and synthetic artificial groundwater (AGW)) are shown in Figure 5.3. Mass transfer coefficients (k_{att})

increased with ionic strength within both the simple KCl suspensions and AGW suspensions from 1 to 100 mM in KCl and 3.3 to 10 mM in AGW. However, there was not a statistical difference between deposition at 10 mM KCl and 100 mM KCl ($p > 0.05$). The attachment rate reaches a maximum at an ionic strength of 10 mM for both the KCl system and the AGW system, and the KCl system plateaus above this level. This agrees with previously reported trends for bacterial adhesion onto mineral and metal surfaces (20, 42). The bacterial adhesion demonstrated minimal sensitivity to ionic strength between $10^{-3} – 10^{-1}$ M as compared to previous studies that observed significant increase in adhesion to quartz with increasing ionic strength in KCl solutions (43).

Despite both the bacteria and the leaf surface being of like charge (negative), the generated DLVO models for these systems indicated favorable attachment. The DLVO profiles generated (see Figure S2, Appendix D) display an energy maximum at 1 mM KCl that is significant enough to retard attractive forces (~4.2 kT). At greater ionic strength, the energy maxima are insufficient (~0.5 kT and below) to inhibit cell interaction with the epicuticle layer, suggesting that favorable interaction conditions exist. Yet, there was not a substantial increase in the observed k_{att} (Figure 5.3), indicating that electrostatic forces are not the dominant mechanism involved in the cell interaction with the spinach surface. These results indicate that electrostatic and van der Waals forces govern interaction between the cells and the epicuticle layer only within a narrow range (1-10 mM KCl and 3.3-10 mM AGW) and, in contrast to several previous studies, water chemistry did not significantly impact the extent of attachment (43, 44).

It was expected that bacterial attachment in a model groundwater system (AGW) would significantly increase in contrast to the ideal KCl systems because of the presence of divalent cations (i.e. Ca^{2+}, Mg^{2+}) (45). Ca^{2+} ions lead to greater electrical double layer compression than monovalent ions due to the larger outer valence shell size and charge screening, which reduces the Debye length (39). However, when evaluating the mass transfer rate coefficients (k_{att}) for cells attaching to the epicuticle layer, this was not observed to be the case.

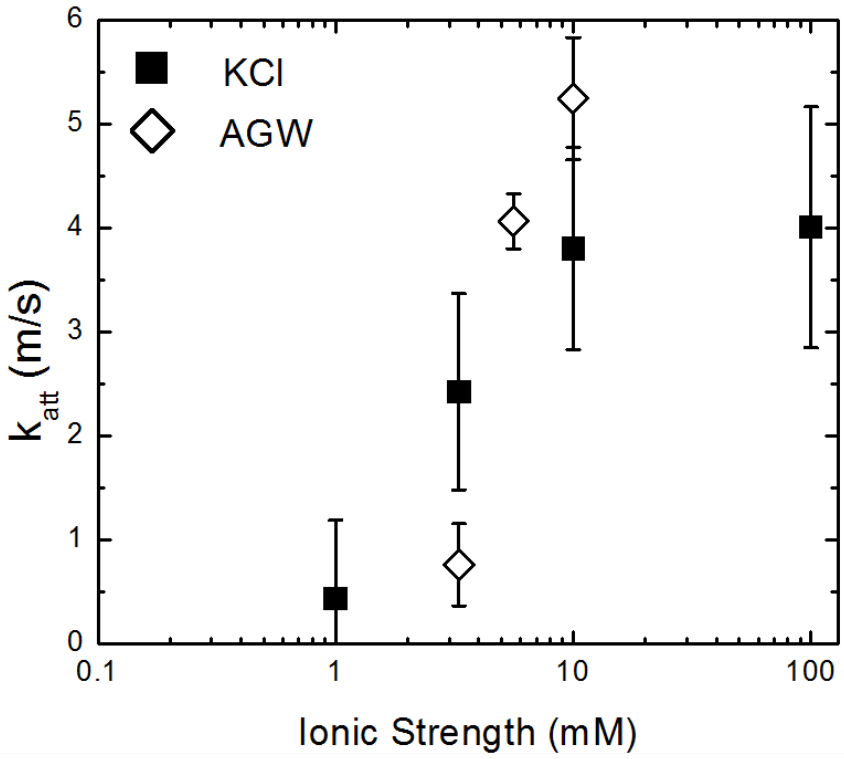

Figure 5.3 Mass transfer rate coefficients (k_{att}) of *E. coli* O157:H7/pGFP attachment.

Bacterial adhesion onto the epicuticle as a function of ionic strength and solution chemistry (KCl and artificial groundwater (AGW)) in the parallel plate flow chamber. Error bars indicate standard deviation.

Cell surface appendages and organelles and their conformations have been observed to play a significant role in bacterial attachment to a variety of surfaces (46, 47). Surface appendages of *E. coli* O157:H7/pGFP are known to selectively adhere to specific surface moieties on hosts' cells for leaf colonization (13, 26, 48). The unanticipated low rate of attachment observed at 3.3 mM AGW (in contrast to 3.3 mM KCl) may be attributed to these surface appendages undergoing conformational changes due to complexation, specifically with calcium (29, 34, 49). This is reflected in the increase in hydrophobicity (related to hydrophobic moieties of the surface appendages) by nearly 15% in the AGW system, whereas only a 7% increase is observed in the KCl system. Although, in AGW systems, the presence of divalent cations (Ca^{+2}, Mg^{+2}) have been shown to competitively absorb to the negative moieties on the epicuticle surface, blocking the specific binding sites of such hydrophobic surface proteins (50) and thus masking any significant difference in attachment between the two systems (AGW vs. KCl). Hydrophobicities of the spinach leaf and *E. coli* cells are presented in Table 5.1 as contact angle and percent relative hydrophobicity values, respectively.

5.3.4 Mechanisms of bacterial detachment from spinach epicuticle surface.

To evaluate the potential for cross-contamination during agricultural rinsing processes, epicuticle surfaces with attached *E. coli* O157:H7/pGFP were subsequently rinsed with DI water. The impact of initial attachment parameters (such as water chemistry and ionic strength) on bacterial detachment during DI water rinsing was investigated.

In previous studies, reversible attachment has been defined as cells attaching for several seconds or swimming, tumbling, or walking along the surface (34, 51). Irreversible attachment was defined as cells attaching and remaining in place. In contrast, we are defining reversibly attached cells as cells that remained attached through the duration of 30-minute attachment segment and background salt rinse (Stages 1 and 2) and subsequently released due to the introduction of a new solution (Stage 3). For all source waters investigated (1-100 mM KCl and 3.3-10 mM AGW), the subsequent DI water rinsing caused some cells to detach from the surface. The mass transfer rate of cells released from the surface was reduced by nearly half when the original attachment solution ionic strength was increased from 3.3 to 10 mM AGW ($k_{det,DI}$ were $12.4 \pm 4.4 \times 10^{-11}$ m/s and $5.7 \pm 2.8 \times 10^{-11}$ m/s, respectively). Attachment in KCl systems (between 3.3 and 10 mM) did not significantly alter initial detachment rate ($p > 0.05$) and $k_{det,DI}$ for initial detachment averaged $11.2 \pm 6.7 \times 10^{-11}$ m/s. However, increasing ionic strength to 100 mM KCl reduced the initial detachment to $6.9 \pm 2.5 \times 10^{-11}$ m/s ($p < 0.05$). There was only a slight difference in the zeta potential of the bacteria in DI water (-7.0 ± 0.5 mV), as compared to KCl and AGW (Table 5.1). Nor was the zeta potential of the epicuticle layer impacted substantially. Hence, DLVO profiles (Figure S2, Appendix D) suggest that this change in solution chemistry did not contribute sufficiently to the interaction forces and electrostatic repulsion was not the mechanism for detachment.

Detachment mass transfer coefficients (k_{det}) were nearly the same between KCl and AGW. However, detachment (Stage 3) lasted 18 ± 4 minutes and 4 ± 2 minutes for cells initially deposited in KCl and AGW, respectively. Increasing ionic strength had

little to no impact on duration of this stage, indicating that the ions present during attachment (such as Ca^{+2}) played a significant role in the cells' ability to remain attached to the surface when the solution ionic strength was reduced. The overall reduced k_{det} and time to plateau in the AGW system compared to the simple KCl system is attributed to the presence of divalent ions (Ca^{+2}, Mg^{+2}) in solution, and the resulting electrostatic interactions, wherein the compression of the electric double layer and reduced Debye length thus reduce the amount of interfacial (52). Also, the bond between bacterium and the substrate has been observed to strengthen over time due to further removal of interfacial water, unfolding of surface structures, or rotation to relax into most favorable site (not including metabolic processes of microorganisms and biofilm formation) (52, 53).

Additionally, water chemistry has been shown to impact the conformation of surface polymers and appendages (29, 54). Divalent ions, such as Ca^{+2}, have been observed to form calcium bridges and form complexations with organics in various systems (26, 47, 51, 55, 56). The removal of the calcium from the system during the rinse stage did not rapidly induce detachment from the surface, indicating that attachment in complex waters were more persistent than in the simple KCl system. This suggests that divalent ions involved in the attachment mechanisms (either through surface appendage conformation changes, complexation with the epicuticle waxes, compression of the electrical double layer, or a combination) are not easily diffused from the surface structure-epicuticle matrix. This implies that microbial contamination within waters with high concentrations of divalent ions should be expected to be more difficult to remove

(i.e. groundwater) and additional processes may be needed to ensure effective microbial removal and disinfection. In contrast, waters with lower divalent ion concentrations (i.e. surface water) could have a greater likelihood of microbial cross-contamination to produce and other surfaces, potentially increasing the scale of foodborne illness outbreaks.

5.3.5 Impacts of bleach on bacterial detachment phenomena.

Relevant bleach concentrations were selected to evaluate potential detachment and disinfection of attached bacteria. As a reminder, these detachment and disinfection experiments were conducted only for cells first deposited at 10 mM KCl in Stage 1. By increasing the bleach concentration within the rinse solution (Stage 3) by 2 orders of magnitude, the $k_{det,bleach}$ increased 20 times ($5.7 \pm 0.7 \times 10^{-11}$ m/s to $112.1 \pm 26.8 \times 10^{-11}$ m/s from 10 ppb to 1000 ppb, respectively), as shown in Figure 5.4B. Increasing bleach concentration resulted in reduced detachment duration from 16 ± 3 minutes at 100 ppb to 3 ± 1 minute at 1000 ppb.

At this point, all observable (fluorescing) attached cells are assumed to be alive due to the continued production of GFP. To rule out the possibility that fluorescence fading impacted the quantitative analysis of the cell attachment and release, control experiments were conducted by rinsing with the attachment background salt solutions for extend time periods (>60 minutes post attachment), under which fading was not observed. Further details on these control experiments are including in the Appendix D. Although diminishing fluorescence was not a direct indicator of cell death, it clearly

indicated oxidation stress within the cell via direct GFP oxidation or internal cellular damage. Although GFP fading may be indicative of cell changes over time, absolute tests of cell viability using live/dead stain at the end of Stage 4 showed that fewer live cells remained following rinsing with increasing bleach concentration (Figure 5.4C). Both aqueous species of bleach (sodium hypochlorite), hypochlorous acid (HOCl) and hypochlorite ion ($^{-}$OCl) are strong oxidizers that react with a wide variety of biological molecules and may lead to detachment, disinfection, or a combination. At the concentrations investigated, the pH ranged between 5 and 7 where HOCl is the predominant species (57). Since HOCl readily diffuses across lipid bilayers, it likely caused either direct oxidation of GFP or internal cellular oxidation thus halting the production of GFP, which was observed as fading. HOCl is a more effective biocide than $^{-}$OCl since it causes cellular damage through many internal oxidations. In contrast, $^{-}$OCl is limited to external oxidation of the cell membrane surface, since the charged molecule is not able to penetrate the lipid bilayer (57). Increasing detachment with increased bleach concentration is attributed to this oxidation and possible cleavage of surface proteins that were adhered to the epicuticle.

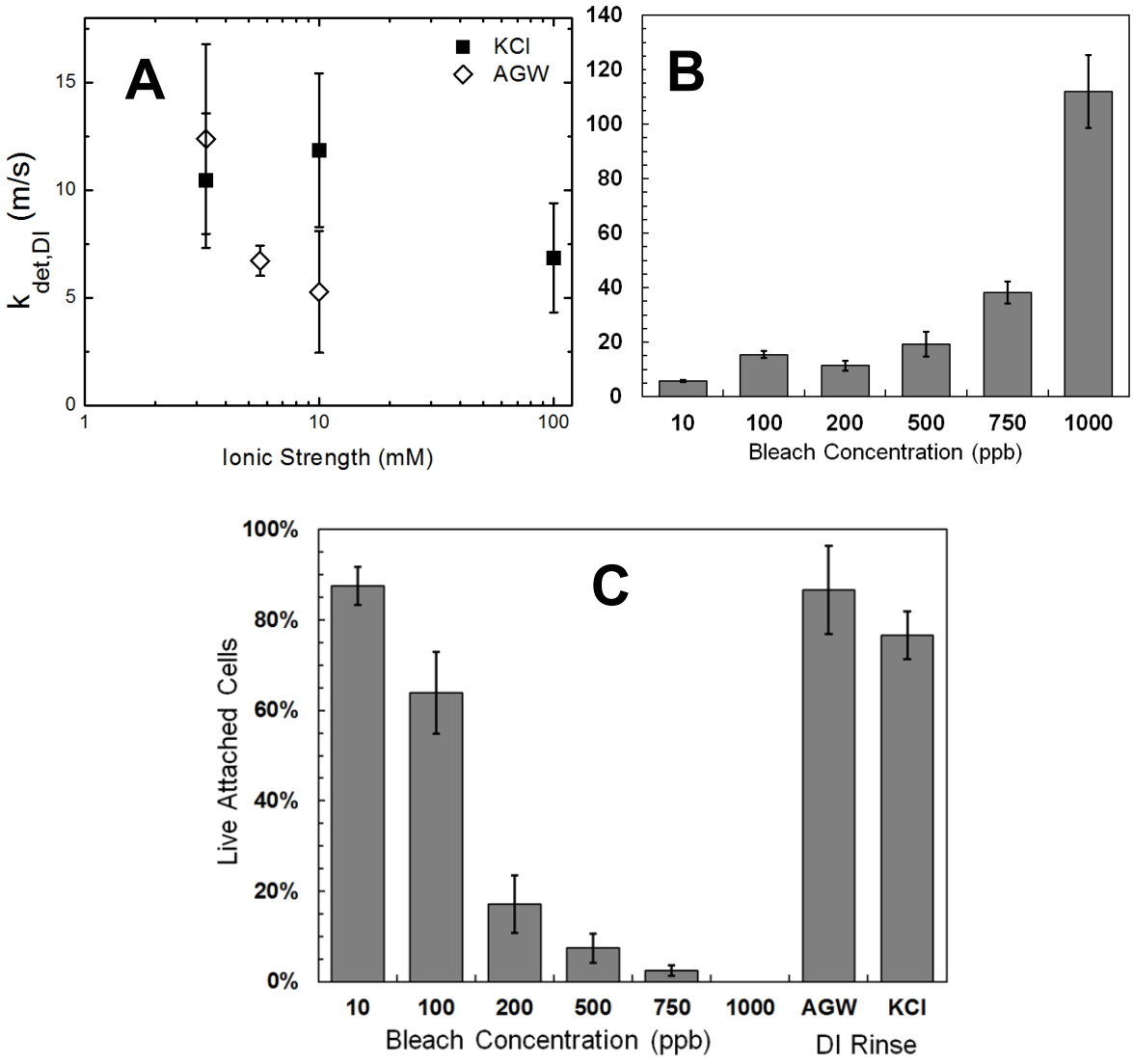

Figure 5.4 Mass transfer coefficients (k_{det}) for *E. coli* O157:H7/pGFP detachment as a function of ionic strength and bleach concentration.

Mass transfer coefficients (k_{det}) for *E. coli* O157:H7/pGFP detachment from immobilized epicuticle on polycarbonate due to rinsing with A) DI water as a function of deposition ionic strength and source water (KCl and AGW) and B) bleach as a function of concentration. C) Percent of live attached cells at the end of Stage 3, as a function of bleach concentration and compared with plain KCl and AGW rinses. Rinsing experiments were conducted for 30 min following the rinse with the background salt solution. In (C), all bleach rinsing experiments were conducted on bacteria that were attached to epicuticle in 10 mM KCl. Additionally, the KCl and AGW column labels represent the attachment conditions. The results of bacteria attached in KCl and AGW and rinsed with DI are shown for comparison to bleach rinsing.

5.3.6 COMSOL model of bleach concentrations.

Preliminary disinfection experiments were designed and conducted to identify how the chlorine rinse impacts the attached cells – whether it is a physical-chemical removal/release, cell death, or other transformation. These experiments were conducted at typical industry bleach concentrations (>10 ppm); however, under these conditions attached bacteria instantaneously detached from the epicuticle surface (data not shown). Under ideal conditions, such concentrations are adequate to remove surface contamination.

It is documented that *E. coli* O157:H7/pGFP seek stomas and damaged sections of the leaf to survive and infect hosts upon consumption (25, 26, 58). Therefore, the nature of the surface (topography and roughness) of spinach leaves were characterized and incorporated into a COMSOL model to evaluate the disinfectant concentration across the leaf surface. The spinach leaf topography was imaged and quantified using atomic force microscopy (AFM). The AFM image reveals rolling peaks and valleys across the surface of the leaf (Figure S3, Appendix D) with amplitudes exceeding 11 μm and exceeding the measurable range of the AFM (exceeded range of AFM cropped from image). The overall roughness of spinach leaves was 2.27 ± 0.65 μm; analyzed using the overall mean squared roughness, based on four areas that measured 50 μm by 50 μm. Surface roughness is a critical parameter that has been observed to increase bacterial attachment, specifically on the micron scale (20, 41).

A simplistic 2-dimensional representation of the leaf surface was designed based on AFM observations. The computational domain used was a 2D cross-section section

incorporating topography and surface features (x) and the water column above the surface of the leaf (y). The 2D model provides valuable insight into the effectiveness of the disinfection rinsing process and the potential for bacterial survival. The rolling peaks and valleys were represented by a repeating wave with amplitude of 10 μm, representative peak height (valley to peak) measured via AFM (several regions exceeded measureable range), and a wavelength of approximately 100 μm to simulate the topography of the leaf. A representation of a stoma was also incorporated within the model since *E. coli* O157:H7/pGFP are known to preferentially accumulate within the stoma and potentially internalize within the leaf (2, 25, 26). A representative image of the model (evaluated at 50 ppm NaOCl, 60 seconds) is shown in Figure 5.5. Concentrations were evaluated over 60 seconds; a typical industry rinse time (38).

The COMSOL model revealed that nearly 15% of the spinach leaf surface may be exposed to significantly (nearly three orders of magnitude) reduced bleach concentrations during disinfection rinses. The model was evaluated at concentrations ranging from 4 ppm (maximum bleach concentration allowable in drinking water (59) and for organic produce rinses (60)) to 200 ppm (maximum bleach concentration typically used in fresh produce processing (59)). These significantly reduced concentrations of bleach occurred in crevices (valleys) and stomas on the leaf surface. The inset of Figure 5.5 charts the minimum bleach concentration observed at each of the bleach rinse concentrations modeled. Due to the short timeframe of typical rinsing processes (1-2 minutes), steady state is probably not achieved, and therefore the actual bleach concentration across the leaf surface will be even less than that predicted by the model. This model is based on

ideal laminar flow, across a flat leaf with micron scale roughness (based on AFM characterization in Appendix D, Figure S3). Under realistic produce rinsing conditions, the leaves are not entirely flat or immobilized (as the leaves were for AFM analysis). This may increase the number and magnitude of peaks and valleys, which were observed in the model to significantly reduce the local surface bleach concentration.

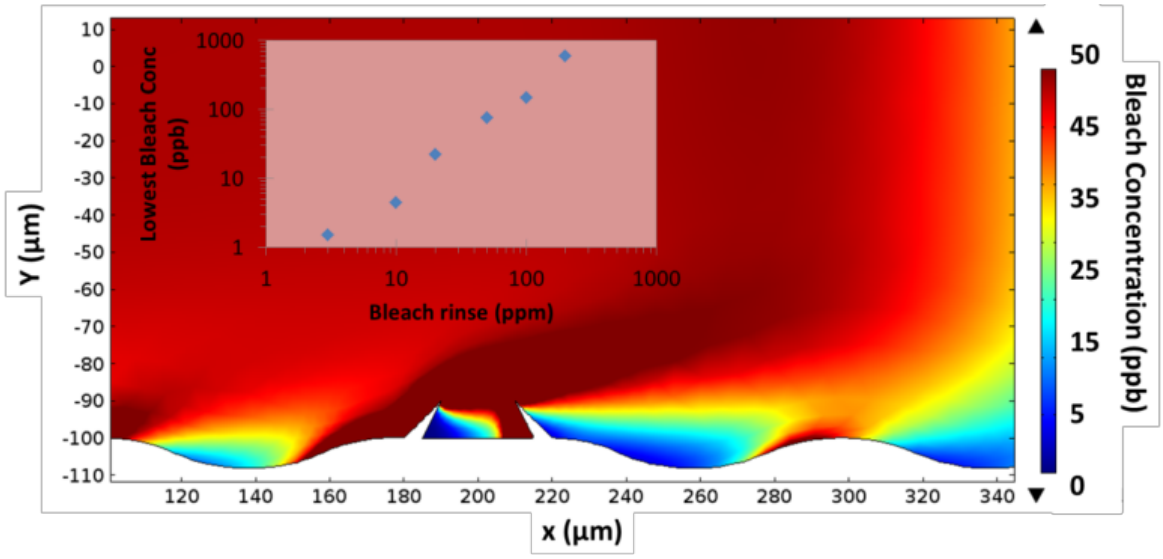

Figure 5.5 Simple 2D COMSOL model of spinach leaf surface under relevant flow conditions during produce rinsing processes.

X: cross section of leaf in flow direction, Y: water column above leaf surface. Evaluated at 60 s and 50 ppm bleach, illustrating the concentration gradient along leaf due to leaf micro-features (scale suppressed to highlight low concentration regions). Inset: Lowest bleach concentration measured during the model at the respective bulk bleach concentrations after 60 s.

In all of the previously discussed DI water rinsing experiments (with bacteria attached in both KCl and AGW), bacteria were qualitatively observed close to the epicuticle surface that did not become irreversibly attached to the epicuticle surface (bacteria cells that were in focus at the surface level, but continued to move). Since *E. coli* O157:H7/pGFP can swim in a series of (relatively) straight runs or tumbles (34), increased attachment to a real spinach leaf would be expected due to the rougher surface, in contrast to the smoother, immobilized epicuticle layer. In the COMSOL model, the most significant reduction in bleach concentration was observed within the stoma. *E. coli* O157:H7/pGFP are known to infiltrate leaves through the stoma, introducing additional disinfection challenges (however, this was not a focus of the current study).

Nonetheless, the valley regions are also shown to have significantly reduced chlorine concentration below the bulk fluid concentration, as shown in Figure 5.5. Since disinfection is a function of concentration and exposure time, these areas of notably reduced bleach concentration represent regions of significantly reduced disinfection efficacy. While the observed increase in detachment rate and reduction in number of live cells remaining with increasing bleach concentration is not unexpected, the experimental results clearly indicate that the reduced concentrations observed in crevices, valleys, and stomas of spinach leaves were inadequate to insure remove and disinfection of microbial contamination.

5.4 Conclusions

This study utilized a microfluidic flow cell to observe and assess pathogen attachment and detachment mechanisms, and the efficacy of standard bleach disinfection on bacterial death and removal from the produce surface. While microbial attachment and detachment mass transfer rate coefficients did not vary significantly between simple and complex waters chemistries (KCl and AGW, respectively), the reduced period of observed detachment in AGW rinses shows that the presence of divalent ions during attachment can make detachment more difficult. Our results demonstrate the importance of preventing this initial microbial contamination, since it required almost four times more concentrated bleach to achieve the same level of disinfection of attached cells versus planktonic cells. Additionally, since bleach disinfection is dependent on both bleach concentration and exposure time, these results indicate that disinfection of attached cells may require additional rinsing time when compared to the typical rinsing times of 1-2 minutes (38). Most detachment from the epicuticle was observed prior to GFP fading, indicating that cells are still intact and may survive to contaminate other leaves and surfaces. Those cells that did remain attached were observed to withstand and survive these disinfection rinses, in contrast to planktonic cells, of which nearly 90% were dead within 1 minute of exposure at 200 ppb (data not shown). Considering this in combination with surface modeling results that show significantly reduced bleach concentration across 15% of the leaf surface, it seems likely that bleach rinses cause detachment, but do not kill cells. This may be cause for significant concern for public health, since many human pathogens (such as *E. coli* O157:H7) have been shown to

require as few as ten cells to cause food borne illness (61). Further research is needed to ensure that the very rinsing and disinfection processes performed to clean, remove, and prevent contamination are not pathways for cross-contamination that could amplify foodborne illness outbreaks and public health risks.

5.5 References

1. Cuthbert JA. 2001. Hepatitis A: Old and New. Clinical Microbiology Reviews, 14(1):38-58.
2. Olaimat AN and Holley RA. 2012. Factors influencing the microbial safety of fresh produce: a review. Food Microbiology, 32(1):1-19.
3. Centers for Disease Control and Prevention (CDC). 2014. CDC and Food Safety. Atlanta, GA, USA. http://www.cdc.gov/foodsafety/cdc-and-food-safety.html (Accessed March 6, 2016).
4. Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. Journal of Food Protection, 75(1):123-131.
5. Food and Drug Administration (FDA). 2008. Guidance for industry: Guide to minimize microbial food safety hazards for fresh-cut fruits and vegetables. Silver Spring, MD, USA.
6. Cooley M, Carychao D, Crawford-Miksza L, Jay MT, Myers C, Rose C, Keys C, Farrar J, Mandrell RE. 2007. Incidence and tracking of *Escherichia coli* O157: H7 in a major produce production region in California. PLoS One, 2(11):e1159.
7. Stuart D. 2008. The illusion of control: Industrialized agriculture, nature, and food safety. Agriculture and Human Values, 25(2):177-181.
8. Fujisawa T, Shin S, Aikawa K, Takahashi T, Yamai S, Shimada T. 2001. Evaluation of sorbitol-salicin MacConkey medium containing cefixime and tellurite (CT-SSMAC medium) for isolation of *Escherichia coli* O157:H7 from raw vegetables. International Journal of Food Microbiology, 74(1-2):161-163.

9. LeBlanc JJ. 2003. Implication of virulence factors in *Escherichia coli* O157:H7 pathogenesis. Critical Reviews in Microbiology, 29(4):277-296.

10. Cálix-Lara TF, Rajendran M, Talcott S., Smith SB, Miller RK, Castillo A, Sturino JM, Taylor TM. 2014. Inhibition of *Escherichia coli* O157: H7 and *Salmonella* enterica on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. Food Microbiology, 38:192-200.

11. Escalona V, Aguayo E, Martinez-Hernandez GB, Artes F. 2010. UV-C doses to reduce pathogen and spoilage bacterial growth in vitro and in baby spinach. Postharvest Biology and Technology, 56(3):223-231.

12. Han Y, Sherman DM, Linton RH, Nielson SS, Nelson PE. 2000. The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157: H7 to green pepper surfaces. Food Microbiology, 17(5):521-533.

13. Macarisin D, Patel J, Bauchan G, Giron JA, Sharma VK.,2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157: H7 to spinach leaves. Foodborne Pathogens and Disease, 9(2):160-167.

14. Markland S, Shortlidge KL, Hoover DG, Yaron S, Patel J, Singh A, Sharma M, Kniel KE. 2013. Survival of pathogenic *Escherichia coli* on basil, lettuce, and spinach. Zoonoses and Public Health, 60(8):563-571.

15. O'Beirne D, Gleeson E, Auty M, Jordan K. 2014. Effects of processing and storage variables on penetration and survival of *Escherichia coli* O157: H7 in fresh-cut packaged carrots. Food Control, 40:71-77.

16. Singh N, Singh R, Bhunia AK, Stroshine RL. 2002. Effect of inoculation and washing methods on the efficacy of different sanitizers against *Escherichia coli* O157: H7 on lettuce. Food Microbiology, 19(2):183-193.

17. Zhang G, Ma L, Phelan VH, Doyle MP. 2009. Efficacy of antimicrobial agents in lettuce leaf processing water for control of *Escherichia coli* O157:H7. Journal of Food Protection, 72(7):1392-1397.

18. Busscher HJ, Norde W, Sharma PK, van der Mei HC. 2010. Interfacial re-arrangement in initial microbial adhesion to surfaces. Current Opinion in Colloid & Interface Science, 15(6):510-517.

19. Cai P, Huang, Q, Walker SL. 2013. Deposition and survival of *Escherichia coli* O157: H7 on clay minerals in a parallel plate flow system. Environmental Science & Technology, 47(4):1896-1903.

20. Chen G, Beving DE, Bedi RS, Yan YS, Walker SL. 2009. Initial bacterial deposition on bare and zeolite-coated aluminum alloy and stainless steel. Langmuir, 25(3): 1620-1626.

21. Chowdhury I and Walker SL. 2012. Deposition mechanisms of TiO_2 nanoparticles in a parallel plate system. Journal of Colloid and Interface Science, 369(1):16-22.

22. Kline TR, Chen G, Walker SL. 2008. Colloidal deposition on remotely controlled charged micropatterned surfaces in a parallel-plate flow chamber. Langmuir, 24(17):9381-9385.

23. Orgad O, Oren Y, Walker SL, Herzberg M. 2011. The role of alginate in Pseudomonas aeruginosa EPS adherence, viscoelastic properties and cell attachment. Biofouling, 27(7):787-798.

24. Vanoyan N, Walker SL, Gillor O, Herzberg M. 2010. Reduced bacterial deposition and attachment by quorum-sensing inhibitor 4-Nitro-pyridine-N-oxide: The role of physicochemical effects. Langmuir, 26(14):12089-12094.

25. Niemira BA and Cooke PH. 2010. *Escherichia coli* O157:H7 biofilm formation on romaine lettuce and spinach leaf surfaces reduces efficacy of irradiation and sodium hypochlorite washes. Journal of Food Science, 75(5):M270-M277.

26. Saldana Z, Sanchez E, Xicohtencatl-Cortes J, Puente JL, Giron JA. 2011. Surface structures involved in plant stomata and leaf colonization by shiga-toxigenic *Escherichia coli* O157:H7. Front Microbiology, 2:119.

27. Haznedaroglu BZ, Kim HN, Bradford SA, Walker SL. 2009. Relative transport behavior of *Escherichia coli* O157:H7 and *Salmonella* enterica serovar pullorum in packed bed column systems: Influence of solution chemistry and cell concentration. Environmental Science & Technology, 43(6):1838-1844.

28. Marcus IM, Bolster CH, Cook KL, Opot SR, Walker SL. 2012. Impact of growth conditions on transport behavior of *E. coli*. Journal of Environmental Monitoring, 14(3):984-991.

29. Kim HN, Hong Y, Lee I, Bradford SA, Walker SL. 2009. Surface characteristics and adhesion behavior of *Escherichia coli* O157:H7: Role of extracellular macromolecules. Biomacromolecules, 10(9): 556-2564.

30. Kim HN, Walker SL, Bradford SA. 2010. Macromolecule mediated transport and retention of *Escherichia coli* O157:H7 in saturated porous media. Water Research, 44(4):1082-1093.

31. Ensikat HJ, Neinhuis C, Barthlott W. 2000. Direct access to plant epicuticular wax crystals by a new mechanical isolation method. International Journal Plant Science, 161(1):143-148.

32. Jetter R, Schäffer S, Riederer M. 2000. Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from Prunus laurocerasus L. Plant, Cell & Environment, 23(6):619-628.

33. Walker SL, Bhattacharjee S, Hoek EMV, Elimelech M. 2002. A novel asymmetric clamping cell for measuring streaming potential of flat surfaces. Langmuir, 18(6):2193-2198.

34. McClaine JW and Ford RM. 2002. Reversal of flagellar rotation is important in initial attachment of *Escherichia coli* to glass in a dynamic system with high- and low-ionic-strength buffers. Applied Environmental Microbiology, 68(3):1280-1289.

35. Patton JT, Mentor DG, Benson DM, Nicolson GL, McIntire LV. 1993. Computerized analysis of tumor cells flowing in a parallel plate chamber to determine their adhesion stabilization lag time. Cell Motility and the Cytoskeleton, 26(1):88-98.

36. van Kooten TG, Schakenraad JM, van der Mei HC, Busscher HJ. 1992. Development and use of a parallel-plate flow chamber for studying cellular adhesion to solid surfaces. Journal of Biomed Materials Research, 26(6):725-738.

37. Bolster CH, Mills AL, Hornberger GM, Herman JS. 1999. Spatial distribution of deposited bacteria following miscible displacement experiments in intact cores. Water Resources Research, 35:1797-1807.

38. Food and Drug Administration (FDA). 2013. Methods to reduce/eliminate pathogens from produce and fresh-cut produce. Safe Practices for Food Processes. Silver Spring, MD, USA.

39. Elimelech M, Jia X, Gregory J, Williams R. 1998. Particle Deposition & Aggregation: Measurement, Modelling and Simulation. Elsevier Science.

40. Derjaguin BV and Landau L. 1941. Theory of stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solution of electrolytes. Acta Physicochim U.S.S.R., 14:633-62.

41. Tang H, Cao T, Liang X, Wang A, Salley SO, McAllister J, Ng KY. 2009. Influence of silicone surface roughness and hydrophobicity on adhesion and colonization of Staphylococcus epidermidis. Journal of Biomedical Materials Research, Part A, 88(2):454-463.

42. Chen G, Bedi RS, Yan SY, Walker SL. 2010. Initial colloid deposition on bare and zeolite-coated stainless steel and aluminum: Influence of surface roughness. Langmuir, 26(15):12605-12613.

43. Walker SL, Hill JE, Redman JA, Elimelech M. 2005. Influence of growth phase on adhesion kinetics of *Escherichia coli* D21g. Applied and Environmental Microbiology, 71(6):3093-3099.

44. Rijnaarts HHM, Norde W, Lyklema J, Zehnder AJB. 1999. DLVO and steric contributions to bacterial deposition in media of different ionic strengths. Colloids and Surfaces B: Biointerfaces, 14(1–4):179-195.

45. Benjamin MM. 2002. Water Chemistry, McGraw-Hill Higher Education.

46. Walker SL, Redman JA, Elimelech M. 2004. Role of cell surface lipopolysaccharides in *Escherichia coli* K12 adhesion and transport. Langmuir, 20(18):7736-7746.

47. Rossez Y, Holmes A, Wolfson EB, Gally DL, Mahajan A, Pedersen HL, Willats WG, Toth IK, Holden NJ. 2014. Flagella interact with ionic plant lipids to mediate adherence of pathogenic *Escherichia coli* to fresh produce plants. Environmental Microbiology, 16(7):2181-2195.

48. Collinson SK, Emödy L, Muller KH, Trust TJ, Kay WW. 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella* enteritidis. Journal of Bacteriology, 173(15):4773-4781.

49. Chen GX and Walker SL. 2012. Fecal Indicator Bacteria Transport and Deposition in Saturated and Unsaturated Porous Media. Environmental Science & Technology, 46(16):8782-8790.

50. Khemakhem W, Ammar E, Bakrouf A. 2005. Effect of environmental conditions on hydrophobicity of marine bacteria adapted to textile effluent treatment. World Journal of Microbiology and Biotechnology, 21:1623-1631.

51. Vigeant MAS, Ford RM, Wagner M, Tamm LK. 2002. Reversible and irreversible adhesion of motile *Escherichia coli* cells analyzed by total internal reflection

aqueous fluorescence microscopy. Applied and Environmental Microbiology, 68(6):2794-2801.

52. Xu LC, Vadillo-Rodriguez V, Logan BE. 2005. Residence time, loading force, pH, and ionic strength affect adhesion forces between colloids and biopolymer-coated surfaces. Langmuir, 21(16):7491-7500.

53. Vadillo-Rodríguez V, Busscher HJ, Norde W, de Vries J, van der Mei HC. 2004. Atomic force microscopic corroboration of bond aging for adhesion of Streptococcus thermophilus to solid substrata. Journal of Colloid and Interface Science, 278(1):251-254.

54. Chen G and Walker SL. 2007. Role of solution chemistry and ion valence on the adhesion kinetics of groundwater and marine bacteria. Langmuir, 23(13):7162-7169.

55. Chen KL and Elimelech M. 2007. Influence of humic acid on the aggregation kinetics of fullerene (C60) nanoparticles in monovalent and divalent electrolyte solutions. Journal of Colloid and Interface Science, 309(1):126-134.

56. Chowdhury I, Cwiertny DM, Walker SL. 2012. Combined factors influencing the aggregation and deposition of nano-TiO2 in the presence of humic acid and bacteria. Environmental Science & Technology, 46(13):6968-6976.

57. Fukuzaki S. 2006. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Science, 11(4):147-157.

58. Han SD, Yang H, Wang L, Kim JW. 2000. Preparation and properties of vanadium-doped SnO_2 nanocrystallites. Sensors and Actuators B-Chemical, 66(1-3):112-115.

59. U.S. Environmental Protection Agency (EPA). 1975. National Primary Drinking Water Regulations: Maximum Residual Disinfectant Levels. Title 40. Code of Federal Regulations. 141.65.

60. U.S. Department of Agriculture (USDA). 1990. Organic Foods Production Act. Title 21. Code of Federal Regulations.

61. Harris LJ, Farber JN, Beuchat LR, Parish ME, Suslow TV, Garrett EH, Busta FF. 2003. Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. Comprehensive Reviews in Food Science and Food Safety, 2:78-141.

Chapter 6

Disrupting Irreversible Bacterial Adhesion and Biofilm Formation with an Engineered Enzyme

Mayton, H.M., Berger, B.W., Walker, S.L. Disrupting irreversible bacterial adhesion and biofilm formation with an engineered enzyme. In preparation (2019).

Abstract

Biofilm formation is one of the main causes of post-harvest pathogenic bacteria persistence on leafy green surfaces. These pathogens may lead to foodborne illnesses due to enhanced microbial resistance to common sanitizers, such as bleach. In this study, an enzyme-based disinfectant was developed and validated as a promising alternative to common disinfection practices for preventing bacterial adhesion and removing mature biofilms. Biofilm assays in 24-well polystyrene plates revealed that 100 ppm of enzyme inhibited up to 40% of biofilm formation by *E. coli* O157:H7, *E. coli* 25922, and *Salmonella* Typhimurium. Further, the enzyme was effective at removing mature *Salmonella* biofilms; providing a 90% improvement over rinsing with plain 10 mM KCl. A parallel-plate flow cell was also used to directly observe and quantify the impact of 250 and 1000 ppb enzyme rinses on *E. coli* O157:H7 cells adhered to spinach leaf surfaces. The presence of 1000 ppb enzyme resulted in nearly 6 times greater detachment rate coefficients than a DI water rinse. The total cells removed from the surface with 250 and 1000 ppb of enzyme increased from 15% to 25% over the 30 minute rinse, respectively, representing a reversal in the initial phases of biofilm formation. The mechanisms of enzyme action are characterized herein by measurable reductions in cell surface hydrophobicity and observed degradation of extracellular polymers with electron microscopy. These results present a strong case for further development and optimization of enzyme activity to be applied as a novel alternative to current antimicrobials to minimize pathogenic food safety risks.

6.1 Introduction

Food safety is a growing global challenge in which pathogens are estimated to cause 600 million illnesses and 420,000 deaths annually (1). Recent high-profile foodborne illness outbreaks associated with leafy greens have raised public awareness about the serious health risks of improper food handling, processing, and packaging, as well as driven increasing demands for safe and effective solutions to prevent future outbreaks (2-4). Biofilm formation on produce is considered a major causes of post-harvest pathogen persistence that leads to foodborne illnesses, as well as spoilage organism persistence that leads to product loss (5, 6). Biofilm is a secreted matrix, made up largely of polysaccharides, nucleic acids and proteins, which encapsulates bacteria cells and protects them from chemical and mechanical disruption, as well as enables adhesion to food, equipment, and packaging surfaces (7-9). Biofilms have been shown to protect cells from chlorine, the most commonly employed disinfectant in the produce industry (10, 11).

Increasingly, the fresh produce industry is pursing alternatives to bleach and other antimicrobials as bacteria have demonstrated to capacity to resist (12). Currently used chemical sanitizers, including bleach, hydrogen peroxide, and peracetic acid, are also restricted in their use due to environmental and public health concerns, as well as customer preferences for organic, minimally-processed materials (13, 14). Although a diversity of alternatives to bleach have been proposed and developed, there are still significant limitations in terms of their efficacy against biofilms. For example, ultraviolet (UV) irradiation is an effective method for eliminating bacteria on produce surfaces

during packaging, but still does not kill bacteria embedded in protective biofilms (15). Additionally, UV radiation and some organic chemical treatments can significantly affect food texture, taste, and appearance, presenting a challenge to consumer acceptance (16, 17). Natural antimicrobials, such as essential oils, and biocontrol agents, such as bacteriophages and lactic acid bacteria, are novel techniques for sterilization that can preserve food texture, flavor, and appearance, but are demonstratively less effective at removing biofilm-embedded pathogens and long-term bacterial reduction (18).

Recently, enzymes have gained attention as alternatives to chemical disinfectants due to their ability to directly degrade components present in microbial biofilms, act specifically on biofilm without modifying food properties, and function under ambient conditions in water without a need for high temperatures, pressures, or chemical sanitizers (19, 20). Dispersin B is one such example; it is a glycosyl hydrolase that degrades poly-N-acetylglucoseamine (PNAG), which is a key polysaccharide found in biofilms formed by the oral pathogen *Aggregatibacter actinomycetemcomitans* (21). Other examples include alginate lyase AlgL from *Pseudomonas aeruginosa* and human DNAse I, both of which have been shown to be effective at removing *P. aeruginosa* biofilms from cystic fibrosis patients (22, 23). This methodology was thus used to design an enzymatic disinfectant to prevent and remove microbial biofilm and surface polysaccharides. A candidate enzyme, referred to as "CAase", which has *E. coli* biofilm-degrading activity and stability to improve performance was developed using a homology-based search based on glycosyl hydrolases with activity against bacterial biofilm. The results presented herein indicate the enzymatic effectiveness at disrupting

mature biofilm formation and production, as well as initial bacterial attachment in a microfluidic model of rinsing produce surfaces.

6.2 Materials and Methods

6.2.1 Enzyme expression

The expression plasmids and methods for enzyme overexpression utilized in this work have been previously described in detail (24). In brief, the CAase gene was subcloned into a pET28a plasmid and transformed into *E. coli* BL21 cells by electroporation. Kanamycin-selective plates (50 μg/mL working concentration) were used to isolate individual colonies; these colonies were then inoculated in 10 mL Luria Bertani (LB) cultures containing kanamycin, and grown overnight at 37 °C in a shaking incubator (Innova R26 at 200 rpm). Cells from saturated cultures were then transferred to 100 mL of fresh LB media containing kanamycin and grown at 37 °C with shaking at 200 rpm for 1 hr, such that the cell density measured at 600 nm reached 0.6. To induce protein production, IPTG was added to the 100 mL culture at a working concentration of 1 mM, and the growth temperature changed to 20 °C. After 16 hours of growth at 20 °C with 200 rpm agitation, cells were harvested by centrifugation at 3000 rpm for 10 min.

6.2.2 Enzyme purification

Cell pellets from 100 mL of induced culture were resuspended in 40 mL of lysis buffer (100 mm HEPES, 500 mm NaCl, 10% w/v glycerol, 10 mm imidazole) and then sonicated (Misonix 3000 Ultrasonic Cell Disruptor, 15 W, 20 min process time, 20 s

on/20 s off pulses) in order to lyse the cells while in an ice bath. The 40 mL lysis mixture was centrifuged at 10000 rpm for 10 min and the soluble supernatant containing enzyme was collected. Centrifugation and disposal of insoluble material was repeated three times.

The enzyme was purified using immobilized metal ion affinity chromatography (IMAC) with 15 mL of Profinity resin, as previously described (25). In summary, one column volume of 0.2 M nickel chloride solution was added to charge the column, followed by three column volumes of deionized water and one column volume of lysis buffer. The cell lysate was then added to the column, allowed to mix gently for 10 min, and then washed with increasing concentrations (10-500 mM) of imidazole, primarily using imidazole concentrations of 250 mM and 500 mM to elute the protein. Eluent was collected in 5 mL fractions and SDS-PAGE was used to confirm purification and purity of final enzyme product. Protein samples (20 µL) were mixed with 5 µL of SDS-PAGE running buffer and heated for 10 min at 90°C to denature proteins before loading 15 µL aliquots onto a 4 % stacking, 12 % separating acrylamide gel with MES running buffer. Precision Plus Protein All Blue Standard (Bio-Rad) was used as a molecular weight standard. The gel was run at 100 V for 15 min and then at 175 V for 40 min. The gel was then stained with Coomassie Blue stain (1 g Coomassie Brilliant Blue (Bio-Rad), 1:4:5 acetic acid, methanol, double-distilled water) for 2 h and then destained with a solution of 1:2:7 acetic acid, methanol and double-distilled water. Collected column fractions that contained purified protein were dialyzed for 24 h at 4°C with a 7000 MWC ThermoFisher Snakeskin dialysis membrane in 4 L of 75 mM pH 8 phosphate buffer, and lyophilized for long-term storage or used immediately. Enzyme concentration was

determined by measuring absorbance at 280 nm, using a calculated extinction coefficient of 121990 M^{-1} cm^{-1} based on primary sequence.

6.2.3 Bacterial preparation and characterization

6.2.3a Bacteria Growth and Harvest

To test enzyme effectiveness on foodborne bacteria, *E. coli* O157:H7 (ATCC 4388), *Salmonella* Typhimurium (ATCC 13311), and *E. coli* ATCC 25922 were used as a model bacteria in this study, obtained from the USDA (Kimberly Cook, USDA-ARS-FAESR, Bowling Green, KY). *E. coli* O157:H7 and *Salmonella* Typhimurium are two pathogens that have been implicated in foodborne illness outbreaks associated with fresh produce (2, 26, 27). *E. coli* 25922 is a non-pathogen surrogate strain that has been identified and used to model pathogens in food safety environments (28). Cells were cultured in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37 °C overnight. For biofilm assays, cells from the overnight culture were diluted 1:100 in 1 mL of M9 minimal media and growth for 48 hr at 37 °C under static conditions. M9 media was created using 6 mg/mL Na2HPO4, 3 mg/mL, KH2PO4, 0.5 mg/mL NaCl, and 1 mg/mL NH4Cl, supplemented with 1% glucose, 2 mM MgSO4, and 0.1 mM CaCl2 in deionized water (29).

For flow cell detachment experiments, *E. coli* O157:H7 cells from overnight culture were transferred to 200 mL fresh LB media and harvested at the mid-exponential cell growth phase by centrifugation at 3000 rpm for 10 min and resuspension in 10 mM KCl three times (30). This simple salt solution chemistry was chosen to represent an

environmentally relevant ionic strength within the realm of possibility for surface and groundwater, and also to maximize observable attachment, as shown by previously reported trends in microbial adhesion to the epicuticle and other solid surfaces (31). Bacterial cell suspensions were adjusted to a final optical density of 0.2 at 600 nm, corresponding to approximately 10^8 cells/mL.

6.2.3b Relative hydrophobicity

Hydrophobicity analysis of the bacteria was done by using the microbial adhesion to hydrocarbon (MATH) test that has previously described in detail (32, 33). In brief, bacteria were first diluted to an optical density of 0.2 at a wavelength of 600 nm in 10 mM KCl. One mL of n-dodecane (Fisher Scientific) was added to three assays of 4 mL of diluted bacteria suspension and each of the assays were vortexed for 3 min. Partitioning of cells between n-dodecane and the electrolyte solution was then determined by measuring absorbance after 45 min. Relative hydrophobicity was calculated as the percent of total cells partitioned into the hydrocarbon layer.

6.2.3c Transmission electron microscopy (TEM)

E. coli PHL628 is known to over-produce extracellular polymeric substances, and was therefore used as a model organism to observe enzyme activity with TEM (34). Solutions containing *E. coli* PHL628 alone and *E. coli* PHL628 plus 100 ppm purified CAase were prepared as described above. Samples were incubated for 30 min at room temperature before 10 μL of each was added to carbon-coated copper TEM disks (Ted

Pella). After incubation for 30 s at room temperature, excess liquid was blotted off, and samples were negatively stained with 1% uranyl acetate for 1 min. Excess liquid was blotted off again, and samples were air-dried for 30 min. Disks were imaged on a JEM-2000FX scanning electron microscope (JEOL).

6.2.4 Biofilm assays

Biofilm growth experiments were conducted using sterile 24-well polystyrene plates (Corning Inc., Corning, NY). Plates were prepared in duplicate, wrapped in alumni foil to minimize evaporation, and incubated at 32 °C for 48 hr. Each plate included four wells of uninoculated M9 minimal media as control wells. After the 48 hr incubation period, 100 μL of 1% crystal violet in 95% ethanol was added to each well and allowed to incubate at room temperature for 20 min. The medium was then removed from wells and microtiter plate wells were washed five times with sterile distilled water to remove loosely associated bacteria. At this point, biofilms were visible as purple rings formed on the side of each well at the air-liquid interface and plates were air dried at room temperature for 45 min. Biofilm production was quantified by adding 2 mL of 20 % acetone/80 % ethanol to destain each of the wells and allowing to mix gently for 20 min. The absorbance was measured at 600 nm to quantify the crystal violet present in the destaining solution. Each assay was performed at least three times and the averages and standard deviations were calculated for all repetitions of the experiment.

6.2.5 Parallel-plate flow cell

Bacterial detachment experiments were conducted in a parallel plate flow chamber (GlycoTech, Rockville, MA) positioned on an inverted fluorescent microscope (BX-52, Olympus) to allow for direct of cells attaching and detaching on the surface (35-37). As shown in Figure 6.1, the inner dimension of the chamber is 6 cm × 1 cm × 0.08 cm and is composed of a Plexiglas® block, mounted to a microscope slide (supporting isolated spinach epicuticle layer on polycarbonate) by a flexible silicone elastomer gasket that is sealed by vacuum grease. The spinach leaf surface was prepared using a freeze-imbedding technique to separate the wax epicuticle layer from the rest of the leaf and transfer to a polycarbonate slide, as previously described (37).

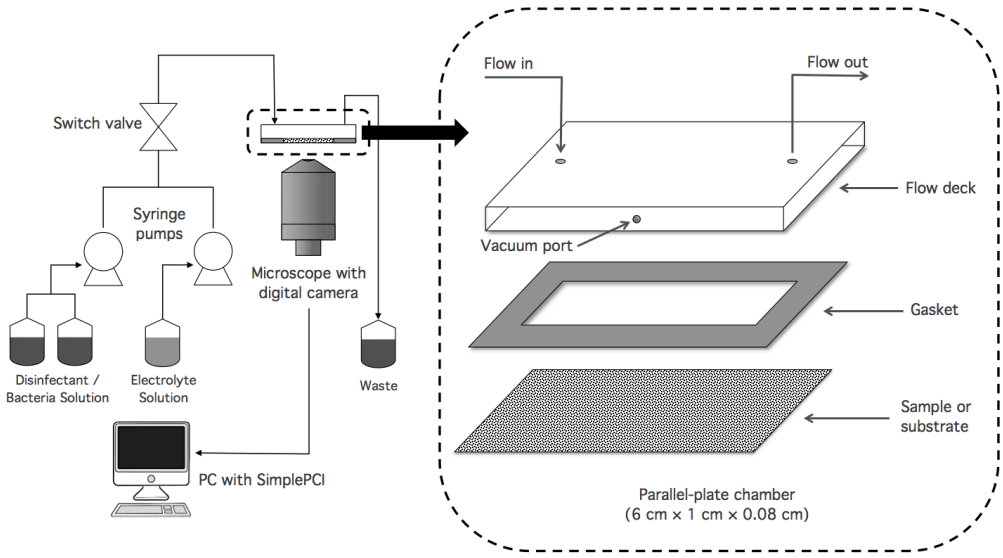

Figure 6.1 Parallel-plate flow cell and supporting materials schematic.

The influent enters the flow chamber from a capillary tube that is connected to a syringe, which is controlled by a syringe pump at a flow rate of 0.1 mL/min, which simulates expected surface conditions in a gentle leafy greens washing process (38). The bacteria were imaged by a 40x long working distance objective (UPlanFl, Olympus), and connected to a computer running SimplePCI to record images with a digital camera (Demo Retiga EXI Monochrome, QImaging). Cells were allowed to attach over a 30 min period, followed by a 30 min rinse with 10 mM KCl solution containing 0, 250, or 1000 ppb CAase enzyme. In order to determine the kinetics of cell detachment, images were recorded every 30 s and enumeration of cells was determined by comparison of successive images.

6.2.5a Mass transfer rate coefficients

During all rinsing experiments, bacterial detachment was negligible beyond a certain time point, resulting in a plateau in the number of remaining, attached bacteria. Detachment mass transfer rate coefficients were calculated using the enumeration of observed cells up the plateau point, using MATLAB (R2015a, Mathworks, Natick, MA) to process collected images. The number of bacterial cells removed from the epicuticle surface was plotted versus time, and bacterial flux, J, was calculated by dividing the slope of the line by the microscope viewing area (230 mm × 170 mm). The mass transfer rate coefficient for the bacteria, k, is calculated using the bacterial flux (number of cells per area per time), and the bulk cell concentration (number of cells per mL), C_0, via (39, 40):

$$k = \frac{J}{C_0}$$

In addition to mass transfer rate coefficients, total number of cells removed from the surface, normalized by the number of cells present at the beginning of the rinse phase, are reported. Each experiment was performed in triplicate using *E. coli* O157:H7.

6.3 Results and Discussion

6.3.1 Enzyme production

Expression and purification of the engineered enzyme (Figure 6.2) indicates that the engineered form can be expressed recombinantly in *E. coli* BL21 cells at high yield and purified using standard IMAC affinity chromatography. Initial attempts at expression resulted in low yields, necessitating optimization of protein sequence to improve expression and recovery. The yields of the enzyme were estimated to be 0.1 g enzyme/L culture, while at least 50% of the material was recovered via IMAC affinity chromatography. Bands at 77 kDa correspond with the molecular weight of the aforementioned enzymes (Figure 6.2), which were collected in relatively high purity in the 250 and 500 mM imidazole washes.

6.3.2 Prevention of biofilm formation

To assess the ability of the enzyme to prevent biofilm formation, *E. coli* 25922, *E. coli* O157:H7, and *Salmonella* Typhimurium were used as model agriculturally relevant

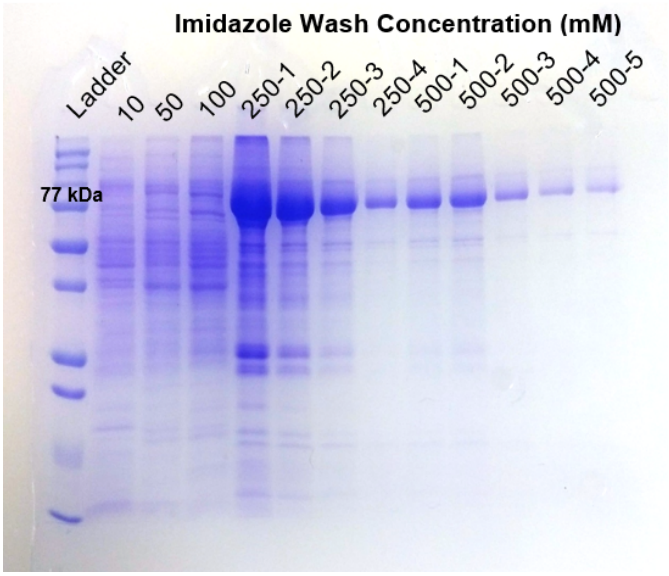

Figure 6.2 SDS-PAGE used to verify expression and purification of CAase enzyme.

bacteria. Biofilm formation of these three strains has been previously investigated as a function of nutrient conditions and provide a representative set of pathogens and a quality control surrogate (41).

As shown in Figure 6.3, the addition of 100 ppm CAase resulted in significant inhibition biofilm formation for all three cell types. Biofilm formation is reduced by 39 ± 6 % for *E. coli* 25922, 41 ± 6 % for *E. coli* O157:H7, and 37 ± 10 % for *Salmonella* Typhimurium. Previous studies have demonstrated reduced removal of mature biofilms as well as reduced biofilm formation when treating with enzymes for both *P. aeruginosa* and *A. actinomycetemcomitans* (21, 23), but broad enzymatic inhibition for multiple pathogens has not been described.

To minimize risks to public health, strategies to prevent biofilm formation are arguably more efficient than removing mature biofilms (42). Other proposed methods for inhibiting biofilm formation in the food industry include modification or treatment of surfaces to discourage bacterial attachment. For example, the potential of increasing surface roughness, hydrophilicity, and zeta potential, as well as the incorporation of antimicrobials like nano-silver, have been demonstrated (42-44). However, these approaches to preventing biofilm formation require industry transition and investment in new materials and processing equipment (18, 45). Additionally, surface modification is often not feasible or safe for addressing bacterial adhesion to produce surfaces.

6.3.3 Removal of mature biofilms

Biofilm removal with 100 ppm CAase was also compared to rinsing with 10 mM KCl solution. The results demonstrate that CAase may be an effective option for enhancing disruption of established biofilms on surfaces, especially in the case of *Salmonella* Typhimurium, in which enzyme exposure resulted in a 90% improvement of biofilms removal compared to the plain electrolyte rinse. Biofilms formed by *E. coli* 25922 and *E. coli* O157:H7 were also slightly reduced after rinsing with CAase, though statistically indistinguishable from control. This suggests that initial attachment and biofilm formation may be specific to a given pathogen type, and these differences are not captured by the enzyme as a broad-spectrum removal agent for biofilms.

The observed differences in efficacy of the enzyme functionality between *Salmonella* and *E. coli* strains may be a function of differences in their respective

mechanisms of attachment and biofilm composition. Cellulose and curli been shown to be crucial components of the extracellular matrix that promote adhesion and biofilm formation in both *E. coli* and *Salmonella* Typhimurium (46-48). Degradation of cellulose and other polysaccharides by the enzyme may disrupt biofilms and restrict adhesion, but curli and other proteins are not susceptible to enzymatic action. Therefore, these results imply that proteins may dominate adhesion mechanisms for *E. coli* cells in these conditions. Previous studies have observed curli expression by various strains of *E. coli* O157:H7 in similar growth conditions; specifically, temperatures below 37 °C and in low salt medium (49-51). Further, curli expression has been correlated with biofilm forming potential by *E. coli* O157:H7 (9, 52), including strains isolated from a spinach-related outbreak in 2006 (53). Macarisin *et al.* (2011) found that curli were essential for attachment of *E. coli* O157:H7 to spinach leaf surfaces, while cellulose was considered dispensable (54). Alternatively, Solano *et al.* (2002) showed that cellulose played a critical role in biofilm formation by *Salmonella enteritidis* (55), which may render its biofilms more susceptible to enzyme treatment.

Overall, removal or weakening of biofilms without physical or mechanical intervention remains a challenge (56). However, planktonic cells are significantly more susceptible to disinfectants, even at relatively low concentrations. These results are especially promising, as they demonstrate the enzyme's ability to disrupt the biofilms both during and after formation, leaving cells planktonic and potentially enhancing the efficacy of disinfectants.

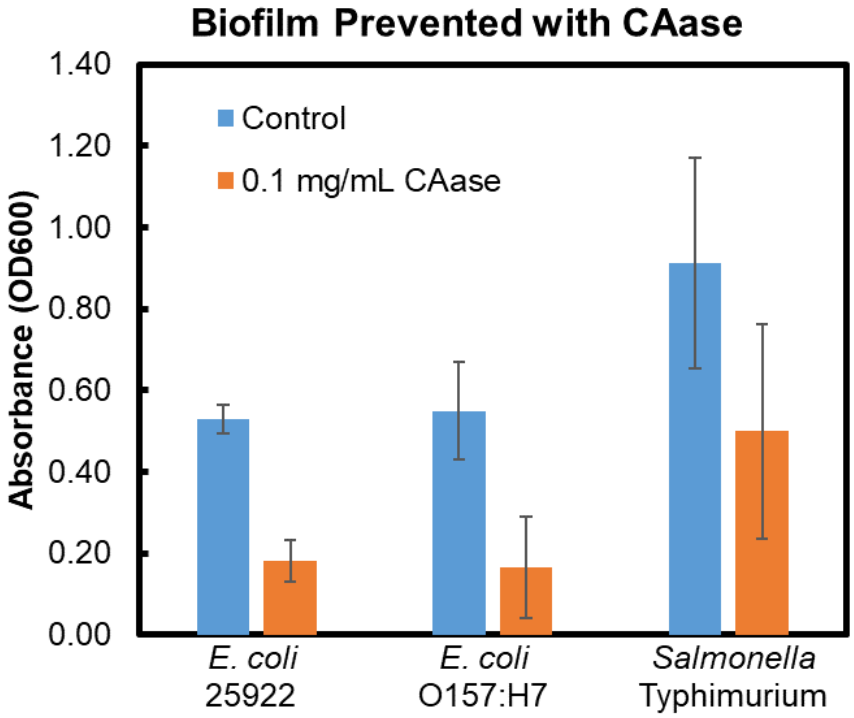

Figure 6.3 Prevention of biofilm formation on polycarbonate with the addition of 100 ppm enzyme.

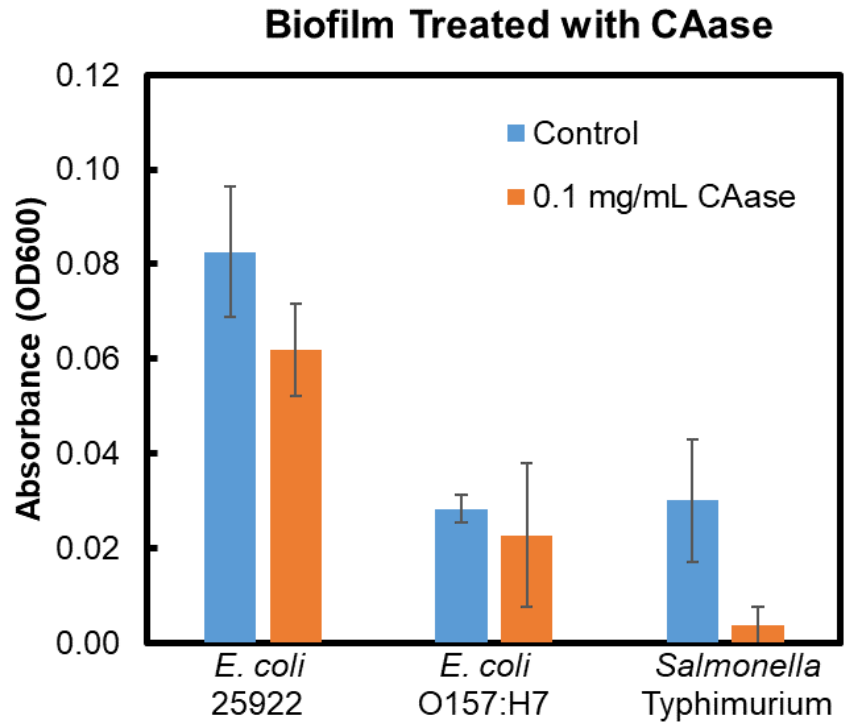

Figure 6.4 Removal of biofilms on polycarbonate with the addition of 100 ppm enzyme

6.3.4 Detachment from spinach leaf surfaces

The initial stages of biofilm formation require a transition from reversible to irreversible bacterial attachment, in order for cells to remain adhered to surface and produce the extracellular matrix that makes up a biofilm. These initial attachment steps were directly observed with the parallel plate flow cell for *E. coli* O157:H7. Based on previous work that utilized surface roughness data and COMSOL modeling to predict minimum disinfectant concentrations on the leaf surface, CAase concentrations three order of magnitude below the relevant bulk concentration were used in the flow cell (250 ppb and 1000 ppb for 0.25 ppm and 1 ppm, respectively) (37).

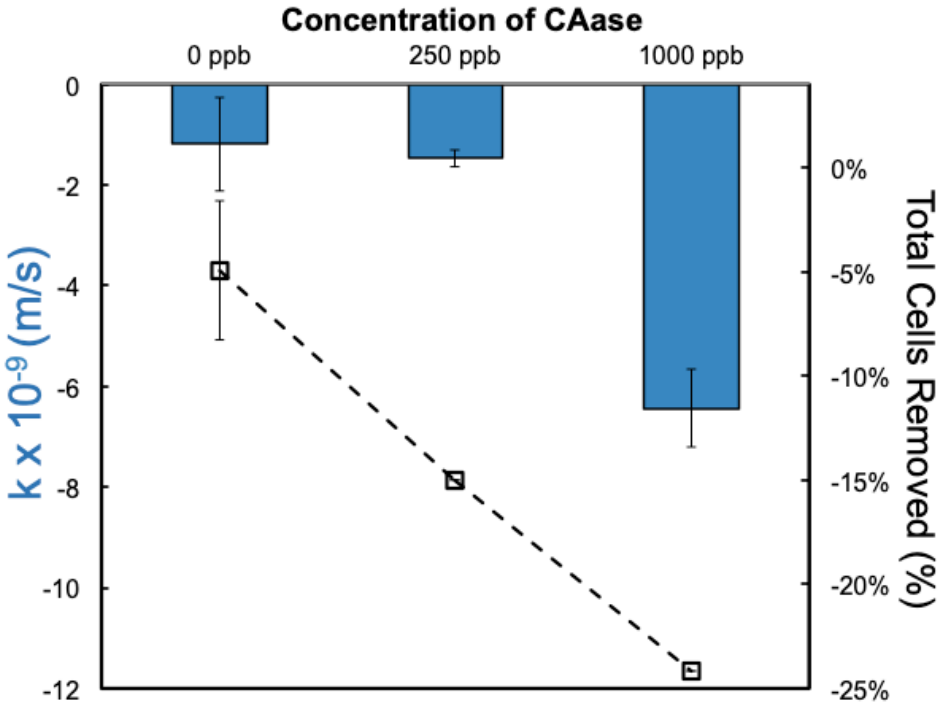

Figure 6.5 Detachment of *E. coli* O157:H7 from spinach leaf surface alone, and with 250 and 1000 ppb enzyme.

Mass transfer rate coefficients for *E. coli* O157:H7 cells did not significantly increase with 250 ppb CAase in the rinse solution, as shown in Figure 6.5. However, total detached cells increased from 5% to 15%, indicating that the time over which detachment is observed was greater with enzyme rinse versus plain 10 mM KCl. Detachment with 1000 ppb was nearly 7 times greater than the DI water rinse, increasing from $-1.19 \pm 0.92 \times 10^{-9}$ m/s to $6.44 \pm 0.77 \times 10^{-9}$ m/s. Additionally, 5%, 15%, and 24% of the total number cells were removed from the surface with 0, 250, and 1000 ppb of CAase over the 30 minute rinse.

6.3.5 Mechanisms of enzyme action

To assess the enzyme impact on cells, the cell surface was probed indirectly through hydrophobicity and directly through electron microscopy. Relative hydrophobicity of cells refers to the percentage of cells remaining in a 10 mM KCl solution versus partitioning into a hydrocarbon. As shown in Figure 6.6, relative hydrophobicity was considerably reduced for all three strains after exposure to 100 ppm of the enzyme. *Salmonella* Typhimurium showed the largest decrease (43.4 ± 14.2 % to 6.7 ± 4.4 % for the control and treated samples, respectively), followed by *E. coli* O157:H7 reduction from 22.3 ± 9.9 % to 1.1 ± 1.9 % and *E. coli* 25922 reduction from 5.7 ± 0.5 % to 0.6 ± 0.7 %.

Hydrophobic interactions are considered a dominant mechanism in the adhesion of bacteria cells to solid surfaces (57, 58). Previous studies using multiple strains of foodborne pathogens, including *E. coli*, *Salmonella*, and *Listeria monocytogenes,* have

demonstrated that reduced hydrophobicity plays a key role in reducing bacterial attachment to surfaces and ultimately biofilm formation (51, 59-61). Specifically, changes in cell surface exopolysaccharides (EPS) and lipopolysaccharides (LPS), which may be degraded by the engineered enzyme in this study, can contribute to measured changes in cell surface hydrophobicity. While the influence of extracellular polymers is debated, several studies have found that the presence of LPS can be correlated with higher cell surface hydrophilicity (62, 63). It is possible that CAase degrades outer polysaccharide regions, leaving inner hydrophilic cell surface structures that make up LPS exposed (64, 65).

Preliminary electron microscopy images corroborate the proposed mechanism of polysaccharide degradation. Images in Figure 6.7 reveal that cells treated with CAase lack extracellular polymers that untreated cells retain. In Figure 6.7A, the cell treated with enzyme appears to be more shriveled and has no extracellular appendages. This result may be attributed to the lack of bacterial cell envelope, which is responsible for the cell shape, protection of external stressors, and facilitating adhesion to solid surfaces. In Figure 6.7B, the untreated cell is rounder and has obvious, fibrous, extracellular appendages. Prigent-Combaret *et al.* (2001) also used electron microscopy to analyze extracellular structures of *E. coli* PHL628 and concluded that similar appendages and round domelike structures seen on the cell surface were extracellular polymeric substances (66).

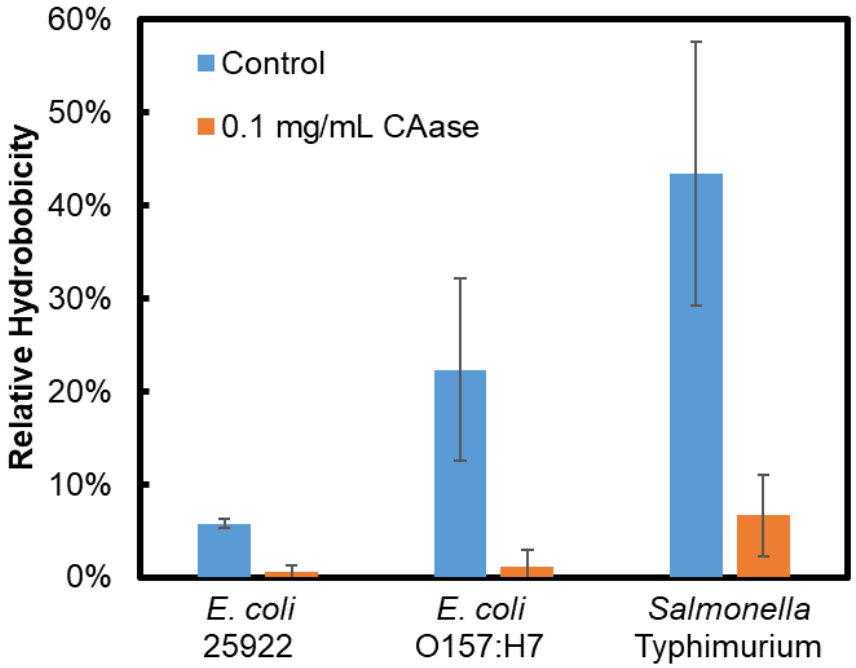

Figure 6.6 Relative hydrophobicity of cells with and without treatment with 100 ppm enzyme.

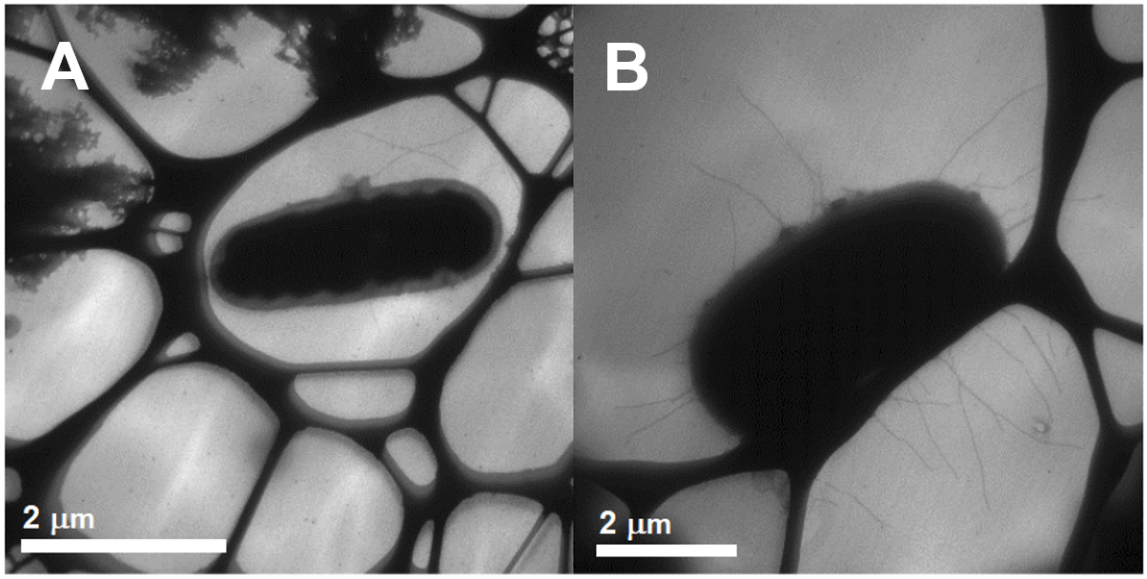

Figure 6.7 Electron microscopy images of cells with (left) and without (right) treatment with enzyme.

6.4 Conclusions

In this study, an enzyme-based disinfectant was developed and validated as a promising option for enhancing or replacing chlorine in food processing applications. To produce the hydrolase enzyme, the protein was expressed, separated, and purified successfully. Then, the detachment of *E. coli* O157:H7 cells from the spinach leaf surface was observed in the parallel-plate flow cell. Detachment rate coefficients and percentage of total detached cells were observed to increase more than 6 times with the addition of 1000 ppb enzyme to the rinse solution. This suggests that the enzyme is able to decrease the amount of irreversibly attached cells from the leaf surface, which represents the reversal of the foundational step in the biofilm formation process. Additionally, biofilm growth by *E. coli* O157:H7, *E. coli* 25922, and *Salmonella* Typhimurium on polystyrene were up to 40% inhibited by the presence of 100 ppm of the enzyme, providing evidence that the hydrolase is able to effectively degrade the extracellular matrix that typically protects cell and supports attachment. The results present a strong case for further development and optimization of enzyme activity as a novel alternative to antimicrobials to prevent pathogenic bacteria from contaminating produce and improving food safety.

6.5 References

1. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleesschauwer B. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLOS Medicine, 12(12):e1001923.
2. Sharapov UM, Wendel AM, Davis JP, Keene WE, Farrar J, Sodha S, Hyytia-Trees E, Leeper M, Gerner-Smidt P, Griffin PM, and Braden C. 2016. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of fresh spinach: United States, 2006. Journal of Food Protection, 79(12):2024-2030.
3. Herman KM, Hall AJ, and Gould LH. 2015. Outbreaks attributed to fresh leafy vegetables, united states, 1973–2012. Epidemiology and Infection, 143(14):3011-3021.
4. Slayton RB, Turabelidze G, Bennett SD, Schwensohn CA, Yaffee AQ, Khan F, Butler C, Trees E, Ayers TL, Davis ML, Laufer AS, Gladbach S, Williams I, and Gieraltowski LB. 2013. Outbreak of shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. Plos One, 8(2):e55300.
5. Korber DR, Mangalappalli-Illathu AK, and Vidović S. 2009. Biofilm formation by food spoilage microorganisms in food processing environments. Biofilms in the food and beverage industries, Woodhead Publishing:169-199, Amsterdam, Netherlands.

6. Blaschek HP, Wang HH, Agle ME, Wang HH, and Agle ME. 2015 Biofilms in the food environment. John Wiley & Sons, Inc. New York, NY, USA

7. Maharjan P, Huff G, Zhang W, and Watkins S. 2017. Effects of chlorine and hydrogen peroxide sanitation in low bacterial content water on biofilm formation model of poultry brooding house waterlines. Poultry Science, 96(7):2145-2150.

8. Ryu JH and Beuchat LR. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: Effect of exopolysaccharide and curli production on its resistance to chlorine. Applied and Environmental Microbiology, 71(1):247-254.

9. Ryu JH, Kim H, and Beuchat LR. 2004. Attachment and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. Journal of Food Protection, 67(10):2123-2131.

10. Corcoran M, Morris D, De Lappe N, O'Connor J, Lalor P, Dockery P, and Cormican M. 2014. Commonly used disinfectants fail to eradicate *Salmonella* enterica biofilms from food contact surface materials. Applied and Environmental Microbiology, 80(4):1507-1514.

11. Meireles A, Ferreira C, Melo L, and Simões M. 2017. Comparative stability and efficacy of selected chlorine-based biocides against *Escherichia coli* in planktonic and biofilm states. Food Research International, 102:511-518.

12. Hoff JC and Akin EW. 1986. Microbial resistance to disinfectants: Mechanisms and significance. Environmental Health Perspectives, 69:7-13.

13. Ölmez H and Kretzschmar U. 2009. Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. Food Science and Technology, 42(3):686-693.

14. Suslow T. 2000. Postharvest handling for organic crops. University of California, Division of Agriculutre and Natural Resources. Publication 7254.

15. Elasri MO and Miller RV. 1999. Study of the response of a biofilm bacterial community to uv radiation. Applied Environmental Microbiology, 65(5):2025-2031.

16. Duncan SE and Chang HH. 2012. Implications of light energy on food quality and packaging selection. Advances in food and nutrition research, Academic Press:25-73. Amsterdam, Netherlands.

17. Martínez-Sánchez A, Allende A, Bennett RN, Ferreres F, and Gil MI. 2006. Microbial, nutritional and sensory quality of rocket leaves as affected by different sanitizers. Postharvest Biology and Technology, 42(1):86-97.

18. Goodburn C and Wallace CA. 2013. The microbiological efficacy of decontamination methodologies for fresh produce: A review. Food Control, 32(2):418-427.

19. Augustin M, Ali-Vehmas T, and Atroshi F. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. Journal of Pharmaceutical Science, 7(1):55-64.

20. Gutiérrez TJ. 2019 Antibiofilm enzymes as an emerging technology for food quality and safety. Enzymes in food biotechnology, Academic Press:321-342. Amsterdam, Netherlands.

21. Izano EA, Wang H, Ragunath C, Ramasubbu N, and Kaplan JB. 2007. Detachment and killing of *Aggregatibacter actinomycetemcomitans* biofilms by Dispersin b and SDS. Journal of Dental Research, 86(7):618-622.

22. Boyd A and Chakrabarty AM. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Applied and Environmental Microbiology, 60(7):2355-2359.

23. Cho H, Huang X, Lan Piao Y, Eun Kim D, Yeon Lee S, Jeong Yoon E, Hee Park S, Lee K, Ho Jang C, and Zhan CG. 2016. Molecular modeling and redesign of alginate lyase from *Pseudomonas aeruginosa* for accelerating CRPA biofilm degradation. Proteins, 84(12):1875-1887.

24. MacDonald LC and Berger BW. 2014. Insight into the role of substrate-binding residues in conferring substrate specificity for the multifunctional polysaccharide lyase Smlt1473. Journal of Biological Chemistry, 289(26):18022-18032.

25. Eckersley E and Berger BW. 2018. An engineered polysaccharide lyase to combat harmful algal blooms. Biochemical Engineering Journal, 132:225-232.

26. Callejón RM, Rodríguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, and Troncoso AM. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and causes. Foodborne Pathogens and Disease, 12(1):32-38.

27. Bennett SD, Littrell KW, Hill TA, Mahovic M, and Behravesh CB. 2015. Multistate foodborne disease outbreaks associated with raw tomatoes, United States, 1990–

2010: A recurring public health problem. Epidemiology and Infection, 143(7):1352-1359.

28. Kim JK and Harrison MA. 2009. Surrogate selection for *Escherichia coli* O157:H7 based on cryotolerance and attachment to romaine lettuce. Journal of Food Protection, 72(7):1385-1391.

29. M9 minimal medium (standard). 2010(8):pdb.rec12295.

30. Haznedaroglu BZ, Kim HN, Bradford SA, and Walker SL. 2009. Relative transport behavior of *Escherichia coli* O157:H7 and *Salmonella* enterica serovar pullorum in packed bed column systems: Influence of solution chemistry and cell concentration. Environmental Science & Technology, 43(6):1838-1844.

31. Rapicavoli JN, Kinsinger N, Perring TM, Backus EA, Shugart HJ, Walker S, Roper MC, and Goodrich-Blair H. 2015. O antigen modulates insect vector acquisition of the bacterial plant pathogen Xylella fastidiosa. Applied and Environmental Microbiology, 81(23):8145-8154.

32. Pembrey RS, Marshall KC, and Schneider RP. 1999. Cell surface analysis techniques: What do cell preparation protocols do to cell surface properties? Applied and Environmental Microbiology, 65(7):2877-2894.

33. Rosenberg M, Gutnick D, and Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters, 9(1):29-33.

34. Vidal O, Longin R, Prigent-Combaret C, Dorel C, Hooreman M, and Lejeune P. 1998. Isolation of an *Escherichia coli* k-12 mutant strain able to form biofilms on

inert surfaces: Involvement of a new ompr allele that increases curli expression. Journal of Bacteriology, 180(9):2442-2449.

35. Chen G, Beving DE, Bedi RS, Yan YS, and Walker SL. 2009. Initial bacterial deposition on bare and zeolite-coated aluminum alloy and stainless steel. Langmuir, 25(3):1620-1626.

36. McClaine JW and Ford RM. 2002. Reversal of flagellar rotation is important in initial attachment of *Escherichia coli* to glass in a dynamic system with high- and low-ionic-strength buffers. Applied and Environmental Microbiology, 68(3):1280-1289.

37. Kinsinger NM, Mayton HM, Luth MR, and Walker SL. Efficacy of post-harvest rinsing and bleach disinfection of *E. coli* O157:H7 on spinach leaf surfaces. Food Microbiology, 62:212-220.

38. Huang K and Nitin N. Enhanced removal of *Escherichia coli* O157:H7 and *Listeria innocua* from fresh lettuce leaves using surfactants during simulated washing. Food Control, 79:207-217.

39. Chowdhury I, Cwiertny DM, and Walker SL. 2012. Combined factors influencing the aggregation and deposition of nano-TiO_2 in the presence of humic acid and bacteria. Environmental Science & Technology, 46(13):6968-6976.

40. Elimelech M, Gregory J, and Jia X. 2013 Particle deposition and aggregation: Measurement, modelling and simulation. Butterworth-Heinemann. Oxford, United Kingdom.

41. Cook KL, Givan EC, Mayton HM, Parekh RR, Taylor R, and Walker SL. 2017. Using the agricultural environment to select better surrogates for foodborne pathogens associated with fresh produce. International Journal of Food Microbiology, 262:80-88.

42. Eleftheriadou M, Pyrgiotakis G, and Demokritou P. 2017. Nanotechnology to the rescue: Using nano-enabled approaches in microbiological food safety and quality. Current Opinion in Biotechnology, 44:87-93.

43. Jansen B and Kohnen W. 1995. Prevention of biofilm formation by polymer modification. Journal of Industrial Microbiology, 15(4):391-396.

44. Arnold JW and Bailey GW. 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: Scanning electron and atomic force microscopy study. Poultry Science, 79(12):1839-1845.

45. Brooks JD and Flint SH. 2008. Biofilms in the food industry: Problems and potential solutions. International Journal of Food Science and Technology, 43(12):2163-2176.

46. Zogaj X, Nimtz M, Rohde M, Bokranz W, and Römling U. 2001. The multicellular morphotypes of *Salmonella* typhimurium and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Molecular Microbiology, 39(6):1452-1463.

47. Solano C, García B, Valle J, Berasain C, Ghigo JM, Gamazo C, and Lasa I. 2002. Genetic analysis of *Salmonella* enteritidis biofilm formation: Critical role of cellulose. Molecular Microbiology, 43(3):793-808.

48. Castelijn GAA, van der Veen S, Zwietering MH, Moezelaar R, and Abee T. 2012. Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* typhimurium strains of different origin in high and low nutrient medium. Biofouling, 28(1):51-63.

49. Saldaña Z, Xicohtencatl-Cortes J, Avelino F, Phillips AD, Kaper JB, Puente JL, and Girón JA. 2009. Synergistic role of curli and cellulose in cell adherence and biofilm formation of attaching and effacing *Escherichia coli* and identification of fis as a negative regulator of curli. Environmental Microbiology, 11(4):992-1006.

50. Kim JY, Kim SY, Kim JM, Kim YK, Yoon KY, Kim JY, Lee BC, Kim JS, Paek SH, Park SS, Kim SE, and Jeon BS. 2009. *Spinocerebellar ataxia* type 17 mutation as a causative and susceptibility gene in parkinsonism. Neurology, 72(16):1385-1389.

51. Patel J, Sharma M, and Ravishakar S. 2010. Effect of curli expression and hydrophobicity of *Escherichia coli* O157:H7 on attachment to fresh produce surfaces. Journal of Applied Microbiology, 110(3):737-745.

52. Pawar DM, Rossman ML, and Chen J. 2005. Role of curli fimbriae in mediating the cells of enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. Journal of Applied Microbiology, 99(2):418-425.

53. Uhlich GA, Sinclair JR, Warren NG, Chmielecki WA, and Fratamico P. 2008. Characterization of shiga toxin-producing *Escherichia coli* isolates associated with two multistate food-borne outbreaks that occurred in 2006. Applied Environmental Microbiology, 74(4):1268-1272.

54. Macarisin D, Patel J, Bauchan G, Giron JA, and Sharma VK. 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodborne Pathogens and Disease, 9(2):160-167.

55. Solano C, Garcia B, Valle J, Berasain C, Ghigo JM, Gamazo C, and Lasa I. 2002. Genetic analysis of *Salmonella* enteritidis biofilm formation: Critical role of cellulose. Molecular Microbiology, 43(3):793-808.

56. Gibson H, Taylor JH, Hall KE, and Holah JT. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. Journal of Applied Microbiology, 87(1):41-48.

57. Palmer J, Flint S, and Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. Journal of Industrial Microbiology & Biotechnology, 34(9):577-588.

58. Hood SK and Zottola EA. 1995. Biofilms in food processing. Food Control, 6(1):9-18.

59. Di Bonaventura G, Piccolomini R, Paludi D, D'Orio V, Vergara A, Conter M, and Ianieri A. 2008. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: Relationship with motility and cell surface hydrophobicity. Journal of Applied Microbiology, 104(6):1552-1561.

60. Walker SL, Hill JE, Redman JA, and Elimelech M. 2005. Influence of growth phase on adhesion kinetics of *Escherichia coli* D21g. Applied and Environmental Microbiology, 71(6):3093-3099.

61. Wang HH, Ye K-P, Zhang Q-Q, Dong Y, Xu X-L, and Zhou G-H. 2013. Biofilm formation of meat-borne *Salmonella* enterica and inhibition by the cell-free supernatant from *Pseudomonas aeruginosa*. Food Control, 32(2):650-658.

62. Al-Tahhan RA, Sandrin TR, Bodour AA, and Maier RM. 2000. Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates. Applied Environmental Microbiology, 66(8):3262-3268.

63. Park KM and So JS. 2000. Altered cell surface hydrophobicity of lipopolysaccharide-deficient mutant of *Bradyrhizobium japonicum.* Journal of Microbiological Methods, 41(3):219-226.

64. Walker SL, Redman JA, and Elimelech M. 2004. Role of cell surface lipopolysaccharides in *Escherichia coli* K12 adhesion and transport. Langmuir, 20(18):7736-7746.

65. Madigan MT, Martinko JM, and Parker J. 1997 Brock biology of microorganisms. Vol. 11. Prentice Hall. Upper Saddle River, NJ, USA.

66. Prigent-Combaret C, Prensier G, Le Thi TT, Vidal O, Lejeune P, and Dorel C. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: Role of flagella, curli and colanic acid. Environmental Microbiology, 2(4):450-464.

Chapter 7a

Identifying Common Ground for Sustainable Water Data Management: The Case of California

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Abstract

Natural resource management will continue to be increasingly important in the face of impending climate change and population growth, respectively impacting supply-side and demand-side constraints. Water resources, the subject of this paper, require sustainable management to provide drinking water for society, habitat and ecological water for the environment, and a myriad of industrial water uses, including agriculture, power generation, and manufacturing. In addition to technologies that increase water supply or reduce demand, the effective management of data, specific to water resources, will be crucial in the immediate and long-term future. With diverse water data generators, collectors, synthesizers, users, and policymakers, an integrated system of water data management has potential to ensure sustainable water resource management. To bring this potential to fruition, this work synthesizes published recommendations, as well as those of water experts, and best practices from examples of water data management to provide a preliminary assessment for larger ongoing efforts to improve data for water decision-making in California. Stakeholder collaboration, data standardization, increased data collection, and data transparency and accessibility are amongst the most common and most important recommendations for sustainably developing and managing an integrated water data management system.

7.1 Introduction

Decisions regarding natural resource management are bound by many constraints. Water resource management in particular affects a variety of stakeholders, both human and non-human, and therefore is influenced by the needs and priorities of competing voices. More than simply balancing supply and demand, effective water management decisions must take into account the severity of insufficient action or inaction, and consider quality as well as quantity of available water sources. Therefore, water management strategies are often regionally specific, yet may take inspiration from similar sites around the world.

One common requirement for all water management decisions is the availability of sufficient information. Regardless of water source, climate, natural infrastructure, type of water uses, or population, decision makers rely on data to make the most informed choices for water management. However, copious amounts of data are useless if unable to be found and interpreted by those who wish to utilize them, thereby making *water data* management a crucial component of water resource management.

Sato et al. (2013) found that of 181 countries surveyed (representing 92% of countries on Earth), about ⅓ collected and curated data for the generation, treatment, and use of wastewater, while another ⅓ had data for one or two of those three components, and the final ⅓ had no data on wastewater whatsoever (1). Of the data available, only 37% of it was recent (within 5 years preceding the publication of the article). While wastewater is only one element of water resource management, this finding illustrates

that the availability of sufficient water data is not a foregone conclusion; on the contrary, it is a luxury that not even some developed countries can claim.

Water data management is a global issue, one that can conceivably span political borders and be a source of collaboration (or dissention) among entities whose water resource management decisions are interdependent. This work focuses on California (CA), USA, as a case study for water data management, inspired by recent legislation AB-1755, *The Open and Transparent Water Data Act* (2016).

AB-1755 mandates that the CA Department of Water Resources (DWR) create, operate, and maintain a statewide water data platform. The platform will integrate existing water and ecological data from multiple databases and provide data on completed water transfers and exchanges, and is required to be operational by September 2019. AB-1755 also calls for protocols for data sharing, documentation, quality control, public access, and promotion of open-source platforms, as well as decision support tools related to water data. These protocols and tools are intended to help decision-makers find and utilize water data, and identify data gaps more easily. DWR is required to conduct these activities in consultation with the CA State Water Resources Control Board (SWRCB), the CA Water Quality Monitoring Council (CWQMC), and the Department of Fish and Wildlife (DFW) (2). The bill is primarily focused on improving how water data is published and accessed, in addition to providing some support for how data is collected, verified, and interpreted, and represents state-level recognition of the importance of data for decision-making in water policy.

With the sixth largest economy in the world, the most populated state in the country, and a high level of geographical diversity, California's prosperity is intrinsically tied to water (3, 4). Between 120-370 trillion cubic metres (100-300 million acre feet) of water is moved in California annually for the agricultural demands on irrigation, the sustenance of human activity, and the necessary environmental flows for countless ecosystems (5). On average, 40% of this water is used by agriculture, 10% by urban centers, and 50% by the environment, although these proportions vary drastically across the state's high geographic variability (6).

This heavy reliance on water infrastructures amongst a diversity of stakeholders with unique priorities can create significant and unique policy challenges for California. Over the past five years of drought, these challenges have been brought to the forefront of the water policy arena. With rivers and wetlands reaching dangerously low levels, many agricultural fields fallowed, and some Central Valley communities without access to any drinking water, the state has had to react quickly to avoid crisis (7).

This type of water stress in California is expected to increase throughout the 21st century with the impacts of climate change and population growth. Therefore, being able to respond quickly, effectively, and efficiently is a priority for state water policy. This has forced the State of California to recently review its water information and data management practices, and many water experts have previously called for changes and updates to state water data systems.

Reasons for the lack of useful water data, both in California and around the world, are plenty. Although rarely is there a complete lack of information regarding water

quality, data recorded by different entities are often recorded differently, rendering them incomparable or incompatible. The same holds true for water availability, transfers among water rights holders, and environmental data (8). For example, some data collection is automated, and the sheer volume of transmitted data and its format can be cumbersome to compare to manually recorded measurements. Temporally and geographically distributed data provide additional layers of complexity that can be useful, but have to be accounted for when managing and using such data. With so much data being recorded by such disparate collectors and contexts all across California, rendering data useful requires a robust framework and management plan that carefully considers who requires data for decision making, and what data and format is needed to accomplish this.

In response to these challenges and the passage of AB-1755, a "Data for Water Decision-Making" initiative has been launched and led by UC Water, DWR, and the California Council on Science and Technology (CCST). This systematic evaluation of previously published recommendations for water data management was performed as a preliminary step to identifying and implementing major policy recommendations. These results provide a snapshot of the ongoing larger, systemic work to improve data-driven decision-making for California's water. While the focus of this case study was California, these recommendations are translational across various entities, including sub-national (e.g., CA), national, and international water systems.

7.2 Materials & Methods

In order to identify and validate the most common and agreed-upon priorities for California's future water data management efforts, a three-pronged approach was employed in this study: (1) a literature review of recent published recommendations for water data management, (2) an assessment of best practices from past water data management strategies, both within California and in other geographic contexts, and (3) informal interviews of California water data stakeholders and experts. Additional details about these three methods are described below. In brief, the literature review and assessment of best practices were conducted in parallel. The set of generalized common recommendations, which were identified from recent literature, was used to characterize key features of past water data management strategies. Based on the combination of most common recommendations from literature and prevalence in observed best practices from past water data management efforts, a subset of key recommendations was synthesized and used to inform the stakeholder interviews. After aggregating responses to the informal interview questions, the final set of shared key recommendations for water data management was generated.

7.2.1 Literature Review

Various, diverse stakeholders across California have inherently different needs in regards to water, which results in different recommendations for how best to manage water data. These recommendations have been published in the form of academic journal articles, white papers, policy briefs, and conference or other event proceedings. In order

to identify common ground and priority actions for water data management in California, 13 publications from a variety of stakeholder groups in the last decade (since 2006) were reviewed. These publications were chosen based on a combination of online searches and recommendations from California water data and policy experts. To the authors' knowledge, this analysis includes a nearly comprehensive sample of the most highly cited and well-known resources pertaining to California water data management recommendations at the time of preparation. Of the resources evaluated, authors and organizers included local, state, and federal agencies, non-profits/NGOs, and research institutions, representing a combination of water data generators, regulators, and users. Documents were evaluated via several criteria, including:

- Author organization and sector (academic, nonprofit, governmental, etc.)
- Intended audience
- Motivation
- Recommended actions
- Key findings

The results of the literature review are summarized in Table 7.1.

Table 7.1 Summary of literature review. Non-governmental organizations (NGO), California government (CAG), academia (Acad), national labs (NL).

Date	Title	Author (Type)	Audience	Motivation	Recommendations	Key Findings
Oct. 2016	California's Water *(9)*	Public Policy Institute of CA (NGO)	Water experts, policymakers	Drought; environment; response to SGMA	More data; modeling; use by policymakers	Improved data management is amongst several pressing priorities for decision-makers
Sept. 2016	California Groundwater Briefing *(10)*	Stanford Water in the West (Acad)	Policymakers, public citizens	Response to SGMA	Transparency; standardization;	Challenges to data management are human and organizational, rather than technical
Aug. 2016	Unanswered Questions for Implementation of the Sustainable Groundwater Management Act *(11)*	UC Water (Acad)	Academia	Response to SGMA	Collaboration; standardization; security	Data collection and sharing requirements under SGMA need a public data framework
July 2016	Accounting for California's Water *(12)*	Public Policy Institute of CA (NGO)	Water experts, policymakers	Drought; environment; response to SGMA	More data; modeling; use by policymakers	Developing a common water accounting framework should be adopted as a statewide priority
April 2016	Establishing a Cloud-based Water and Energy Data Platform *(13)*	UC Davis (Acad)	Resource managers, policymakers, academia	Water-energy nexus	Transparency; standardization; security; data platform	Benefits and technical feasibility of integrating water and energy data in shareable, secure way
April 2016	Strategic Vision and Framework for Integrated Water Management Data and Tools *(14)*	CA Dept. of Water Resources (CAG)	Public citizens	Response to SGMA and California WAP	More data; collaboration; standardization;	Outlines specific goals for consistency and quality control in data management through watershed-based water budgets
May 2015	Enhancing the Vision: Managing California's Environmental Information *(15)*	Delta Stewardship Council (CAG)	Resource managers, academia, public	Environment; response to CA WAP	Transparency; data platform	Accessibility is major barrier to environmental data utilization
Dec. 2014	Assessment of Surface Water Quality Data *(16)*	Surface Water Ambient Monitoring Program (CAG)	Water experts, policymakers	N/A	More data; collaboration;	Ongoing monitoring needs to be expanded and better coordinated, but lacks resources
Oct. 2014	California State Water Plan (2013 Update) *(5)*	State of CA (CAG)	Public citizens	Drought; environment; water-energy nexus	Transparency; collaboration; standardization; use by water managers	Methods to share data effectively between entities is a priority, rather than single data repository
Aug. 2014	100 Years of California Water Rights System *(8)*	UC Water (Acad)	Academia	N/A	N/A	California must reconcile water rights and supply with better accounting
April 2014	Achieving a Sustainable California Water Future Through Innovation in Science and Technology *(17)*	CA Council on Science and Technology (NGO)	Policymakers	Environment; energy use; response to CA WAP	More data; integration; data platform	Strategic policy should be used to leverage current innovations for sustainable water management
Jan. 2014	California Water Action Plan (WAP) *(18)*	State of CA (CAG)	Public Citizens	N/A	More data; integration	Data on water use, availability, and changes will be essential for refining water transfer processes
July 2012	Climate and Water: Knowledge of Impacts to Action on Adaptation *(19)*	UC Water (Acad)	Academia	N/A	Transparency; more data; collaboration; standardization; modeling; use by policymakers, water managers, and public	More complete data of climate impacts on water systems strengthens adaptation responses
Dec. 2009	Maintenance and Dissemination of Water Transfer Database for 12 Western States *(20)*	CA Institute of Water Resources (Acad)	Academia	N/A	N/A	Large variability in patterns, size, and reporting of water transfers
Sept. 2007	Assessing Security Needs of the Multifaceted Relationships of Energy and Water Providers *(21)*	Lawrence Livermore National Lab (NL)	Academia	Drought; water-energy nexus	N/A	Understanding stakeholder relationships, data needs, and cultural contexts are first steps

Name	Organization	Context	Key Features	Current Status
California Integrated Water Quality System (CIWQS)	State and Regional Water Quality Control Boards	Launched in 2005, this regulatory information tracking system manages information about environment, permits, inspections, violations and other activities, following a $1.5 million investment by the U.S. Environmental Protection Agency and the State Water Board.	Data platform; use by resource managers and public	Due to insufficient resources to address challenges such as the poor user interface, data compatibility, and perceived integrity of its data, usage has been limited *(22)*.
Surface Water Ambient Monitoring Program (SWAMP)	State Water Resources Control Board	As mandated by AB 982, SWAMP was established in 1999 with the goal of integrating surface water monitoring data from the state and regional water quality control boards and exists to inform management decisions, and maintain data collection.	Transparency; standardization; collaboration; use by resource managers	While limited to surface water data, SWAMP continues to enable comprehensive assessment of the conditions throughout California *(23)*.
California Water Science Center	U.S. Geological Survey (USGS)	Basic hydrologic data on groundwater levels, surface water flows, and water quality is collected throughout the state to contribute to flood and water-supply forecasts, planning and design, regulation, research investigations, and more via annual reports since 1961.	More data; use by policymakers	Data is relatively limited in its scope and user interface, but USGS continues to provide reliable historic and present day data *(24)*.
Hobbes Project	Center for Watershed Sciences at University of California, Davis	Launched in 2013, HOBBES takes a bottom up approach to improve and organize data for water modeling efforts by providing a web-based platform to ultimately make modeling California water systems more transparent and participatory.	Transparency; standardization; modeling; data platform	This project is essentially complete and provides an example of a useful framework for water data management and modeling *(25)*.
California Water Planning Information Exchange (Water PIE)	Conservation Biology Institute	This basin-based platform was developed in 2016 and aims to make water data more widely usable and accessible by allowing users to search for datasets, visualize projects, and learn more about conservation science and design.	Transparency; data platform; use by policymakers, resource managers, and public	Expansion of Water PIE continues to be supported by the CA Department of Water Resources, as mandated by the 2013 CA Water Plan Update *(26)*.
New Jersey Water Monitoring Council (NJWMC)	State of New Jersey	This Council, established in 2003, includes representation from a variety of state and federal agencies and facilitates the coordination, collaboration and communication of ambient water quality and quantity data to support effective environmental management.	Collaboration; use by policymakers	This body, along with the State's Office of Water Resources Management Coordination, enables New Jersey to provide one of the most rigorous and comprehensive water data management programs in the U.S. *(27)*.
European Union Water Framework Directive (WFD)	European Union	Since 2000, the WFD has used natural geographical and hydrological units to govern water, as opposed to possibly arbitrary administrative boundaries, and facilitates information exchange between countries using a web-based platform.	Collaboration; data platform	Shortcomings in WFD's data monitoring, assessment, and management have been identified by various EU research groups and will be utilized in reorganizing the WFD in the coming years *(28, 29, 30)*.
Australia National Water Initiative (NWI)	Australia	In response to the "Millennium drought", Australia unlocked water rights from land ownership in 2004, providing statutory force for environmental water and substantial federal investments in water science and data a part of the NWI.	More data; use by policymakers and resource managers	Australia's Productivity Commission found that NWI significantly improved national management of water resources, and future improvements will work on usefulness in the water policy sector and integration of climate change and population growth challenges *(31)*.

7.2.2 Best practices within and beyond California

While California currently lacks a comprehensive water data management program, water data are collected, synthesized, and displayed by various organizations, for various purposes, and in various ways. This work also considers best practices and lessons learned from previous water data management projects. Within the state of California, data management efforts have previously been initiated by water-focused academic, state agency, and federal organizations with widely varying levels of success. We also consider the best practices from three unique established water data managements systems that operate at the statewide, national, and international level. Each example of water data management was characterized by:

- Directing organization and location
- Intended user(s)
- Notable features
- Operating status

These water data management examples are summarized in Table 7.2. Selection of notable features was informed by the recommendations identified during the literature review process, with an emphasis on recommendations that appeared in more than one report or publication.

7.2.3 Synthesis

From this collection of recommendations and examples of water data management, 11 themes were identified as being notable in multiple publications or data management systems. These themes are outlined in Table 7.3, along with a visual representation of the number of times that each one appears in our review (Figure 7.1). Based on the distribution of themes, a subset of key recommendations for sustainable water data management in California was identified and used to inform informal interviews that followed. Recommendations that were explicitly mentioned in more than three unique publications and were present as key features of success in previous water data management efforts were included. These key recommendations are detailed in the following Results & Discussion section.

7.2.4 Informal interviews

In order to validate the range of publications considered and key recommendations identified, a series of semi-structured interviews were conducted with several California water experts (Table 7.4). Interviewees were selected based on authorship or involvement in previously published recommendations and/or water data management efforts within the state, and are intended to represent the range of stakeholder groups involved in California water data management (including state government, academic institutions, and non-governmental organizations). The repository of documents was made available to subjects of the informational interviews for their reference, and to solicit recommendations for supplementary materials to be considered.

The interview questions that provided the basis for the discussions included:

- "Are there clear discrepancies between what has been recommended for water data management and what has been put into action? Why do you think that is?"
- "Over the course of your career, have you observed any trends or changes in water and water data management?"
- "In an ideal world, what would be your vision of a perfect water data management system in California?"

The results of these interviews were incorporated into the following conclusions of this work.

Table 7.3 Summary of 11 most common recommendations

Recommendation	Description
Transparency	Expansion of accessibility of existing data amongst data users and data generators
More data	Increase the amount of data being collected through monitoring and reporting
Collaboration	Stakeholders working together to address water data management
Standardization	Shared, common processes and metrics for collection and reporting of data
Modeling	Integrating predictive data technologies to inform and/or utilize data
Integration	Combining water data management with other resources, like land, energy, and air
Security	Sharing or storing data provides protection and privacy for users
Data platform	Creation of an all-in-one, comprehensive data management platform
Use by policymakers	Main users of data are policymakers
Use by water managers	Main users of data are water managers
Use by public	Main users of data are public citizens

Table 7.4 Participants of semi-structured interviews

Interviewee	Affiliation(s)
Roger Bales Distinguished Professor Director	 UC Merced UC Water Security and Sustainability Research Initiative
Gary Darling Operations Research Specialist	Bay-Delta Office, Department of Water Resources
Jay Lund Professor Director	 University of California, Davis Center for Watershed Sciences
Greg Smith	Division of Statewide Integrated Water Management, Department of Water Resources
Soroosh Sorooshian Distinguished Professor Director	 University of California, Irvine Center for Hydrometeorology and Remote Sensing (CHRS)
Stephen Weisberg Executive Director	 Southern California Coastal Water Research Project Authority

7.3 Results and Discussion

Recommendations for better water data management in California over the past decade came from within the California state government, non-governmental organizations, academic institutions, and national labs, as shown in Table 7.1. For those that explicitly mentioned a motivation for their report, reasons could be summarized by five major themes: (1) the California drought, including how it impacts or is impacted by population growth; (2) environmental concerns related to climate change and urban sprawl; (3) the symbiotic and conflicting interactions of water and energy resources throughout the state; (4) in response to the Sustainable Groundwater Management Act

(SGMA); and (5) in response to the Governor's California Water Action Plan (WAP). These motivations are also summarized in Table 7.1 and highlight a notable emphasis on macro-scale data amongst these publications. While improved water data management will ultimately be useful at all scales, from individual water use to hydrologic water cycles, one emergent theme from recent publications and interviews with select experts was a focus on large-scale water data in California. Therefore, these results largely refer to a subset of all water data that seems to be the primary focus of current water data experts and policy change within the state, rather than comprehensively addressing every aspect of water data.

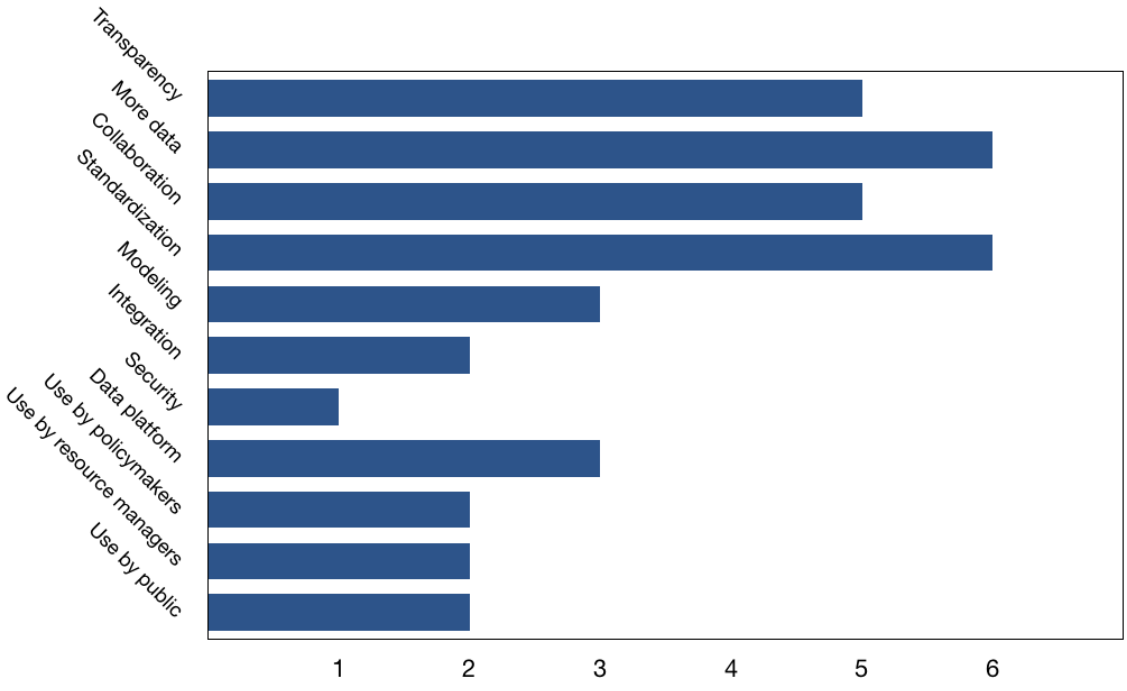

Figure 7.1 Number of times each recommendation specifically appears within literature review.

7.3.1 Key Recommendations

The methodological approach of synthesizing advice for water data management resulted in four specific recommendations identified as the most commonly suggested and most applicable to governmental policy (Figure 7.1):

1. Increased collaboration and data sharing between data users and generators
2. Standardization of metrics used in ambient water, water supply, and wastewater industries
3. Greater investment in data collection and water monitoring systems
4. Data curation that is accessible and transparent

Increased collaboration and data sharing between data users and generators.

Stakeholder collaboration was explicitly called for by multiple state-level organizations and research institutions, including DWR, CA Surface Water Ambient Monitoring Program, the CA Water Action Plan, and Lawrence Berkeley National Laboratory / Lawrence Livermore National Laboratory. An efficient water data management system will require a comprehensive set of statewide data from various agencies. Thus, cooperation between these groups is necessary to identify overlapping efforts and shared goals, and will ensure that future monitoring and initiatives are most efficient. Greg Smith of DWR pointed out the need to start with collaboration in order to identify and utilize data systems that already exist and are effective. DWR's Strategic Vision and Framework for Integrated Water Management Data and Tools (2012)

recommends efficient cooperation by establishing watershed-based water budgets, rather than using political boundaries (14).

This is similar to the approach taken by the European Union when developing river basin management plans to implement the Water Framework Directive (WFD) (28). As part of the WFD, managing one the largest aquifers in southern Europe, the Mancha Oriental Aquifer, requires that governments, private stakeholders, and other social actors share correct and credible data amongst themselves in order to facilitate meaningful engagement by all (29). Much of Europe's international policies were based on best practices from French water laws in 1964 and 1992, which mandated that states create water policy in partnership with local stakeholders, including industrial businesses, large regional developers, farmers, water suppliers, fishermen, and environmental organizations. As recently as 2016, France has continued to systematize water management by establishing the multi-institutional French Biodiversity Agency, which manages the national information system on water and all water resources monitoring data. This concept translates to the whole of the European Union within the Water Governance Initiative, which tangibly demonstrates the support for collaborative water policy-making, as it is an association specifically dedicated to 'effective, efficient and inclusive water policies in a shared responsibility with the broader range of stakeholders' (30). As Europe has shown, intentionally creating opportunities for stakeholders to come together throughout the decision-making process can provide a venue for local, regional, and state level data users to ask and answer questions, ultimately building capacity and necessary trust for water data management.

Standardization of metrics for ambient water, water supply, and wastewater industries.

Policy, academic, and state organizations such as PPIC, University of California (UC), and DWR have recognized the importance of standardization of data collection and reporting in successfully managing water resources in the future. The results of a workshop held in Davis, California, Establishing a Cloud-based Water and Energy Data Platform, identified data standardization as the main challenge to establishing comprehensive water data management (13). This is because water data can be highly variable in pattern, size, units, terminology, and organization structure. In addition, the non-technical aspects of water data integration are often the most neglected. For example, Mark Cowin, director of DWR, noted as a panelist in the California Groundwater Briefing forum, that the most pressing needs for sustainable groundwater management are human and organizational in nature (10). Even when all necessary technical information exists, data remain useless to water management if not efficiently translatable between fields and users. Dr. Jay Lund, Director of the UC Davis Center for Watershed Sciences, reiterated that any common statewide framework would need the capability to sync with existing and future data systems. CCST (2014) takes this a step further in Achieving a Sustainable California Water Future through Innovations in Science and Technology by recommending integration of future water, energy, and land-use planning (17).

One way that this has been previously addressed for hydrologic data at the national level is through a foundational quality management framework for water data, such as the one created and employed by the U.S. Geological Service (USGS). Through

periodic publication of updated data collection techniques, usage of data quality ratings, and providing raw data in addition to standardized data sets, USGS has been able to set a world precedent for effective data aggregation and presentation. While the USGS hydrologic standards provide an example of the importance of comprehensive data comparability in the utilization and reputation of water data sources (32), California will face the additional challenge of incorporating more disperse and autonomous water data sources. In order to achieve successful standardization in California, several recommendations do indeed include the specific need to establish and enforce a protocol or framework for collecting compatible data.

Greater investment in data collection and water monitoring systems.

Many organizations that take a statewide perspective on water data management identified the need for additional water data to be collected in order to effectively manage water resources, including PPIC, DWR, CCST, and the University of California. Specifically, California has yet to capitalize on the "big data" field, along with other new technologies, such as land- and remote-sensing to create opportunities for accurate data collection (14). The California Water Action Plan (2014) points out that increasing the amount of high quality water data available will ultimately enable better modeling and predictive research to be done, which can enhance California's water future (18). However, as Dr. Steve Weisberg of the Southern California Coastal Water Research Project said, identifying true water data gaps is a necessary first step to ensure that investments aren't made in data just for data's sake.

Investment in data collection can result in improved water-use efficiency, integrated statewide resource management, data driven decision-making, and the ability to meet new regulatory needs (i.e. SGMA, surface water management, and water rights accounting), as exemplified by Australia's commitment to the National Water Initiative in response to their "Millenium drought" in 2004 (31, 33). In contrast, Garcia (2008) found that the need for large amounts of reliable, regional data and predictive models was a major hurdle for water management practitioners to overcome in several Latin American countries that attempted to implement integrated water resources management (34). With the involvement of strong leadership groups, like the Inter-American Development Bank, overcoming these challenges was logistically feasible. However, financial investments to enhance data collection capacity and translate results into action at the local level were lacking and proved to be prohibitive for most stakeholders to perceive any benefits. Within North America, similar barriers exist to expanding the potential benefits of modern data management to multiple levels of the water system.

Data curation that is accessible and transparent.

From a variety of stakeholder perspectives, transparency and accessibility are essential throughout the development of plans to improve California's water data management. Specifically, the University of California, the Delta Stewardship Council, and DWR all argued that making accessibility and transparency should be a priority, allowing stakeholder collaboration to be encouraged, litigation and misinformation to be avoided, and decision-making to be simplified. Further, Naik and Glickfeld (2017) found

that transparency and verifiability were essential elements in the implementation of a successful water data management system in southern California (35). Following the Environmental Data Summit, convened by the Delta Stewardship Council's Delta Science Program, a final report (2015) cited the massive amount of inaccessible environmental water data in California as an example of this need (15). One option to achieve this goal, as referenced by institutions like the Delta Stewardship Council (2015) and the University of California, Davis (2016), is the creation of a single, user-friendly platform for housing and accessing California water data (13, 15). DWR's Gary Darling highlighted the need for any future data management infrastructure to enable users to address real world problems, in addition to the technical and scientific data accessibility priorities.

Previous statewide water data management efforts like the California Integrated Water Quality System (CIWQS) have faced major challenges due to insufficient resources to address challenges such as the poor user interface, data compatibility, and perceived integrity of its data. The resulting obsolescence of this potential water data management resource, exemplifies the importance of accessibility and transparency (22). Once again, the example of Latin America shows that this challenge is not unique to California. The 2006 Global Water Partnership's survey of integrated water management strategy implementation in 95 countries found significant progress in water management policy implementation amongst Latin American countries, but this did not necessarily translate to operational benefits for water data generators or users. In several cases, barriers such as perceived bias of the implementation agencies, discrepancies in

interpretation of water data, and lack of clarity amongst the on-the-ground resource managers were cited as major difficulties (34). Considering and overcoming socio-political hurdles requires significant time and economic investment, but has been shown to be an essential component to translating technical progress in water data management to operational success.

7.3.2 Remaining Questions

After identifying common recommendations among water data generators, regulators, and users, the next step is to consider potential mechanisms for implementing these priorities. However, there were many discrepancies in motivation and perspectives on water data management amongst the publications that were analyzed and amongst the opinions of the stakeholders that were interviewed. These differences are not simple to succinctly and accurately put in words, but they are a notable outcome of this analysis. Therefore, we summarize these potential challenges as a list of questions that should be answered before making future decisions about water data management and/or policy.

Who are the end users of water data? End users should be the first and foremost consideration in conversations regarding data accessibility. Users may include water policymakers, resource managers, the public, and more.

What form should stakeholder collaboration take, and who should be included? Collaboration can occur through workshops or forums, creation of a coalition or neutral

liaison to act as coordinator, or a written agreement or understanding between stakeholders. Collaboration should include continued open communication. Data users and generators at every level should be represented, but an efficient approach may be to start with the largest data users and contributors.

Who should decide the standardized metrics and protocols for data management? While the stakeholder collaboration process should help inform the answer to this question, a neutral facilitator may lead the identification of the most common current collection and reporting practices, while also ensuring that the data are easily accessible by a variety of users.

Which technologies are optimal for homogenizing and comparing water data? Big data management platforms have the ability to translate numbers, units, and terminology, in addition to alleviating the burden of changing practices at the data generation level. Data management tools, such as those being developed within the U.S. Department of Energy (Environmental System Science Community Cyberinfrastructure), are facing challenges of "ingestion, curation, archiving, long-term preservation, and publication" (21). Other modern technologies can automatically create accuracy and precision thresholds to differentiate data reporting quality. Regardless of the technology, it will be important to maintain the context of each data set during standardization and translation.

Which water sectors have the greatest need for more monitoring and data collection? Groundwater, surface water, water rights, environmental water, and flood-vulnerable

areas are some examples of areas mentioned throughout this analysis. However, data collection and monitoring priorities may ultimately be determined by system vulnerability and largest current inefficiencies or unknowns in California's water system.

Additional water data should be collected by whom, and for whom? A state-level investment of resources may be required for maximum improvement of water data collection and monitoring, which can be for the benefit of and use by water managers, users, policymakers, as well as the public.

How can water data security be ensured for data contributors? To create necessary trust, facilitate effective collaboration, and manage uncertainties in data reporting and usage, water data security must be integrated from the beginning of the decision-making process. Answering this question requires consideration of proprietary data. For example, the California State Water Plan (2013 Update) recommends a written agreement between data-sharing institutions that contributes to understanding at local, regional, and state levels as one mechanism for establishing boundaries and mutual expectations, and that can help identify opportunities for building trust between relevant parties (5).

7.4 Conclusions

Through compilation and comparison of recent recommendations made by diverse stakeholders in California's growing water data management infrastructure, this

study has identified several key aspects of water data management that should be prioritized. Further, we have demonstrated that there are tangible examples of success and failure in water data management, which can be utilized. By ensuring that water data in California is managed collaboratively, commonly, reliably, and transparently, modern data management technology can significantly contribute to water sustainability and resilience in the face of challenges posed by climate change and population growth throughout the state.

While the resulting primary recommendations are not surprising, it is useful to be able to present the priorities in a semi-quantitative way to inform ongoing larger projects that are a part of "Data for Water Decision-Making". These common recommendations by diverse stakeholder entities can also elucidate opportunities for common ground and collaboration in the future. Importantly, it enables us to define and begin the process of answering the necessary questions to accomplish these priorities. The technology for efficiently managing natural resource data is sufficiently in place, and proven benefits are evident. However, the political feasibility of a comprehensive water data management system in the state of California has been lacking. Given the recent passage of the Open and Transparent Water Data Act and movement to facilitate conversations about this subject amongst stakeholders, the results of this work are timely in informing policy implementation. While data management remains a salient issue in water policymaking in California, the key recommendations outlined in this article will be important for both establishing and maintaining a sustainable system.

7.5 References

1. Sato T, Qadir M, Yamamoto S, Endo T, Zahoor A. 2013. Global, regional, and country level need for data on wastewater generation, treatment, and use. Agricultural Water Management, 130:1–13.

2. California State Assembly. 2016. A.B. 1755, The Open and Transparent Water Data Act. Division 6 California Water Code, Part 4.9, Sec. 12400-12420.

3. California Department of Finances. 2017. Gross State Product in California: Annual from 1963. https://www.dof.ca.gov/Forecasting/Economics/Indicators/Gross_State_Product/

4. United States Census, 2010: Resident Population Data. Washington, D.C.: Government Printing Office, 2011.

5. California Department of Water Resources (DWR). 2013. California Water Plan Update. https://www.water.ca.gov/waterplan/

6. Mount J and Hanak E. 2016. Water use in California. Public Policy Institute of California. https://www.ppic.org/main/publication_show.asp?i=110

7. Environmental Defense Fund (EDF). 2016. Better Access. Healthier Environment. Prosperous Communities. Recommended Reforms for the California Water Market. https://www.edf.org/sites/default/files/california-water-market.pdf

8. Grantham TE and Viers JH. 2014. 100 Years of California's water rights system: Patterns, trends, and uncertainty. Environmental Research Letters, 9(8):1-10.

9. Public Policy Institute of California, Water Policy Center. 2016. California's Water. http://www.ppic.org/publication/californias-water/

10. Stanford University, Water in the West. 2016. California Groundwater Briefing: Findings and Implications for the Future of California's Water. https://www.waterinthewest.stanford.edu/news-events/events/california-groundwater-briefing-findings-and-implications-future-californias

11. Kiparsky M. 2016. Unanswered Questions for the implementation of the Sustainable Groundwater Management Act. California Agriculture, 70(4):165-168.

12. Public Policy Institute of California, Water Policy Center. 2016. Accounting for California's Water. http://www.ppic.org/publication/accounting-for-californias-water/

13. University of California, Davis, Center for Water-Energy Efficiency. 2016. Establishing a Cloud-Based Energy and Water Platform: A White Paper Summary and Road Map. https://www.cwee.ucdavis.edu

14. California Department of Water Resources (DWR). 2012. Strategic Vision and Framework for Integrated Water Management Data and Tools. https://ww.water.ca.gov/irwm/stratplan/

15. Delta Stewardship Council, Delta Science Program. 2015. Enhancing the Vision: Managing California's Environmental Information. https://www.deltacouncil.ca.gov/enhancing-vision-managing-california-s-environmental-information

16. Surface Water Ambient Monitoring Program (SWAMP). 2014. Review of the Surface Water Ambient Monitoring Program. https://www.waterboards.ca.gov/water_issues/programs/swamp/docs/reports/2014_swamp_review_rpt.pdf

17. California Council on Science and Technology (CCST). 2014. Achieving a Sustainable California Water Future through Innovations in Science and Technology. https://ww.ccst.us/publications/2014/2014water.php

18. California Natural Resources Agency (CNRA). 2014. California Water Action Plan. https://www.resources.ca.gov/california_water_action_plan/

19. Kiparsky M, Milman A, Vicuna S. 2012. Climate and Water: Knowledge of Impacts to Action and Adaptation. Annual Review of Environment and Resources, 37:163-194.

20. Libecap G and Donohew Z. 2009. Maintenance and Dissemination of a Water Transfer Database for 12 Western States, 1987-2008. University of California Water Resources Center.

21. Goldstein N, Newmark R, Burton L, May D, McMahon JE, Whitehead CD, Ghatikar R. 2007. Assessing security needs of the multifaceted relationships of energy and water providers. Lawrence Berkeley and Lawrence Livermore National Labs. Report number LLNL-UCRL-TR-234269.

22. Southern California Coastal Water Research Project (SCCWRP). 2007. Preliminary Report of the California Integrated Water Quality System Review Panel. https://www.waterboards.ca.gov/ciwqs/

23. Southern California Coastal Water Research Project (SCCWRP). 2006. Review of California's Surface Water Ambient Monitoring Program (SWAMP). https://www.waterboards.ca.gov/water_issues/programs/swamp/

24. U.S. Geological Survey (USGS). California Water Data homepage. https://ca.water.usgs.gov/ Accessed October 18, 2016.

25. Center for Watershed Science, University of California, Davis. HOBBES Project homepage. https://hobbes.ucdavis.edu/ Accessed October 18, 2016.

26. Conservation Biology Institute. California Water Planning Information Exchange (Water PIE) homepage. https://cadwr.databasin.org/ Accessed October 18, 2016.

27. New Jersey Department of Environmental Protection. New Jersey Water Monitoring Council homepage. https://www.nj.gov/dep/wms/wmcchome.html Accessed October 18, 2016.

28. Grizzetti B, Lanzanova D, Liquete C, Reynaud A, Cardoso AC. 2016. Assessing water ecosystem services for water resource management. Environmental Science & Policy, 61:194-203.

29. Sanz D, Calera A, Castaño S, Gomez-Alday JJ. 2016. Knowledge, participation and transparency in groundwater management. Water Policy, 18(1):111-125.

30. Colon M, Richard S, Roche PA. 2017. The evolution of water governance in France from the 1960s: Disputes as major drivers for radical changes within a consensual framework. Water International, 43(1):109-132.

31. Australia National Water Commission (ANWC). 2014. 10 Years of Water Wins: Australia's National Water Initiative. https://www.apo.org.au/node/40198

32. Larson S, Hamilton S, Lucido J, Garner B, Young D. 2016. Supporting Diverse Data Providers in the Open Water Data Initiative: Communicating Water Data Quality and Fitness of Use. Journal of the American Water Resources Association, 52(4):859-872.

33. Baldwin C, Tan P, White I, Hoverman S, Burry K. 2012. How scientific knowledge informs community understanding of groundwater. Journal of Hydrology, 474:74-83.

34. Garcia L. 2008. Integrated Water Resources Management: A 'Small' Step for Conceptualists, a Giant Step for Practitioners. Water Resources Development, 24(1):23-36.

35. Naik KS and Glickfeld M. 2017. Integrating water distribution system efficiency into the water conservation strategy for California: A Los Angeles perspective. Water Policy, 19(6):1030-1048.

Chapter 7b

Conceptualizing Sustainable Diets in Vietnam: Minimum Metrics and Potential Leverage Points

Mayton, H.M., Beal, T., Rubin, J., Sanchez, A., Heller, M., Hoey, L., de Haan, S., Duong, T.T., Huynh, T., Dhar Burra, D., Khoury, C.K., Jones, A.D. Conceptualizing sustainable diets in Vietnam: Minimum metrics and potential leverage points. In preparation for *Food Policy* (2019).

Abstract

A major barrier to achieving sustainable diets is the lack of clear interventions points that will positively influence multiple sectors of the food system. There is an urgent need to understand what policy approaches and interventions will most effectively enhance the sustainability of diets in rapidly urbanizing low- to middle-income countries. To address this need, this work combines the input of diverse stakeholders and analysis of existing datasets to develop a sophisticated conceptual framework for sustainable diets that is locally relevant to Vietnam, using a process that is generalizable to other developing countries. The resulting framework includes 235 unique, measurable indicators within eight domains: (1) environmental health, (2) sociopolitical context, (3) nutrition, (4) food production, (5) food processing and distribution, (6) food access and consumption, (7) food loss and inorganic waste, and (8) food safety and water quality. This conceptual framework was employed in a participatory workshop that brought together 50 stakeholders from diverse areas of expertise to identify and prioritize specific metrics for measuring sustainable diets. Based on the results of the stakeholder workshop, expert interviews, and characterized datasets, a comprehensive set of minimum metrics for each domain was produced and key leverage points that are likely to have an impact across multiple sectors were identified. Leverage points include food quality and safety, agricultural chemical usage, food waste, and water. These formative findings are an essential starting point for enhancing evidence-based policymaking in Vietnam, centered on cultivating more sustainable diets.

7.6 Introduction

Diets in low- and middle-income countries (LMICs) and the food systems that underlie them are changing rapidly alongside rapid changes in economic development, globalization, and urbanization (1, 2). The concept of a "sustainable diet" has received increasing attention in recent decades given recognition of the numerous, interconnected ways that food systems influence not only food availability, diet quality, and health outcomes, but also ecosystems, use of natural resources, livelihoods, and social equality (3, 4). Sustainable diets have been defined as those with "low environmental impacts, which contribute to food and nutrition security and to healthy life for present and future generations. They are protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable; nutritionally adequate, safe and healthy; while optimizing natural and human resources" (5). Interest in the concept of sustainable diets has been spearheaded by both government and NGO efforts, and has resulted in incorporation of environmental elements into the dietary guidelines of national governments including, among others, the Netherlands, United Kingdom, Brazil, and Qatar (6, 7). Still, policy approaches to promote sustainable diets remain largely uncoordinated, as a clear understanding of how to operationalize this broad, advocacy-oriented definition, has not yet emerged (8).

A variety of conceptual frameworks have been developed to define and inform the measurement of sustainable diets and food systems more broadly (9-13). These conceptual frameworks vary according to the context and their intended use, the priorities of the stakeholders that conceived the framework, as well as the included domains (e.g.,

environmental, social, economic) and the level of granularity and abstraction within each domain. Domains of food systems overlap, so assigning boundaries between them is problematic, and the decision to include or exclude particular components from a sustainable diets framework is subjective and may result in frameworks that are incompatible with bureaucratic policies and priorities. Decision-makers must understand the roles and interactions between food system domains to define concrete actions that effectively address specific constraints to sustainable diets. They must also have access to and understand the metrics that can be used to measure these different domains and the data sources needed to construct such metrics.

To our knowledge, most sustainable diets conceptual frameworks have been developed from a global perspective, with rare exceptions. Given the large heterogeneity in food system contexts between countries, global-level frameworks are unable to account for unique political, geographical, and cultural contexts, limiting the usefulness of these approaches at the national level, where most of the practical decisions that influence sustainable diets are made (14). Contextually relevant frameworks for individual countries or somewhat homogeneous regions would allow for more actionable decision-making at the national level. For example, Downs *et al.* (2017) developed a framework for the purpose of assessing the sustainability of national policies in Nepal (15). However, they developed their framework based on existing literature and did not interact with national policy makers or include feedback from local stakeholders, who are most influenced by national policies. Regional or national-level frameworks are needed that are both contextually appropriate and informed by inputs from local decision-makers

and other stakeholders who are directly impacted by policies, especially since tradeoffs often occur that benefit one geography at the expense of another.

Given the limitations of current conceptual frameworks, we designed a contextually relevant sustainable diets framework for Vietnam, incorporating input from national decision-makers and other local stakeholders. Like many countries, the Vietnamese government and international aid organizations in Vietnam have considerable interest in achieving the Sustainable Development Goals (SDG) (16), which are inextricably linked with sustainable diets. We grounded the development of our conceptual framework in the Vietnamese context from the beginning of the study, while also drawing from the scientific literature to incorporate the breadth of knowledge and theory already developed on sustainable diets.

Our resulting framework includes 8 essential domains of sustainable diets, including the interactions between them, and nearly 250 non-overlapping metrics divided according to these domains. We used this framework and set of metrics alongside stakeholder engagement to identify preliminary leverage points that emerged across multiple domains. Leverage points refer to specific policy areas that can improve multiple aspects of the sustainability of diets in Vietnam, which represent opportunities in which actions like stakeholder collaboration and data sharing can result in maximum impact for national dietary sustainability efforts.

7.7 Methods

We developed a conceptual framework and associated metrics for sustainable diets in Vietnam with the purpose of enabling decision-makers to identify intervention points that would create positive improvements across multiple sectors of the food system. Through a review of the literature, this framework was organized to allow for identification and understanding of interactions that exist within domains of sustainable diets. In particular, the framework includes components from sustainable food system frameworks described by Gustafson *et al.* (2016) and unpublished literature by Melesse *et al.* (in progress) (11). In addition to reviewing relevant literature, we used three distinct but complementary approaches: (1) data characterization of existing data sources relevant to sustainable diets, (2) informal interviews with decision-makers, and (3) a stakeholder workshop of national experts representing diverse domains of sustainable diets. While these processes generally occurred in sequential order, there was considerable overlap between iterations of data characterization and interviews. The resulting conceptual framework for sustainable diets in Vietnam is therefore based on a mix of both data dependent and data independent approaches, which are described in detail below.

7.7.1 Data characterization

We reviewed and characterized existing datasets on food, agriculture, the environment and nutrition at the global, national and local levels in Vietnam. These included national and global surveys, academic articles, white papers, and reports. To identify sources, we began by collectively listing known resources on relevant topics. We

then conducted a non-systematic keyword search on Google and Google Scholar to identify additional sources. Lastly, we conducted informal interviews with approximately a dozen Vietnamese decision-makers who had expertise in disparate areas of sustainable diets to obtain further resources.

Each dataset was initially characterized by a set of keywords, the organization that collected the data, year the study was conducted, public availability, geographic location, sample size, and other characteristics. After characterizing 40 data sources, we collaboratively categorized the keywords into eight domains: (1) food production, (2) food processing and distribution, (3) food loss and inorganic waste, (4) food access and consumption, (5) food and water safety, (6) nutrition, (7) sociopolitical context, and (8) environmental health. The definition of each domain is presented in Table 7.5.

7.7.2 Informal interviews

To build a more robust understanding of current research and policy priorities that should inform the chosen domains within the framework, we conducted informal interviews with food system actors from eight agencies in Vietnam, including non-government organizations, national and international research institutions, private companies, and government agencies. These interviewees were chosen based on their diversity of expertise within the Vietnamese food system and interest in data-driven decision-making towards sustainability. They included representatives from the following institutions: (1) General Statistics Office; (2) Hanoi University of Public Health, Center for Public Health and Ecosystem Research; (3) Institute for Agriculture and the Environment; (4) Institute

Table 7.5 Definitions of each of the eight domains of sustainable diets.

Domain	Working definition
Food production	This domain includes production enablers, practices, inputs, and outputs. The enablers considered are primarily environmental enablers including precipitation and temperature. The domain is then broken up into practices such as the use of crop rotations or cover crops, use of technology, inputs including water usage, fertilizers, or antibiotics and finally the agricultural outputs.
Food processing and distribution	This domain considers the points of process and distribution of the agricultural outputs from post- harvest to the distribution channel which can be represented by either collectors, traders, retail buyers, farmer groups, or direct sales to consumers. This domain also considers forms of food processing including fortification or additives, the energy and water used to distribute and process, and distribution infrastructure such as packing houses or cold storage.
Food loss and inorganic waste	This domain considers the various points of food loss along the supply chain from the pre-harvest stage to final waste management practices. At pre-harvest food loss can occur as a result of economic shocks, weather shocks, or political shocks. The domain then includes crop loss from pests, weeds and others, post-harvest loss from storage, transportation or others, and waste management such as landfill, compost, or others.
Food access and consumption	This domain includes the metrics *consumer food access*–both physical access and socioeconomic factors that influence access; *food preparation*, including preparation methods and knowledge of these methods as well as associated energy use; and *diet quality*, which includes fiber and the nutrients in food and water (also represented through dietary diversity) and child feeding (feeding practices and the quality of complementary foods).
Food safety and water quality	This domain includes food and water safety at various stages along the supply chain where food and waterborne illness can occur. It considers sanitation and hygiene to include sewage infrastructure, latrine quality, hygiene practices, and potable water access. The domain considers the microbial pathogens and chemical contaminants that can affect the safety of food. Water quality includes solids, microbial measures, and chemicals. Disease burden from food safety includes outbreaks, enteric infections, mortality, and antimicrobial resistance.
Nutrition	This domain represents nutritional status and associated physiological consequences. Metrics include micronutrient status, anthropometric measures, nutrition-related non-communicable diseases, and nutrition-related birth outcomes and morbidity.
Sociopolitical context	This domain includes socioeconomic factors, gender equity, community health, labor, farmer livelihoods, access to services, animal welfare, political factors, regulations, and standards. It is a domain that represents the many and complex elements of the enabling environment of the food system. It includes both the physical and social elements that contribute to the sustainability of the food system.
Environmental health	This domain represents the many and interconnected elements related to environmental health that are impacted or impact the food system. It includes soil health, both its physical and chemical properties, land use, environmental water quality, climate change, GHG emissions, drought, biodiversity, habitat loss, species diversity, and genetic diversity.

of Policy and Strategy for Agriculture and Rural Development; (5) International Livestock Research Institute; (6) National Institute of Nutrition; (7) Rikolto in Vietnam (previously VECO Vietnam); and (8) Vietnam Academy of Agricultural Sciences. Interviews primarily involved the introduction of project idea, discussion of research and policy priorities, and identification of notable datasets pertaining to sustainable diets in Vietnam.

7.7.3 Participatory stakeholder workshop

The third component of our study involved a participatory workshop that we designed and conducted to gather feedback on the defined domains within the framework and identify shared priorities between them. To identify potential participants, we first compiled a list of all in-country authors from the data characterization sources, informal interviewees, and recommended domain experts from the interviewees and our colleagues, resulting in 150 unique food systems stakeholders. We then identified 48 representative stakeholders to invite to participate in the workshop based on equal representation from various sectors of the food system and areas of expertise (Figure 7.1). Six individuals were chosen and invited to represent each of eight domains that were identified through the literature for inclusion in the final conceptual framework. One key stakeholder from the informal interview process was chosen as the group coordinator for each domain group. Prior to the workshop, all participants were asked to provide information about their area of knowledge and expertise via an online form.

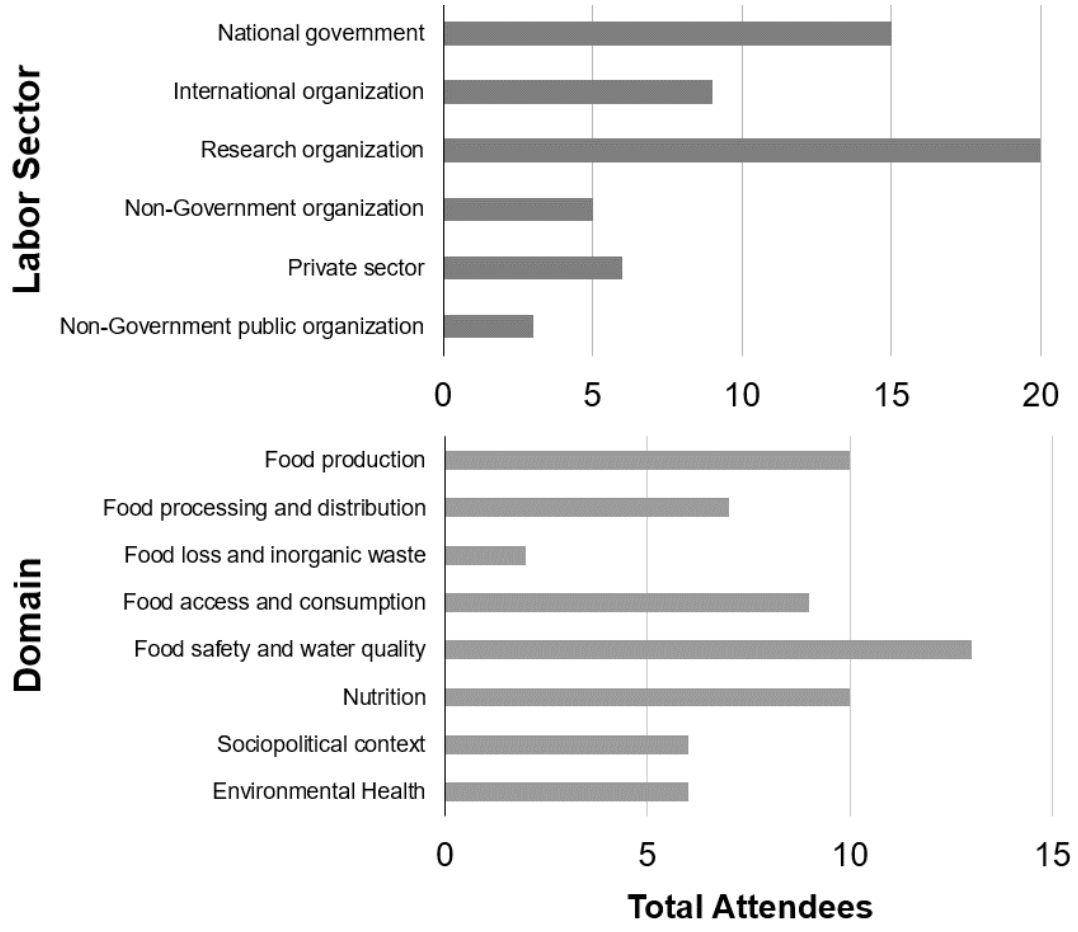

Figure 7.2 Summary of the labor sector (top) and relevant domain of sustainable diets (bottom) represented by the attendees of the stakeholder workshop.

The workshop was designed to facilitate stakeholder discussions both within and between domains of sustainable diets. In each domain group, stakeholders were asked to collectively write a definition of their domain to broadly describe the types of data that would be included in it, and propose priority metrics given the following three criteria: (1) feasibility of data collection (accounting for the technical, political and logistical hurdles and costs for gathering data); (2) representativeness of the domain (data that are more holisitic or are cumulative of other potential metrics within the domain, rather than

highly specific data points were considered more representative); and (3) alignment of the indicators with decision-making priorities in Vietnam (i.e., significant metrics for the policy agenda at various levels of Vietnamese government).

Following this exercise, workshop participants were provided the previously developed conceptual framework. Participants were asked to review the proposed framework definitions for comparison with their own, and submit a final version of the domain definition, along with a list of priority metrics within their domain. Workshop participants were then asked to review the priority metrics of other domains outside of their direct field of expertise. This exercise allowed participants to review the priority indicators of other domains and identify those that seemed the most essential and useful in relation to their own. The results of this exercise are presented in Table 7.6. Participants were encouraged to consider the importance of specific data sets and data sharing between domains to be used for policy development to advance sustainability of diets.

7.7.4 Identifying leverage points

From the collection of metrics and priorities that resulted from the workshop, several themes were identified as being notable in the discussions and outcomes of multiple domains of sustainable diets. Based on the distribution of themes, four distinct facets of sustainable diets were identified as leverage points because of the shared interest

Table 7.6 Summary of outcomes of stakeholder workshop.

Metrics Generated by Domain Group	Selected by: Percent	Total
Food Production		
Climate indicators	31%	19
Cost of production (input/output)	15%	9
Quantity and price by target market	11%	7
Volume of waste / byproducts generated*	10%	6
Proportion meeting quality standards*	8%	5
Proportion of area planted by variety	7%	4
Proportion by farm size	7%	4
Land use intensity composite indicator	5%	3
Proportion for domestic vs. export	3%	2
Total volume of production	3%	2
Food Processing and Distribution		
Quality control protocol*	20%	11
Mass and quality of output products	19%	10
Nutritional quality*	13%	7
Management and capacity of distribution	13%	7
Price of products	11%	6
Mass and quality of input products	9%	5
Number of distribution channels	7%	4
Number of products sold (at scale, per site)	4%	2
Number of farmers and producers	2%	1
Number of customers	2%	1
Food Access and Consumption		
Food affordability	23%	16
Nutritional knowledge	19%	13
Consumer awareness	17%	12
Food traceability*	10%	7
Volume of quality food*	7%	5
Fruit and vegetable consumption	7%	5
Adoption of food safety at home*	6%	4
Distances to quality food market	6%	4
Restaurants and schools	4%	3
Water quality*	1%	1
Food Loss and Inorganic Waste*		
Harvest techniques	27%	12
Storage capacity	18%	8
Processing	18%	8
Restaurant food waste	14%	6
Distribution channels	11%	5
Market factors	5%	2
Nutrient management	5%	2
Infrastructure	2%	1
Irrigation management*	0%	0
Weather factors	0%	0

Metrics Generated by Domain Group	Selected by: Percent	Total
Food Safety and Water Quality		
Chemical contaminants*	25%	14
Hygiene practices	24%	13
Heavy metals	16%	9
Water quality*	13%	7
Outbreaks	9%	5
Bacteria	7%	4
Coliforms	2%	1
Mortality	2%	1
Antimicrobial	2%	1
Latrine quality	0%	0
Nutrition		
Food security index	27%	15
Nutritional status	25%	14
Diet diversity / species richness	16%	9
Micronutrient deficiency	11%	6
Food composition / quality*	11%	6
Under 5 Mortality	5%	3
Dietary intake	2%	1
Morbidity	2%	1
Sociopolitical context		
Food quality control policy and standards*	20%	11
National Plan for sustainable food system	20%	11
Social media	16%	9
Technology transfer for farmers policy	13%	7
Education plan	11%	6
Food security and food safety law*	7%	4
Consumer demand research	7%	4
Import and export law	4%	2
Labor code and social policy	4%	2
Communication plan for decision-makers	0%	0
Environmental Health		
Chemical usage*	25%	15
Water quality*	21%	13
Soil chemistry	21%	13
Air quality	11%	7
Land use	8%	5
Nutrient content	5%	3
VNTC Standards of Vietnam	3%	2
Species population density	3%	2
Environmental yield losses	2%	1
pH	0%	0

between multiple domains. Metrics that were explicitly mentioned in more than one domain and were present as key discussion points by workshop participants were included. These leverage points are detailed in the following Results section.

7.8 Results and Discussion

7.8.1 Conceptual framework

The resulting conceptual framework included eight interconnected domains, which encompass a total of about 250 unique, measurable metrics within them, as shown in Figures 7.2 and 7.3. The domains are: 1) environmental health; 2) sociopolitical context; 3) nutrition; 4) food production; 5) food processing and distribution; 6) food access and consumption; 7) food loss and inorganic waste; and 8) food safety and water quality. These domains reflect adaptation of established theory on the components of sustainable diets, as well as context-specific priorities in Vietnam. For example, Jones *et al.* (2016) and Downs *et al.* (2017) identify ecological (environmental health), social (sociopolitical context), and human health (nutrition) categories as primary considerations in defining and measuring sustainability of diets (6, 15). However, we found that disentangling and explicitly including domains related to the production, processing, and consumption of food was essential within our framework.

Explicitly outlining tangible food chain domains allowed for prioritization of metrics for measuring improvements in food system sustainability that are unique to rapidly developing LMICs. The content for these five domains of food chain outcomes was ultimately decided based on the current needs and context in Vietnam. Food

production, food processing and distribution, and food access and consumption represent the three steps of growing, handling, and eating food. Additionally, data aggregation and informal interviews elucidated the importance of including food waste and food safety as unique domains of sustainable diets in Vietnam. For example, the food and water safety domain represents growing consumer and governmental concerns in Vietnam over chemical and microbial contamination in food markets (17-19).

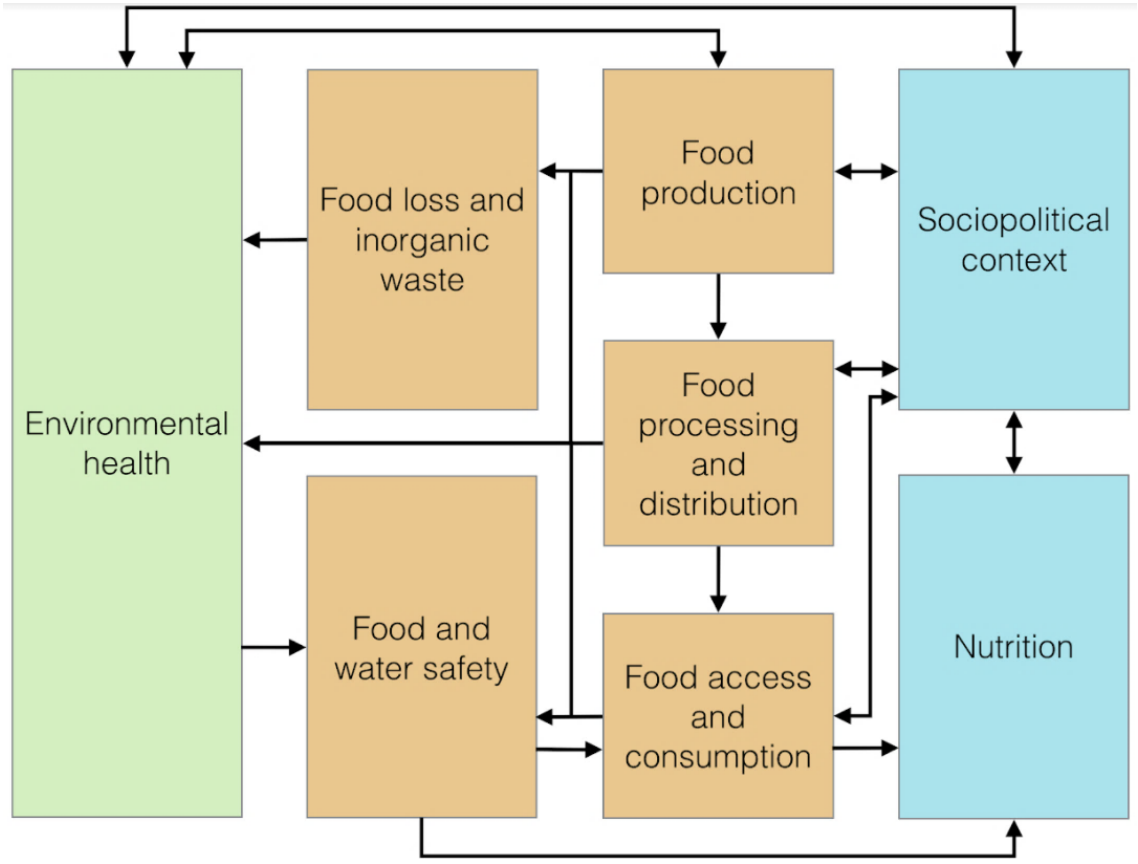

Figure 7.3 An overview of the eight interconnected domains of sustainable diets in Vietnam, including ecological (green), human (blue), and food chain (orange) factors.

By separating these eight domains and eliminating overlap, this study considered specific data-based metrics and stakeholder expertise within the confines of each domain (defined in Table 7.5). This framework also facilitates the identification of potential leverage points at the intersections or connections between each of the domains. Additionally, the quantity and quality of data available to inform decision-making within each domain provides some insight into the existing capacity to collect certain data and priorities among Vietnamese decision-makers and researchers.

7.8.2 Minimum metrics

Based on the data sets used for data characterization, which spanned the various domains of sustainable diets and existing literature on key components of sustainable diets, a comprehensive list of potential indicators was generated. These are summarized in Figure 7.3. The priority metrics within each domain were identified (with systematic stakeholder feedback) based on the three criteria described above, namely: 1) feasibility of collecting the necessary data, 2) ability to represent the domain holistically, and 3) alignment with national policy priorities. A summary of the highlights for each domain follows.

Figure 7.4 Comprehensive list of unique metrics for sustainability of diets within each domain**.**

Food production

Within the food production domain, the top metrics for measuring sustainability can be summarized as scale of production, climate adaptation, regulations and standards, and land use intensity. Scale of production refers to both the total volume produced, and associated costs and income, per farm or other agricultural operation. This metric, along with land use intensity, provides insights on sustainable food production in Vietnam, which is an essential shift in order to meet projected global food demand (20). Climate adaptation refers to both climate indicators (average rainfall, temperature, seasonality) and the prevalence of agricultural practices that provide resilience against extreme weather (21). Regulations and standards refers to the access to, adoption of, and compliance with national food production standards and/or specialty certifications, such as organic, Good Agricultural Practices (GAP), and fair trade (22).

Food processing and distribution

During the workshop, stakeholders opted to split this domain into two separate lists of priorities for food processing and food distribution. Key metrics for measuring sustainability in the food processing and distribution domain include trade and distribution rates, total processed food, energy use, and technology use. The scale of trade and food distribution might be measured by numbers of sales and products for sale, amount and quality of food imports/exports, or capacity of food distribution systems. This broad metric, as well as the total amount of processed food, are important indicators of the efficiency and industrialization of the food system over time (1). The metrics also

represent proxies for assessing resource inputs in the value chain, representing the demand for more cheap, packaged, and often nutrient-poor foods (23, 24). The types and amounts of energy and technology employed in the food processing and distribution sector is also included, as these are specific inputs that are reflective of the costs, waste, and scale of operations.

Food access and consumption

Food security, consumer awareness, and nutritional knowledge were identified as key topics under the domain of food access and consumption. Food security is defined by the UN as "the condition in which all people, at all times, have physical, social and economic access to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (25). Thus, measuring food security involves many individual indicators, such as household poverty rate and dietary diversity, or an aggregate indicator such as the Global Food Security Index (26). Household poverty rate is the proportion of the population with a monthly income below the poverty line for urban and rural areas, adjusted for price changes. It is collected at the national level in Vietnam every two years and analyzed by the General Statistics Office (GSO) and in the Vietnamese Household Living Standards Survey (VHLSS). Household poverty rate is highly representative of this domain and influential to the nutrition domain because it is often correlated with other determinants of malnutrition such as education, nutritional knowledge, sanitation and hygiene, and infectious disease (27-29). Dietary diversity can be measured with validated dietary diversity indicators for adult women and

children 6-23 months (30, 31). Consumer awareness and nutritional knowledge were also identified as priorities by workshop participants, but indicators for these topics are not routinely collected in Vietnam (32).

Nutrition

Stakeholder participants listed food security and dietary diversity/quality as priorities under this domain, but we discussed them in the food access and consumption domains. Other priorities identified that are relevant to the nutrition domain are nutritional status and under-five mortality. Markers of nutritional status include anthropometry and micronutrient status (33). Data on population-level micronutrient status is not easily or affordably collected regularly at the national level and thus is not a realistic priority metric. Anthropometry of children under five and women of reproductive age, however, are easy and affordable to collect and are available annually in Vietnam (32). For children under five, useful anthropometric indicators include stunting (representative of chronic undernutrition and/or infection, and can have long-term developmental consequences), wasting/thinness (indicative of acute malnutrition, such as famine and/or infection, and can lead to death if untreated), and overweight/obesity (associated with high body fatness, and increases risk of current and future noncommunicable diseases as well as socioemotional problems (34). For adult women, useful anthropometric indicators include stunting, thinness, and overweight/obesity. Under-5 mortality rate is the risk of a child dying before the age of

five. The primary causes globally are prematurity, acute respiratory infections, complications during birth, congenital anomalies, and diarrhea (35).

Food safety and water quality

The metrics for assessing sustainability within the food safety and water quality domain included total bacteria and coliforms, heavy metals, turbidity of water sources, and food- and waterborne illnesses/mortality. The presence of bacteria and coliforms (in colony forming units per volume) and heavy metals (in parts per million) on food or in water are relatively simple measures of microbial and chemical contamination, respectively (36). Regulation and measurement of these indicators provide insight on the hygiene quality of the food and water systems in place. Water quality that is used for irrigating, washing, and cooking food can be generally assessed using turbidity (in Nephelometric Turbidity Units). These three metrics are present in World Health Organization guidelines (37), and represent opportunities for significant health and sustainability improvements in Vietnam. Lastly, food- and waterborne illnesses and mortality statistics within the population can capture the improvements or areas of need for food and water quality improvement throughout the country (38).

Food loss and inorganic waste

In the food loss and inorganic waste domain, weather shocks, storage capacities, harvest and management methods, and recycling were the priority metrics for measuring sustainability. Regulation of these indicators allows for measurement of food loss at

several points along the supply chain, form the pre-harvest stage to final waste management practices. Weather shocks (such as number of extreme weather events) as well as harvest practices (e.g. % of land harvested by machines) can provide an indication of the amount of food lost at the pre-harvest stage (39). After leaving the farm, the capacity of processing facilities, wholesalers, and/or distributors to adequately store food (e.g. number of cold storage facilities, size of facilities, or amount of product that moves through facilities per unit of time) before it can be sold is an important indication of food loss, especially when coupled with information regarding demand for an item. For example, it was noted during the workshop that rice is often lost at this stage due to inadequate storage, particularly when demand was low. Recycling is considered to be a priority metric as these services are not currently offered at the municipal level in Vietnam, similar to other LMICs (40).

Environmental health

Soil health, land use, water quality, and pesticide usage were the top metrics for measuring sustainability within the environmental health domain. These metrics represent many interconnected elements related to environmental health that are impacted by or impact the food system. Soil health can be measured in terms of its chemistry and composition, including the microbial diversity, nutrient content, pH, and the presence of heavy metals (41). Soil health represents the long-term capacity of the land to produce nutrient-rich foods for a growing population, as well as its capacity to sequester carbon as atmospheric CO_2 levels continue to rise (42). Use of hazardous chemical compounds

continue to be primary means of pest management among Vietnamese farmers, including organophosphate, pyrethroid, and carbamate compounds. Traces of banned organochloride compounds persist in soil, food and water sources (43). High levels of exposure to pesticides can cause health effects such as skin irritation, headache, eye irritation, dizziness, shortness of breath, vomiting, fever, diarrhea, convulsions, and in severe cases, death (44). While farmers and farming communities face highest risk of harmful exposures, consumers are concerned for the safety of their food due to a widespread perception that pesticides are overused on food products (45). These chemicals and other can leach into waterways, negatively affecting crop, animal, and soil productivity. The phosphates and nitrates that serve to promote plant growth reduces the aerobic capacity of aquatic ecosystems, thus reducing the number of organisms it can accommodate within that ecosystem (46). The pollution of water bodies due to contamination caused by inadequately treated discharge from agricultural activities not only has negative impacts on organisms and plants found in the aquatic ecosystems, but also to the natural biological population as a whole. Water quality thus both impacts and is impacted by agricultural processes, making it an important indicator of sustainability within this framework. Longitudinal measurement of land use (ha, purpose, and kg output) can be used as both a measure of development and to better understand how space is being allocated within the region. As the population continues to grow, this metric will be essential in describing how the country will feed the population, and which markets will dominate.

Sociopolitical context

In the sociopolitical context domain, education, labor, farmer services, and agricultural imports and exports were selected to represent minimum metrics for assessing sustainability. Workshop participants focused on education policy for food system actors, such as researchers, consumers, and traders. However, indicators such as average education level of the entire population or literacy rates might be more readily available measures (47). The same goes for labor policy and regulations, which might be assessed using distribution of labor by gender and age, average wages, and strength of work protections. Services provided to farmers in Vietnam, such as training and education, marketing opportunities, subsidies, and access to land and transportation, are an excellent indication of vested interest in supporting small farms at the national level. Enabling small farms to survive and flourish in parallel with the industrialization of a lower-middle income country's agricultural system can be a hallmark of sustainable development (48, 49). Lastly, while quantities of import and exports were previously discussed as a part of food access and distribution, the trade policy behind those exchanges are an important political aspect of promoting sustainable diets.

7.8.3 Potential leverage points

In order to improve evidence-based decision-making for sustainable diets, it is essential that research and data flows between the different domains of expertise that traditionally operate in isolation. By allowing workshop participants to review the priority metrics of other domains, commonalities could begin to be recognized. Based on

interactions and overlapping priorities between the domains in the conceptual framework, we identified several potential leverage points for improving multiple aspects of sustainable diets, which are denoted with a * in Table 7.6. As opposed to being specific metrics for measuring sustainability, these leverage points are general policy areas in which investment in data sharing, data generation, or data quality may result in benefits for multiple stakeholders, spanning multiple domains of sustainable diets.

Food quality and safety

The World Health Organization recently reported that the Western Pacific region, including Vietnam, ranked second in the world in foodborne illness, with over 125 million people impacted annually (50). Concerns about microbial and chemical food quality and safety are present in the news media, government, and academic sectors of Vietnam (18, 51, 52). In fact, a 2016 survey by VECO Vietnam found that more than 97% of respondents in Hanoi were "worried" or "extremely worried" about the quality of their food (53). This was exemplified within the stakeholder workshop as nutrition and/or hygiene indicators appeared in nearly every domain, while they were expected to be largely confined to the food safety and water quality and nutrition domains. Depending on the audience, food quality might refer to microbial contamination, pesticide residue, or unhealthy composition. However, these are all integrally connected, which presents an opportunity for these parties to collaborate in pursing solutions.

Within the nutrition domain, "food quality and composition" was an explicit priority metric and was in the top five, as voted on by other workshop attendees. "Food

quality control policy and standards" received the most votes in the sociopolitical domain. Similarly, in the food processing and distribution domain, "food quality control protocol" was the top selected metric by the stakeholders. "Proportion meeting quality standards" and "adoption of food safety at home" were two other examples of related metrics from the food production and food consumption domains, respectively.

Food safety risks exist throughout agricultural supply chains and may be exacerbated by prioritizing some sustainable practices (54). Promoting food safety and quality remains an issue even in developed countries (55), but the actual burden of disease is more difficult to measure in low- to middle-income countries with limited reporting infrastructures. However, the fact that many entities are already aware and concerned about this issue creates an opportunity for collaboration in pursuit of sustainable solutions. An important first step will be to clarify the food quality and safety goals in Vietnam, followed by consideration of how achieve them while also being environmentally and socially sustainable. For example, minimizing chemical usage would have to be achieved without increasing product loss, protecting cultural traditions of open-air street markets, and creating access to fresh, local food in an urbanizing environment.

Chemical usage in agriculture

Controlling pesticides and fertilizers is an emergent priority in the environmental and food safety domains. Within the food safety and water quality domain the "chemical contaminants" metric was the clear favorite metric amongst the stakeholder workshop

participants, receiving 25% of the total votes within that domain. In the environmental health domain, “chemical usage” was also the top metric with 25% of the workshop attendees’ votes. While stakeholders working separately on environmental sustainability and food safety issues as they pertain to agricultural chemicals may be addressing the opposite ends of the food chain, opportunities to share data and information could be extremely beneficial to their a shared policy agenda.

Stakeholders at the workshop pointed out the additional impacts of consumer perception and social aspects of chemical applications in agriculture, as communication technology enables a more informed and concerned population. An actualized and advertised reduction in chemical usage could also mitigate the nutritional losses from consumers who may avoid purchasing fresh produce that they perceive as contaminated or dirty. One way to test this and its efficacy as tool for sustainable diets, as participants said, would be to start with a well-advertised and trusted organic or pesticide-free produce label.

In most sustainable food systems literature, the challenge of reducing pesticides, herbicides, and fertilizers in the agricultural system is categorized as a purely environmental pursuit. For example, the Barilla Center “Fixing Food” report (2017) points to this as a specific challenge in addressing SDG: Protecting ecosystems (Target 15) (56). However, the benefits of reducing chemical usage in agricultural benefit both the ecological and human aspects of sustainable diets by minimizing greenhouse gas emissions from agricultural chemical production and human exposure to carcinogenic or otherwise unhealthy chemicals during application and potentially consumption (44).

Water

Water is used for a variety of purposes including hygiene, irrigation, and drinking, and therefore impacts many of these domains of sustainable diets. It is therefore no surprise that it appeared as a priority metric in the food access and consumption, food safety and water quality, and environmental health domains. Arguably, water can be considered in nearly every other domain as a component of "production inputs," "input products," and "irrigation management" in the food production, food processing and distribution, and food loss and inorganic waste domains, respectively. Although Vietnam has improved its water supply infrastructure in the past few decades, many rural parts of the country, who are often the poorest communities, have not seen significant improvement. It is reported that only 60% of the rural population has access to safe water and sanitation (38). Even as a low-middle income country, as much as 90% of wastewater is released to the environment without treatment in Vietnam (57). Similar to food waste, shared investments in water quality and distribution stand to create significant improvements for sustainable and healthy food systems in Vietnam.

Food waste

Out of 40 existing datasets on various domains of sustainable diets, there were numerous datasets with indicators for nutrition and environmental health, but little information on food loss and inorganic waste. Similarly, it was a challenge to identify stakeholders with expertise in this domain. Still, "volume of waste and byproducts" was one of the top five metrics identified in the food production domain. The food loss and

inorganic waste domain priorities ultimately included several metrics that overlap with other domains, such as “harvest techniques”, “processing steps” and “distribution channels”. Therefore, there may be a significant amount of low-hanging fruit within this domain, with many benefits to be gained by investing in food waste data collection and by ensuring that relevant existing stakeholders are integrated into the food system (58).

During discussion, workshop participants identified the category of food loss and inorganic waste as the primary topic that was missing from the overall sets of metrics that had been chosen and presented. In general, there was agreement that initial food waste estimates could come from expected values based on food production or from small-scale surveys that can be extrapolated and scaled up. Several stakeholders proposed existing surveys that food waste questions might be added to, exemplifying the logistical value of collaboration amongst these domains.

Most of the recent literature on sustainable diet and food system frameworks includes a designated food loss and waste component, as it is known to be a primary contributor to greenhouse gas emissions and an important opportunity for redistribution to improve food security (59, 60). It even earned its own SDG: Cut per capita food waste in half by 2030 (Target 12). In the policy sphere, recommendations such as implementation of nationwide recycling programs, education campaigns, and increasing access to cold storage facilities are well-known, but it is difficult to enact and measure progress without major investments in waste management infrastructure. While reducing food loss and inorganic waste is important from an environmental perspective, it will also be essential to sustainable transportation and distribution systems that maintain access to

high quality, nutritious food as Vietnam continues to develop, as opposed to shifting to more processed and preserved food items that tend to reduce nutritional quality (61).

7.9 Conclusions

By including input from diverse stakeholders, analyzing existing datasets, and consulting the scientific literature, a sophisticated conceptual framework for sustainable diets was developed that is locally relevant to Vietnam, using a process that is generalizable to other developing countries. This process revealed important data gaps in Vietnam, such as limited information on food loss and inorganic waste. This conceptual framework enabled identification of key leverage points that are likely to provide benefits across multiple domains of sustainable diets, including food quality and safety, agricultural chemical usage, food waste, and water. These formative findings are an essential starting point for enhancing evidence-based policymaking in Vietnam, centered on cultivating more sustainable diets. Next steps will include the facilitation of in-depth interviews to learn more deeply about policy mechanisms that may be encouraging or hindering actions to support sustainable diets, the clarification of data gaps, and the communication of existing data to decision-makers based on priority indicators to enable more interdisciplinary, evidence-based policy making. Ultimately, this initiative will result in a customizable model useful for defining sustainable diet leverage points in different contexts.

7.10 References

1. Popkin BM. 2014. Nutrition, agriculture and the global food system in low and middle income countries. Food Policy, 47:91-96.
2. Gómez MI and Ricketts KD. 2013. Food value chain transformations in developing countries: Selected hypotheses on nutritional implications. Food Policy, 42:139-150.
3. Auestad N and Fulgoni VL. 2015. What current literature tells us about sustainable diets: Emerging research linking dietary patterns, environmental sustainability, and economics. Advances in Nutrition, 6(1):19-36.
4. Allen T, Prosperi P, Cogill B, and Flichman G. 2014. Agricultural biodiversity, social–ecological systems and sustainable diets. Proceedings of the Nutrition Society, 73(4):498-508.
5. Burlingame B and Dernini, S. 2012 Sustainable diets and biodiversity: Directions and solutions for policy, research and action. FAO Headquarters: Rome, Italy.
6. Jones AD, Hoey L, Blesh J, Miller L, Green A, and Shapiro L. 2016. A systematic review of the measurement of sustainable diets. Advances in Nutrition, 7(4):641-664.
7. Joseph H and Clancy K. 2015. Advancing health and well-being in food systems: Strategic opportunities for funders. Dietary guidelines and sustainable diets: Pathways to progress. 88-107.

8. Mason P and Lang T. 2017 Sustainable diets: How ecological nutrition can transform consumption and the food system. Routledge. New York, NY, USA.

9. Allen T, Prosperi P, Cogill B, Padilla M, and Peri I. 2018. A delphi approach to develop sustainable food system metrics. Social Indicators Research.

10. Fanzo J, Cogill B, and Mattei F. 2012. Metrics of sustainable diets and food systems. Bioversity International, Rome, Italy.

11. Gustafson D, Gutman A, Leet W, Drewnowski A, Fanzo J, and Ingram J. 2016. Seven food system metrics of sustainable nutrition security. Sustainability, 8(3):196.

12. Johnston JL, Fanzo JC, and Cogill B. 2014. Understanding sustainable diets: A descriptive analysis of the determinants and processes that influence diets and their impact on health, food security, and environmental sustainability. Advances in Nutrition, 5(4):418-429.

13. Rutten M, Achterbosch TJ, De Boer IJ, Cuaresma JC, Geleijnse JM, Havlík P, Heckelei T, Ingram J, Leip A, and Marette SJAS. 2018. Metrics, models and foresight for European sustainable food and nutrition security: The vision of the SUSFANS project. Agricultural Systems, 163:45-57.

14. Springmann M, Wiebe K, Mason-D'Croz D, Sulser TB, Rayner M, and Scarborough P. 2018. Health and nutritional aspects of sustainable diet strategies and their association with environmental impacts: A global modelling analysis with country-level detail. The Lancet Planetary Health, 2(10):e451-e461.

15. Downs SM, Payne A, and Fanzo J. 2017. The development and application of a sustainable diets framework for policy analysis: A case study of Nepal. Food Policy, 70:40-49.

16. Vietnam Government. 2015. 15 years achieving the Vietnam millennium development goals. Hanoi, Vietnam.

17. Carrique-Mas JJ and Bryant JE. 2013. A review of foodborne bacterial and parasitic zoonoses in vietnam. EcoHealth, 10(4):465-489.

18. Nguyen-Viet H, Tuyet-Hanh TT, Unger F, Dang-Xuan S, and Grace D. 2017. Food safety in Vietnam: Where we are at and what we can learn from international experiences. Infectious Diseases of Poverty, 6(1):39-39.

19. Wertheim-Heck SC, Vellema S, and Spaargaren G. 2015. Food safety and urban food markets in Vietnam: The need for flexible and customized retail modernization policies. Food Policy, 54:95-106.

20. Tilman D, Balzer C, Hill J, and Befort BL. 2011. Global food demand and the sustainable intensification of agriculture. Proceedings of the National Academy of Sciences of the United States, 108(50):20260-20264.

21. Thoai TQ, Rañola RF, Camacho LD, and Simelton E. 2018. Determinants of farmers' adaptation to climate change in agricultural production in the central region of Vietnam. Land Use Policy, 70:224-231.

22. Oya C, Schaefer F, and Skalidou D. 2018. The effectiveness of agricultural certification in developing countries: A systematic review. World Development, 112:282-312.

23. Minten B and Reardon T. 2008. Food prices, quality, and quality's pricing in supermarkets versus traditional markets in developing countries. Applied Economic Perspectives and Policy, 30(3):480-490.

24. Cadilhon JJ, Moustier P, Poole ND, Tam PTG, and Fearne AP. 2006. Traditional vs. Modern food systems? Insights from vegetable supply chains to Ho Chi Minh City (Vietnam). Development Policy Review, 24(1):31-49.

25. Food and Agriculture Organization of the United Nations (FAO). 1996. Declaration on world food security. http://www.fao.org/docrep/003/w3613e/w3613e00.htm Accessed November 16, 2018.

26. The Economist Intelligence Unit. 2018. Global food security index: Building Resilience in the face of rising food-security threats. New York, NY, USA.

27. Marmot M. 2005. Social determinants of health inequalities. The Lancet, 365(9464):1099-1104.

28. Harrison P. 1981. Inside the third world: The anatomy of poverty. Penguin Books, Middlesex, England.

29. Alkire S and Santos ME. 2010. Acute multidimensional poverty: A new index for developing countries. OPHI Working Papers 38, University of Oxford, England.

30. Food and Agriculture Organization of the United Nations (FAO). 2016. Minimum dietary diversity for women: A guide for measurement. Rome, Italy.

31. World Health Organization (WHO). 2008. Indicators for assessing infant and young child feeding practices: Part 1: Definitions: Conclusions of a consensus meeting held 6-8 November 2007 in Washington D.C., USA. Geneva, Switzerland.

32. Nationsl Institute of Nutrition and UNICEF. 2011. Nutrition surveillance 2010. Vietnam nutrition profile. Hanoi, Vietnam.

33. Maire B and Delpeuch, F. 2005. Nutrition indicators for development. Food and Agircultural Oganization of the United Nations (FAO), Food and Nutritin Division. Rome, Italy.

34. Sahoo K, Sahoo B, Choudhury AK, Sofi NY, Kumar R, and Bhadoria AS. 2015. Childhood obesity: Causes and consequences. Journal of Family Medicine and Primary Care, 4(2):187-192.

35. Chao F, You D, Pedersen J, Hug L, and Alkema L. 2018. National and regional under-5 mortality rate by economic status for low-income and middle-income countries: A systematic assessment. Lancet Global Health, 6(5):e535-e547.

36. Jay JM. 2005. Indicators of food microbial quality and safety. Modern Food Microbiology, Springer:473-495.

37. World Health Organization (WHO). 2011. Guidelines for drinking-water quality. Geneva, Switzerland.

38. World Health Organization (WHO). 2015. Progress on sanitation and drinking water: 2015 update and MDG assessment. Geneva, Switzerland.

39. Gustafson J, Cederberg C, Sonesson U, van Otterdifk R, and Meybeck A. 2011. Global food losses and food waste: Extent, causes and prevention. Food and Agriculture Organization of the United Nations (FAO). Rome, Italy.

40. Troschinetz AM and Mihelcic JR. 2009. Sustainable recycling of municipal solid waste in developing countries. Waste Management, 29(2):915-923.

41. Schloter M, Dilly O, and Munch JC. 2003. Indicators for evaluating soil quality. Agriculture, Ecosystems & Environment, 98(1):255-262.

42. Doran JW and Zeiss MR. 2000. Soil health and sustainability: Managing the biotic component of soil quality. Applied Soil Ecology, 15(1):3-11.

43. Hoai PM, Sebesvari Z, Minh TB, Viet PH, and Renaud FG. 2011. Pesticide pollution in agricultural areas of northern Vietnam: Case study in Hoang Liet and Minh Dai communes. Environmental Pollution, 159(12):3344-3350.

44. Dasgupta S, Meisner C, Wheeler D, Xuyen K, and Thi Lam N. 2007. Pesticide poisoning of farm workers-implications of blood test results from vietnam. International Journal of Hygeine and Environmental Health, 210(2):121-132.

45. Van Hoi P, Mol A, and Oosterveer P. 2013. State governance of pesticide use and trade in Vietnam. Wageningen Journal of Life Sciences, 67:19-26.

46. Okorogbona AO, Denner FD, Managa LR, Khosa TB, Maduwa K, Adebola PO, Amoo SO, Ngobeni HM, and Macevele S. 2018 Water quality impacts on agricultural productivity and environment. Sustainable Agriculture Reviews, 27:1-35.

47. United Nations Educational, Scientific, and Cultural Organization (UNESCO). 2015. Education for all 2000–2015: Achievements and challenges. Paris, France.

48. D'souza G and Ikerd J. 1996. Small farms and sustainable development: Is small more sustainable? Journal of Agricultural and Applied Economics, 28(1):73-83.

49. Fan S and Chan□Kang C. 2005. Is small beautiful? Farm size, productivity, and poverty in asian agriculture. Agricultural Economics, 32:135-146.

50. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, and Devleesschauwer B. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLOS Medicine, 12(12):e1001923-e1001923.

51. Grace D. 2015. Food safety in low and middle income countries. International Journal of Environmental Research and Public Health, 12(9):10490-10507.

52. Van Hoi P, Mol AP, and Oosterveer PJ. 2009. Market governance for safe food in developing countries: The case of low-pesticide vegetables in vietnam. Journal of Environmental Management, 91(2):380-388.

53. Flechet C and Nhung, PTK. 2016 Case study: Habits, concerns and preferences of vegetables consumers in Hanoi. Rikolto Vietnam, Hanoi, Vietnam.

54. Nguyen-the C, Bardin M, Berard A, Berge O, Brillard J, Broussolle V, Carlin F, Renault P, Tchamitchian M, and Morris CE. 2016. Agrifood systems and the microbial safety of fresh produce: Trade-offs in the wake of increased sustainability. Science of The Total Environment, 562:751-759.

55. Callejón RM, Rodríguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, and Troncoso AM. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: Trends and causes. Foodborne Pathogens and Disease, 12(1):32-38.

56. Barilla Center for Food and Nutrition. 2017 Fixing food: Towards a more sustainable food system. Parma, Italy.

57. Mateo-Sagasta J, Raschid-Sally L, and Thebo A. 2015 Global wastewater and sludge production, treatment and use. Wastewater, Springer:15-38.

58. Idris A, Inanc B, and Hassan MN. 2004. Overview of waste disposal and landfills/dumps in Asian countries. Journal of Material Cycles and Waste Management. 6(2):104-110.

59. Garnett T, Appleby MC, Balmford A, Bateman IJ, Benton TG, Bloomer P, Burlingame B, Dawkins M, Dolan L, Fraser D, Herrero M, Hoffmann I, Smith P, Thornton PK, Toulmin C, Vermeulen SJ, and Godfray HCJ. 2013. Sustainable intensification in agriculture: Premises and policies. Science, 341(6141):33-34.

60. Reisch L, Eberle U, and Lorek S. 2013. Sustainable food consumption: An overview of contemporary issues and policies. Sustainability: Science, Practice and Policy, 9(2):7-25.

61. Popkin BM, Adair LS, and Ng SW. 2012. Global nutrition transition and the pandemic of obesity in developing countries. Nutrition Reviews, 70(1):3-21.

Chapter 8

Summary & Conclusions

This overarching goal of this doctoral work was to investigate the effects of various environmental conditions on bacterial adhesion, detachment, and disinfection on leafy green surfaces. The fate and transport of two common foodborne pathogens (*E. coli* O157:H7 and *Salmonella* Typhimurium), a common non-pathogen control strain (*E. coli* 25922), and a suite of environmental *E. coli* isolates were used throughout the technical work presented here. Changes in cell surface characteristics and the resulting impacts on the adhesion and detachment trends were studied over a range of growth conditions, solution chemistries, nanoparticle interactions, and disinfectants. Physiochemical characteristics such as zeta potential, hydrophobicity, cell size, and production of extracellular polymers (EPS) were measured and used to elucidate mechanisms of bacterial transport. A parallel plate flow cell was employed to model gentle washing or rinsing of spinach leaf surfaces and directly observe cell transport in controlled, dynamic conditions.

In Chapter 2, five environmental *E. coli* isolates that may provide improved food safety surrogates for common pathogens were identified. Through collaboration with the USDA, eighteen environmental *E. coli* isolates from manures and surface waters were grown in high- and low-nutrient media and systematically characterized and compared to those of known foodborne pathogens *E. coli* O157:H7 and *Salmonella* Typhimurium. When grown in nutrient-restricted conditions, the zeta potential, relative hydrophobicity, and EPS composition of the current common surrogate strains were all significantly different from those of the pathogens. However, five environmental strains among the isolates were found to have similar surface characteristics to each of the common

foodborne pathogens in this study. Overall, the results from this chapter emphasize the need for environmentally relevant surrogates to be discovered and utilized to accurately predict pathogen behavior in food processing scenarios.

Employing the same pathogens and two of the identified promising surrogates, Chapter 3 showed that growth conditions and solution complexities can have significant effects on mechanisms of bacterial adhesion at interfaces of water and spinach leaf surfaces. Specifically, results demonstrated that deposition of *E. coli* O157:H7 and *Salmonella* Typhimurium on spinach epicuticle layers significantly increases when cells are grown in nutrient-restricted conditions, implying that food safety research that only includes well-nourished cells may underestimate attachment to produce surfaces. This difference in adhesion could be attributed, in part, to increasing cell surface charge heterogeneity, as characterized by changes in EPS composition and minimal changes in overall cell surface charge. The most significant differences in high- and low-nutrient conditions were observed with suspension in multivalent artificial groundwater versus two simple salt solutions. This illustrated the role of complex, environmentally relevant water chemistries in magnifying changes on the cell surface. Additionally, this project demonstrated that transport of environmental isolates are less impacted by nutrient conditions, which presents a potential challenge to using stress-tolerant environmental microbes as food safety surrogates. Broadly, the results of this project contribute to understanding the effects of realistic environments on bacterial adhesion and ultimately improving removal of foodborne pathogens.

Beyond natural variables of agricultural environments, Chapter 4 considers the impacts of introducing nanoparticles as pesticides or soil supplements. The results demonstrated that the presence of 10 mg/mL of nano-TiO_2 particles resulted in an unchanged or slightly increased release of pathogenic *E. coli* O157:H7 from leaf and sand surfaces. This suggests that the application of nano-TiO_2 may promote reversible bacterial attachment and present a safety consideration due to bacteria release with a change in solution chemistry, such as in a food rinsing process or rain event. In contrast, nano-CuO caused an increase in irreversible bacterial attachment to both leaf and sand surfaces, possibly fostering increased food illness risk by enhancing the early stages of the biofilm formation process. The effects of these nanomaterials were observed to be more pronounced on the transport of pathogenic *E. coli* O157:H7 than the common non-pathogenic quality control strain, *E. coli* 25922. This implied that the more neutral surface charge and lesser EPS production of the pathogen allowed cells to be more sensitive to stress from interacting with the nanoparticles. This work revealed important considerations for managing microbial risks throughout the food system that may result from increased use of nanomaterials in agricultural operations.

Chapter 5 shifts from the field to the processing plant and considers the impact of a common food industry disinfectant: chlorine. Still using the microfluidic flow cell, *E. coli* O157:H7 attachment and detachment was evaluated, along with the efficacy of standard bleach disinfection on bacterial death and removal from the produce surface. Results demonstrated the importance of preventing this initial microbial contamination, since it required almost four times more concentrated bleach to achieve the same level of

disinfection of attached cells versus planktonic cells. Additionally, bleach disinfection was dependent on both bleach concentration and exposure time, which indicated that disinfection of attached cells may require additional rinsing time when compared to the typical rinsing times. Those cells that did remain attached through the detachment rinse were observed to withstand and survive disinfection rinses, in contrast to planktonic cells, of which nearly 90% were dead within 1 min of exposure at 200 ppb. Overall, it seems likely that bleach rinses cause detachment from the spinach leaf surface, but do not kill adhered cells. This may be cause for significant concern for public health, since many human pathogens have been shown to require as few as ten cells to cause food borne illness.

In Chapter 6, an enzyme-based disinfectant was developed and validated as a promising option for enhancing or replacing chlorine in food processing applications. To produce the hydrolase enzyme, the protein was expressed, separated, and purified successfully. Then, the detachment of *E. coli* O157:H7 cells from the spinach leaf surface was observed in the parallel-plate flow cell. Detachment rate coefficients and percentage of total detached cells were found to increase more than 6 times with the addition of 1000 ppb enzyme to the rinse solution. This suggests that the enzyme is able to decrease the amount of irreversibly attached cells from the leaf surface, which represents the reversal of the foundational step in the biofilm formation process. Additionally, biofilm growth by *E. coli* O157:H7, *E. coli* 25922, and *Salmonella* Typhimurium on polycarbonate were up to 40% inhibited by the presence of 100 ppm of the enzyme, providing evidence that the hydrolase is able to effectively degrade the extracellular matrix that typically protects cell

and supports attachment. The results present a strong case for further development and optimization of enzyme activity as a novel alternative to antimicrobials to prevent pathogenic bacteria from contaminating produce and improving food safety.

In addition to the fundamental scientific research presented in this dissertation, a secondary objective was to investigate the perception, management, and translation of technical data (such as the data presented in Chapters 2-6) to inform food and water policy decisions. Chapter 7*a* presents the results of a compilation and comparison of recent recommendations made by diverse stakeholders in California's growing water data management infrastructure. Stakeholder collaboration, data standardization, increased data collection, as well as data transparency and accessibility were among the most common and most important recommendations for sustainably developing and managing an integrated water data management system for the state. Focusing more broadly on the interpretation and organization of food systems data in Vietnam, the development of a sophisticated conceptual framework for evidence-based decision-making on sustainable diets is covered in Chapter 7*b*. Through input from diverse stakeholders, analysis of existing datasets, and consultation of the scientific literature, multiple data-based leverage points for advancing sustainable diets were identified, including food quality and safety, agricultural chemical usage, food waste, and water. Both of these projects ultimately revealed both challenges and opportunities in the communication of science and data to decision-makers and importance of enabling more interdisciplinary, evidence-based policy making at the state, national, and even international levels.

The research presented herein that comprises this dissertation demonstrates that colloidal transport theory and fundamental research models can elucidate the impacts of specific environmental conditions, components, or disinfectants on foodborne bacteria fate. Specifically, the findings indicate that the likelihood of bacterial attachment to produce surfaces will vary throughout the watering, washing, and rinsing processes that take place from farm to fork. Factors like nutrient availability, water chemistry, and the presence of nanoparticles in suspension have the potential to significantly impact the transport of foodborne bacteria and therefore warrant scientifically rigorous food safety policy and regulations to account for them. The efficacy of standard bleach disinfection processes on the removal and inactivation of bacteria on leafy greens' surfaces has been called into question, and the potential of an enzyme-based enhancer or alternative have been demonstrated. Furthermore, this doctoral research has identified specific opportunities and mechanisms for translating scientific data into public policy change in the areas of food and water policy, including food safety and water quality.

Appendix A

Supplementary Material for Chapter 2

Table S1
Antibiotic zones of inhibition measurements of surrogates and control strains.

Source	Isolate	Antibiotics by Class										
		Aminocoumarins	Aminoglycosides	Antimycobacterials		Beta lactams			Lincosamides	Macrolides	Sulfonamides	Tetracyclines
		Novobiocin (5µg)	Streptomycin (10µg)	Isoniazid (5µg)	Rifampin (25µg)	Ampicillan (10µg)	Chloramphenicol (5µg)	Amoxicillin/ Clavulanic Acid (20/10µg)	Clindamycin (2µg)	Erythromycin (15µg)	Sulfamethoxazole/ Trimethoprim (23.75/1.21µg)	Tetracycline (30µg)
		mm										
Poulty	P1	7	18	R	22	13	15	13	R	12	34	28
	P2	R	19	R	22	15	18	15	R	25	32	26
	P3	R	19	R	21	12	12	13	R	12	22	26
	P4	R	20	R	21	15	13	14	R	12	34	28
Dairy	D1	R	18	R	25	17	15	19	R	12	34	28
Swine	S1	7	20	R	21	17	20	17	R	18	30	7
	S2	7	17	R	17	14	12	13	R	10	30	10
	S3	10	19	R	19	7	15	7	R	13	24	7
	S4	R	17	R	20	12	15	14	R	16	31	R
	S5	R	9	R	21	R	13	7	R	12	30	R
	S6	R	R	R	18	R	R	R	R	R	20	8
Surface water	DE1	R	18	R	23	14	12	11	R	9	30	25
	DE2	7	18	R	22	13	12	11	R	10	33	25
	DE3	R	18	R	22	12	14	9	R	13	29	25
	DE4	R	18	R	21	13	14	11	R	11	30	25
	DE5	R	18	R	25	11	15	12	R	12	31	25
	WE1	R	18	R	19	10	16	9	R	R	30	25
	WE2	R	17	R	19	12	15	11	R	12	29	24
Controls	QC Strain	R	17	R	20	18	17	11	R	10	28	25
	E. coli O157:H7	R	20	R	21	13	10	13	R	11	30	25
	Salmonella	R	18	R	17	25	29	22	R	13	32	30

Zones of inhibition (mm) corresponds to bacterial susceptibility to the dose (µg) of antibiotic and no measureable zone indicated by R shows complete bacterial resistance to the antibiotic.
Each antibiotic tested using antimicrobial disk susceptibility tests based on Hudzicki (2009).

Table S2
Summary of cell surface characteristics of surrogates and control strains.

Source	Isolate	Zeta Potential		Effective Diameter		Relative Hydrophobicity		Surface Charge Density		EPS Composition	
		LB[a]	LM[b]	LB	LM	LB	LM	LB	LM	LB	LM
		----------mV----------		----------nm----------		----------% ----------		----------µC/cm^2----------		----------Sugar/Protein----------	
Poulty	P1	-31.15 ± 0.69	-29.02 ± 1.65	1822.44 ± 99.55	1434.26 ± 64.91	44.22 ± 7.71	51.24 ± 9.95	218.41	1582.56	0.029 ± 0.0023	0.073 ± 0.0051
	P2	-6.20 ± 1.48	-3.20 ± 0.79	1247.96 ± 106.99	1031.92 ± 24.91	42.83 ± 7.64	33.33 ± 1.04	1068.80	3225.08	0.025 ± 0.0009	0.050 ± 0.0070
	P3	-7.79 ± 1.53	-8.77 ± 0.64	1182.04 ± 220.99	1203.28 ± 93.10	37.62 ± 9.90	43.00 ± 14.65	1381.10	1336.34	0.028 ± 0.0018	0.043 ± 0.0007
	P4	-7.65 ± 0.39	-8.92 ± 1.88	1334.98 ± 52.97	1148.50 ± 44.32	30.83 ± 6.55	47.14 ± 5.06	3860.18	8575.68	0.019 ± 0.0017	0.039 ± 0.0020
Dairy	D1	-12.43 ± 1.46	-2.80 ± 0.93	1415.80 ± 82.43	972.86 ± 115.75	46.70 ± 6.23	44.95 ± 12.34	3169.39	1844.38	0.031 ± 0.0010	0.047 ± 0.0027
Swine	S1	-7.98 ± 0.86	-2.78 ± 1.09	1444.32 ± 60.50	1130.44 ± 51.79	9.21 ± 6.75	27.33 ± 3.40	306.57	12938.07	0.025 ± 0.0020	0.044 ± 0.0059
	S2	-40.34 ± 1.65	-35.86 ± 0.73	1859.02 ± 116.30	1347.00 ± 74.27	1.16 ± 0.76	-0.83 ± 1.04	479.88	6137.98	0.040 ± 0.0188	0.042 ± 0.0003
	S3	-2.50 ± 0.63	-1.67 ± 1.88	1229.32 ± 126.42	1073.52 ± 26.15	23.43 ± 1.59	30.32 ± 4.21	1031.37	18974.43	0.028 ± 0.0012	0.027 ± 0.0041
	S4	-8.03 ± 1.84	-11.87 ± 0.26	1166.64 ± 39.76	1051.02 ± 26.75	38.64 ± 8.96	52.09 ± 1.54	2425.16	4253.81	0.026 ± 0.0011	0.047 ± 0.0013
	S5	-62.84 ± 2.45	-58.54 ± 2.10	1383.54 ± 99.19	1282.14 ± 44.23	32.66 ± 2.30	1.84 ± 0.58	866.17	7101.96	0.025 ± 0.0009	0.057 ± 0.0044
	S6	-6.45 ± 1.22	-6.29 ± 0.86	1454.12 ± 78.17	1170.06 ± 29.81	61.81 ± 8.45	38.05 ± 16.75	4686.90	6101.85	0.029 ± 0.0018	0.033 ± 0.0007
Surface water	DE1	-4.65 ± 1.69	-6.81 ± 0.73	1510.68 ± 46.69	1406.24 ± 66.03	43.28 ± 5.54	83.42 ± 3.53	638.44	704.06	0.077 ± 0.0010	0.054 ± 0.0053
	DE2	-6.29 ± 0.50	-9.03 ± 0.69	1548.56 ± 75.16	1330.60 ± 70.43	45.05 ± 18.99	74.04 ± 10.17	2759.94	21913.90	0.032 ± 0.0007	0.040 ± 0.0054
	DE3	-31.76 ± 1.50	-33.06 ± 2.71	1420.18 ± 59.03	984.90 ± 191.32	22.89 ± 2.63	6.73 ± 0.00	454.26	---	0.029 ± 0.0058	0.029 ± 0.0019
	DE4	-29.82 ± 1.59	-20.77 ± 1.27	1411.92 ± 55.96	1295.04 ± 159.13	13.40 ± 5.37	16.75 ± 1.60	2911.73	1147.57	0.033 ± 0.0038	0.030 ± 0.0021
	DE5	-56.18 ± 2.80	-50.39 ± 2.00	1489.42 ± 35.79	1341.00 ± 66.59	13.27 ± 5.46	1.49 ± 0.50	4868.81	1991.59	0.036 ± 0.0014	0.060 ± 0.0128
	WE1	-12.07 ± 1.38	-6.27 ± 1.32	2228.62 ± 187.03	1198.48 ± 54.19	8.42 ± 1.49	60.94 ± 2.59	619.46	12144.95	0.026 ± 0.0008	0.030 ± 0.0021
	WE2	-38.85 ± 1.02	-42.23 ± 1.89	1510.64 ± 95.16	1243.40 ± 101.96	22.94 ± 3.37	1.16 ± 0.29	10404.03	4988.91	0.031 ± 0.0023	0.036 ± 0.0063
Controls	QC Strain	-45.07 ± 1.88	-45.93 ± 0.80	1665.46 ± 117.93	1470.38 ± 37.78	23.83 ± 3.06	1.82 ± 1.04	1728.75	2575.89	0.038 ± 0.0065	0.087 ± 0.0135
	E. coli O157:H7	-3.64 ± 0.03	-0.97 ± 3.05	1450.54 ± 64.53	908.64 ± 136.42	38.86 ± 3.35	28.36 ± 7.51	12622.25	3905.84	0.028 ± 0.0024	0.058 ± 0.0043
	Salmonella	-19.74 ± 1.32	-9.88 ± 1.47	1350.24 ± 28.58	1087.38 ± 95.89	43.67 ± 4.37	41.54 ± 9.35	929.88	16052.21	0.036 ± 0.0036	0.042 ± 0.0023

[a]LB, Luria-Bertani broth (considered high nutrient)

[b]LM, lettuce lysate with minimal salts media added (considered low nutrient)

Table S3
Motility, curli expression, biofilm, growth rate and virulence gene results of surrogates and control strains.

Source	Isolate	Motiliy[a]	Curli[b]	Biofilm[c]	Growth Rate[c]	Virulence Genes[d]							
						LT	STa	STb	*stx* 1	*stx* 2	*eae*	*cnf1*	*cnf2*
Poulty	P1	LM	Other1	+									
	P2	HM	Rdar		++								
	P3	GM	Rdar	++									
	P4	LM	Rdar	++									
Dairy	D1	GM	Bdar		++								
Swine	S1	GM	Bdar										
	S2	NM	SAW	+	+								
	S3	GM	Bdar		++								
	S4	LM	Rdar		++								
	S5	NM	Other2	+	+								
	S6	LM	Bdar	+							+		
Surface water	DE1	LM	Bdar	++									
	DE2	GM	Bdar	++									
	DE3	NM	Rdar	+									
	DE4	NM	Rdar	+	+								
	DE5	LM	bdar									+	
	WE1	GM	Rdar		+								
	WE2	HM	Bdar	+	++								
Controls	QC Strain	LM	other		++								
	E. coli O157:H7	LM	Other3		++						+		
	Salmonella	LM	Bdar										

[a]NM indicates no motility; LM indicates low motility; GM indicates good motility; HM indicates high motility

[b]Rdar indicates Curli/Cellulose postive; Bdar indicates Curli positive/Cellulose negative; SAW indicates Curli/Cellulose negative

[c]No entry indicates below average value; (+) indicates average value; (++) indicates above average value

[d]Virulence genes tested by Pennsylvania State University *E. coli* Reference Center, (+) indicates positive result

Appendix B

Supplementary Material for Chapter 3

Bacterial Growth and Preparation

Cultures were grown in Luria-Bertani broth (LB) or minimal media (M9) at 37°C for 3.5 or 6 hours, respectively, to reach mid-exponential phase (1, 2). The corresponding growth curves are shown in Figure S1. Cells were harvested through centrifugation methods (accuSpin 3R centrifuge, Fisher Scientific, Pittsburgh, PA). Centrifugation for 10 minutes at 3700xg was alternated with rinsing the pelleted cells in salt solutions (KCl. $CaCl_2$, or AGW) three times to remove traces of growth medium[40]. The salt solutions were prepared with reagent-grade salt (Fisher Scientific) and DI Water (Millipore, Billerica, MA), and were used for characterization and flow cell experiments with unadjusted pH (5.6 - 5.8).

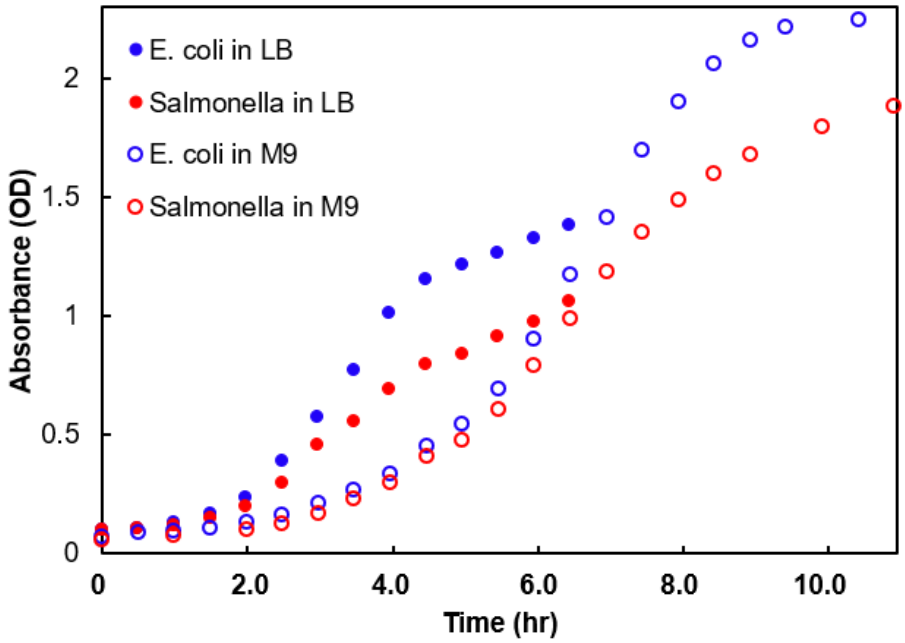

Figure S1. Growth curves. Filled circles (●) indicate growth in LB media and open circles (○) indicates growth in M9 media.

DLVO Theory

In order to predict the theoretical results of planktonic bacterial interaction with and adhesion to spinach surfaces when suspended in various water chemistries in the parallel plate flow cell (Figure S2), Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was used. Total interaction energies between bacteria cells and spinach leaf surface (or substrate), as a function of bacteria-substrate separation distance, were calculated using DLVO theory and modeled as a sphere-plate system (3). Zeta potentials for *Escherichia coli* (*E. coli*) O157:H7 cells and the spinach leaf surface were used to calculate interaction energy profiles. Electrostatic double layer (EDL) forces can be calculated according to the equation (4):

$$V_{el} = \pi\varepsilon_0\varepsilon a_p \left\{2\psi_p\psi_c \ln\left[\frac{1+\exp(-\kappa h)}{1-\exp(-\kappa h)}\right] + \left(\psi_p^2+\psi_c^2\right)\ln\left[1-\exp(-2\kappa h)\right]\right\}$$

where h = separation distance between bacteria and substrate; ψ_p and ψ_c = surface potentials of *E. coli* O157:H7 and spinach leave, respectively; a_p = radius of bacteria; ε_0 = permittivity of vacuum; ε = the dielectric constant of water.

The van der Waals attraction energy was calculated according to the equation (5):

$$V_{vdW} = \frac{A_{102}a_p}{6h}\left[1+\frac{14h}{\lambda}\right]^{-1}$$

where λ = characteristic wavelength of the dielectric and A_{102} = Hamaker constant.

The Hamaker constant is function of material properties, and the constant used was 6×10^{-21} J, and was calculated using the known Hamaker constants for water and bacteria, as well as the contact angle of the spinach leaf surface.

Table S1. Bacteria cell surface characteristics and mass transfer rate coefficients (k) for non-pathogen surrogates suspended in artificial groundwater[a].

Growth media	Cell Radius (nm)[b]	Zeta Potential (mV)[c]	Relative Hydrophobicity (%)[d]	EPS Composition (Sugar/Protein ratio)	$k \times 10^{-8}$ (m/s)
		E. coli B01			
LB	969 ± 416	-3.81 ± 1.73	29.3 ± 20.7	20.41 ± 1.62	18.0 ± 9.40
M9	1416 ± 153	-3.87 ± 1.49	49.3 ± 2.8	9.04 ± 1.89	27.5 ± 3.29
		E. coli B05			
LB	1122 ± 151	-3.15 ± 4.83	29.8 ± 13.9	4.38 ± 0.31	33.9 ± 4.99
M9	1492 ± 78	-8.65 ± 2.28	22.7 ± 8.1	10.97 ± 10.04	21.8 ± 9.39

[a] Ionic strength of all electrolyte solutions was 10 mM.
[b] Spherical radius calculated from experimentally measured length and width of individual cells
[c] Zeta potential calculated from electrophoretic mobility using ZetaPALS analyzer
[d] Relative hydrophobicity as indicated by microbial adhesion to hydrocarbon (MATH) test, which measures the percent of cells partitioned in dodecane versus electrolyte
[e] Based on the Lowry method with BSA as the standard and the phenol−sulfuric acid method with xanthan gum as the standard at 10^8 cells/mL

References

1. Patton JT, Mentor DG, Benson DM, Nicolson GL, McIntire LV. 1993. Computerized analysis of tumor cells flowing in a parallel plate chamber to determine their adhesion stabilization lag time. Cell Motility and Cytoskeleton 26: 88-98.

2. van Kooten TG, Schakenraad JM, Van der Mei HC, Busscher HJ. 1992. Development and use of a parallel-plate flow chamber for studying cellular adhesion to solid surfaces. Journal of Biomedical Materials Researh 26:725-38.

3. Derjaguin BV, Landau L, Verwey EJ, Overbeek JTG. 1948. Theory of the Stability of Lyophobic Colloids. Elsevier: New York, N.Y.

4. Hogg RI, Healey TW, Fuerstenau DW. 1966. Mutual coagulation of colloidal 173 dispersions. Trans. Faraday Soc. 62:1638-1651.

5. Gregory J. 1981. Approximate expression for retarded Van der waals interaction. Journal of Colloid Interface Science 83: 138-145.

Appendix C

Supplementary Material for Chapter 4

Sand Preparation and Column Operation

Sand was prepared as described previously, except that baked sand was not stored under vacuum between baking and rehydration[1]. Briefly, quartz (IOTA) was sieved and the fraction between 250 and 300 μm sieves was retained and soaked in 12 M HCl for at least 24 hours. Acid-soaked sand was washed with DI H2O and baked in an oven at 800 °C for at least 8 hours. The baked sand was rehydrated for at least one hour by boiling in DI water and it was stored in DI water until used when wet packing the column. The column had a length of 5 cm, inner diameter of 1.5 cm, and porosity of 0.45 +/- 0.01.

In preparation for column experiments, at least 40 mL (10 PV) of DI H2O were flowed through the column, followed by at least 40 mL (10 PV) of 10 mM KCl. Once UV-VIS measurements began, 30 mL of the experimental solution (7½ PV), 30 mL of the KCl electrolyte (7½ PV), and 20 mL of DI H2O (5 PV) were flowed through the column. Once each experiment was completed, the column was removed from the flow system and the experimental solution was flowed through the tubing to measure C_0 for the breakthrough curve using the same UV-VIS detector.

The detector scanned at 366 and 600 nm so that the composition of the effluent could be probed. Previous research has used these two wavelengths to study the photocatalytic activity of TiO_2 and measure the optical density of bacteria, respectively[2, 3]. During measurements of experimental C_0 values, suspensions containing *E. coli* O157:H7 alone or in the presence of either particle displayed a ratio of absorbance at 366 nm vs. 600nm that was greater than one. Meanwhile, both ENMs in the absence of bacteria displayed an absorbance ratio less than 1.

Syringe Pumps

Solutions were pumped through the column using syringe pumps (kd Scientific KDS 230) to reduce the pulsation of flow caused by peristaltic pumps, which have been used in past experiments[1]. Experimental solutions were not stirred or sonicated while held in syringes. An additional experiment was run to support the validity of using syringe pumps without stirring or sonication, showing that the C_0 for the *E. coli* O157:H7 and *E. coli* O157:H7 + TiO_2 scenarios changed less than 3% over a length of time similar to an experiment.

Instrument Limitation: Not Allowing Multiple Refractive Indices

For the mixture of *E. coli* O157:H7 and TiO_2, DLS measurements indicate the effective size of the bacteria is much smaller than that for bacteria in the presence of CuO. This is contradicted by SEM images. The contradiction is hypothesized to be due to the higher refractive index of TiO_2, compared to *E. coli* O157:H7 or CuO. TiO_2 may dominate dynamic light scattering measurements. It is hypothesized that the smaller effect of TiO_2 on DLS size measurements of *E. coli* 25922 is due to the TiO_2 being more covered by EPS in this scenario.

DLVO Modeling

The interaction energy profiles for particle-particle, particle-spinach, and particle-quartz systems are presented in figures S1, S2, and S3, respectively. DLVO modeling predicted key aspects of experimental results. As indicated by SEM images and Figure S1, both nanoparticles appear to interact with both bacteria species in suspension. Also, the breakthrough curves for *E. coli* O157:H7 alone and in the presence of CuO are

consistent with the primary minimum deposition predicted by DLVO theory. In addition, more column elution was observed during the DI H_2O rinse of the *E. coli* + TiO_2 scenario than during the other scenarios with the pathogen. UV-VIS adsorption analysis revealed that *E. coli* was the main species appearing in the effluent. Therefore, the pathogen was more stable in the presence of TiO_2, as predicted in Figure S3.

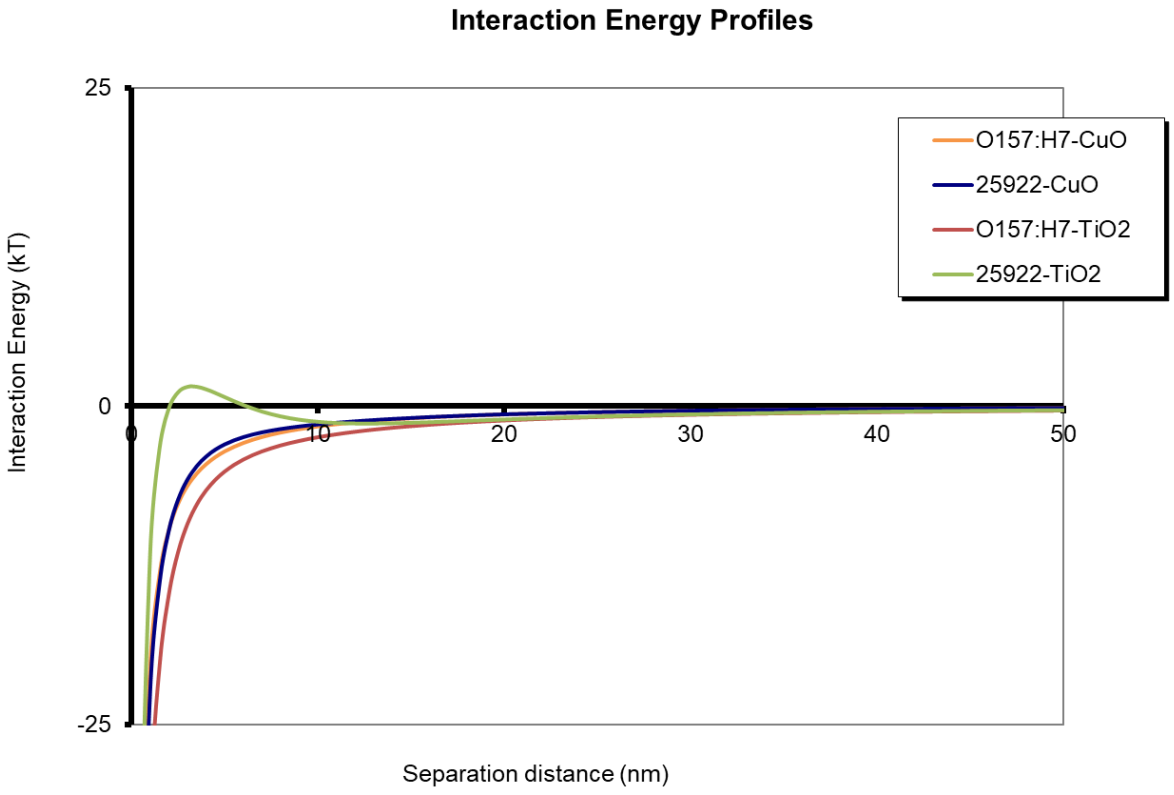

Figure S1. Particle-Particle DLVO for bacteria and ENMs.

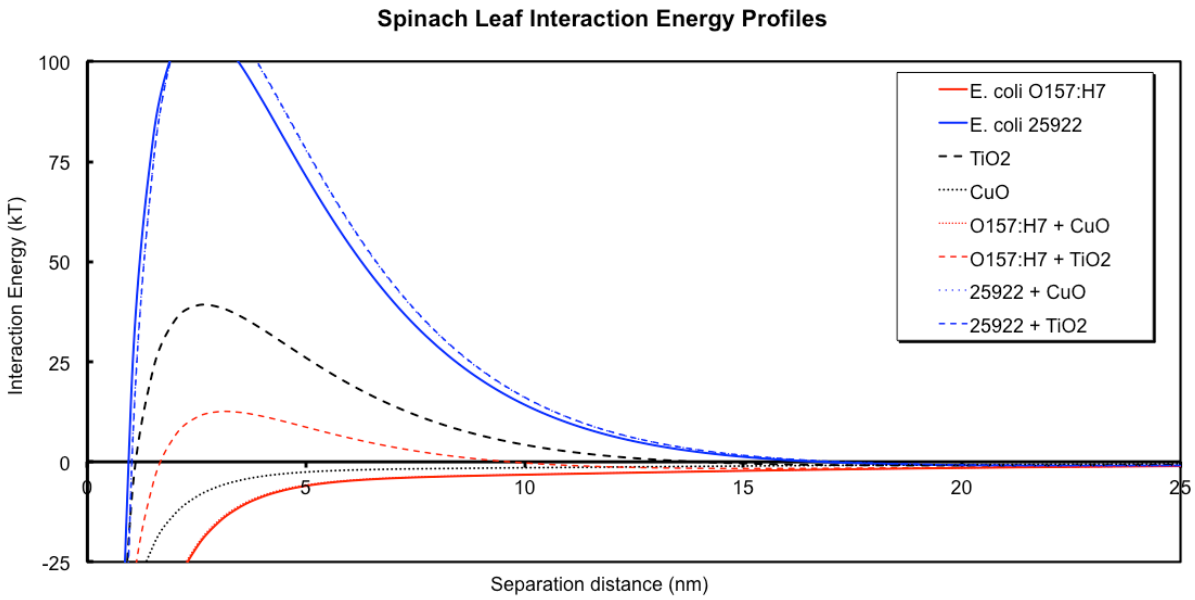

Figure S2. Particle-Plate DLVO for spinach leaf surface.

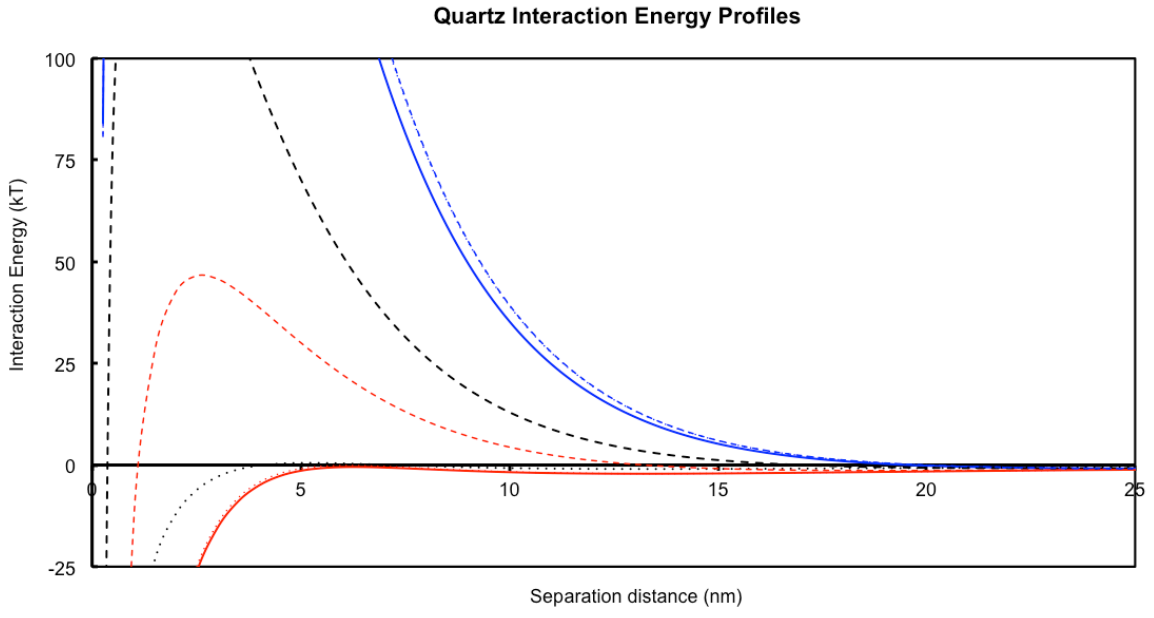

Figure S3. Particle-Plate DLVO for quartz sand surface.

An initial interpretation of DLVO results may lead the reader to conclude that most of the *E. coli* O157:H7 in the presence of TiO_2 should elute during the H_2O rinse. This would be a rational conclusion since a previous study with *E. coli* retained in a saturated sand column under unfavorable deposition conditions showed that the majority of bacteria were eluted from the column upon decreasing the ionic strength of the pore fluid, behavior consistent with secondary minimum deposition[1]. However, in the current study, only a fraction of the *E. coli* in the presence of TiO_2 elutes during the DI H_2O rinse.

It is likely that only a small portion of the *E. coli* O157:H7 cells have meaningful interaction with TiO_2. Based on the hydrodynamic diameter of TiO_2 in the absence of *E. coli*, TiO_2 concentration is estimated to be 10^7 aggregates/mL, while the *E. coli* O157:H7 is present at 10^9 cells/mL. Therefore, it is logical that the majority of the cells exhibit behavior that is consistent with DLVO predictions for *E. coli* in the absence of TiO_2.

DLVO Shortcomings

Based on DLVO predictions, *E. coli* O157:H7 should irreversibly deposit onto the spinach and quartz surfaces, due to its nearly neutral cell surface charge. Meanwhile, more highly charged *E. coli* 25922 should experience greater repulsive forces, so it should attach less and detach more. Instead, attachment rate coefficients onto spinach are around the same magnitude for both the pathogen and non-pathogen scenarios in the flow cell. Also, significant detachment of *E. coli* O157:H7 cells is observed in the flow cell (up to 18% of attached cells), while essentially no detachment of *E. coli* 25922 is

observed. In addition, only partial release of *E. coli* 25922 is observed in the column (62% retention after DI rinse).

Similarly, the presence of TiO2 in suspension with *E. coli* O157:H7 is predicted to hinder irreversible bacteria attachment. However, TiO_2 has little effect on the pathogen's attachment and detachment in the flow cell. Moreover, *E. coli* O157:H7 exhibits a likely increase in retention in the column in the presence of TiO_2.These observations indicate DLVO interactions arenot the primary factors influencing bacterial adhesion. This is in agreement with previous work[4].

A further shortcoming of DLVO modeling is that it predicts the elution of the majority of TiO_2 in the column in the absence of *E. coli*. However, little TiO_2 release was observed. Similar behavior of food grade TiO_2 was previously observed[5]. This behavior may be due to hydrophobic interactions of the TiO_2 nanoparticles.

***E. coli* Deposition Mechanisms**

Some hypotheses for DLVO failing to predict *E. coli* deposition/detachment trends are based on physical shortcomings of the theory. Previous work found that colloid deposition kinetics were insensitive to particle size, despite DLVO theory predicting a decrease in particle deposition with increasing particle size[6]. It is possible that DLVO over-estimates the interaction energy for bacteria (relatively large particles) with surfaces. Furthermore, the equation that was used for van der Waals attraction between *E. coli* and surfaces in DLVO predictions, which assumes sphere-plate geometry, may under-estimate their attraction. Rod-shaped *E. coli* are expected to have greater van der Waals attraction to a surface than their spherical counterpart[7].

Other hypotheses for *E. coli* deposition deviating from DLVO theory are biological in nature. Cell surface heterogeneity and bacteria population heterogeneity may provide for primary minimum deposition in spite of DLVO calculations[1]. Moreover, it has been hypothesized that *E. coli* can form a bridge with a surface via EPS while they remain in a secondary energy minimum, as was demonstrated in previous work (reference). In addition, the larger amount of EPS observed with *E. coli* 25922 (Figure 1) may facilitate additional surface interactions with quartz, irrespective of charge barriers.

Extra EPS may also contribute to the larger *E. coli* 25922 aggregates observed in DLS measurements, and thus physical straining. The straining hypothesis for *E. coli* 25922 in the column is supported by the bumpy dissection curve (data not shown). The curve bumpiness is in contrast to the scenarios with *E. coli* O157:H7, where there is a steep decrease in absorbance with increasing depth, due to favorable deposition conditions. In the hypothetical absence of straining, there is already a hypothesized difference between *E. coli* O157:H7 and *E. coli* 25922 dissection curves: the *E. coli* 25922 dissection curve may be less consistent with classical colloid filtration theory due to repulsive DLVO interactions[8].

However, the bumpy nature of the *E. coli* 25922 dissection curve is different than the hypothesized deviation from classical colloid filtration theory. In previous work, a correlation can be seen between bumpy column dissection curves and straining of *E. coli*[9]. In this work, bumpy column dissection curves often coincided with breakthrough curves that had an upward-sloping breakthrough plateau, as observed in the *E. coli* 25922 scenario. Furthermore, it is logical that the slightly larger *E. coli* 25922 would be strained

in 275 μm sand grains when previous work showed that *E. coli* O157:H7 was strained in 240 μm sand grains[9]. Other potential reasons for nonexponential dissection curves are discussed elsewhere[9].

DLVO Modeling Assumptions and Qualifications

Calculations of effective hydrodynamic diameter and zeta potential were used to generate DLVO predictions. Zeta potentials of -12 and -25mV were used as estimates of net charges for spinach surfaces and quartz grains based on previous work[1, 10].

The Van der Waals equation used in DLVO modeling of sphere-plate interaction is valid for separation distances of up to about 20% of the sphere radius[11]. For the limiting case of CuO nanoparticles, the DLVO calculations are estimated to be valid up to 47 nm (based on hydrodynamic diameter), by which point the DLVO curve is asymptotic.

The electrical double layer (EDL) equation used in the DLVO modeling of sphere-plate interaction is exact for surface potentials less than 25mV[12]. At higher surface potentials, this equation appears to be a good approximation for separation distances that are greater than 3 nm in 10 mM KCl, based on Table 1 of the referenced article[12]. Since the interaction energy barriers of highly charged particles in figures S1 – S3 extend past 3 nm, the EDL equation is considered valid. Another requirement for the validity of the EDL equation is that the double layer thickness be small relative to the particle size, which is easily satisfied for the limiting condition of CuO nanoparticles in 10 mM KCl.

References

1. Redman, J.A., S.L. Walker, and M. Elimelech, *Bacterial Adhesion and Transport in Porous Media: Role of the Secondary Energy Minimum.* Environmental Science & Technology, 2004. **38**(6): p. 1777-1785.

2. Baran, W., A. Makowski, and W. Wardas, *The effect of UV radiation absorption of cationic and anionic dye solutions on their photocatalytic degradation in the presence TiO2.* Dyes and Pigments, 2008. **76**(1): p. 226-230.

3. Begot, C., et al., *Recommendations for calculating growth parameters by optical density measurements.* Journal of Microbiological Methods, 1996. **25**(3): p. 225-232.

4. Mayton, H.M., I.M. Marcus, and S.L. Walker, *Escherichia coli O157: H7 and Salmonella Typhimurium adhesion to spinach leaf surfaces: Sensitivity to water chemistry and nutrient availability.* Food Microbiology, 2019. **78**: p. 134-142.

5. Waller, T., I.M. Marcus, and S.L. Walker, *Influence of septic system wastewater treatment on titanium dioxide nanoparticle subsurface transport mechanisms.* Analytical and bioanalytical chemistry, 2018: p. 1-8.

6. Elimelech, M. and C.R. O'Melia, *Effect of particle size on collision efficiency in the deposition of Brownian particles with electrostatic energy barriers.* langmuir, 1990. **6**(6): p. 1153-1163.

7. Vold, M.J., *Van der Waals' attraction between anisometric particles.* Journal of Colloid Science, 1954. **9**(5): p. 451-459.

8. Tufenkji, N. and M. Elimelech, *Deviation from the classical colloid filtration theory in the presence of repulsive DLVO interactions.* Langmuir, 2004. **20**(25): p. 10818-10828.

9. Bradford, S.A., J. Simunek, and S.L. Walker, *Transport and straining of E. coli O157: H7 in saturated porous media.* Water Resources Research, 2006. **42**(12).

10. Elimelech, M., et al., *Relative insignificance of mineral grain zeta potential to colloid transport in geochemically heterogeneous porous media.* Environmental science & technology, 2000. **34**(11): p. 2143-2148.

11. Gregory, J., *Approximate expressions for retarded van der Waals interaction.* Journal of Colloid and Interface Science, 1981. **83**(1): p. 138-145.

12. Hogg, R., T.W. Healy, and D. Fuerstenau, *Mutual coagulation of colloidal dispersions.* Transactions of the Faraday Society, 1966. **62**: p. 1638-1651.

Appendix D

Supplementary Material for Chapter 5

DLVO Theory

DLVO theory was utilized to calculate total interaction energies between bacteria cells and spinach leaf surface (or substrate), as a function of bacteria-substrate separation distance, and modeled as a sphere-plate system (1). Measured zeta potentials for *Escherichia coli* (*E. coli*) O157:H7/pGFP and spinach leaf surface (Table 5.1) were used to calculate interaction energy profiles. The electrostatic double layer (EDL) forces can be calculated according to the equation (2):

$$V_{el} = \pi\varepsilon_0\varepsilon a_p \left\{2\psi_p\psi_c \ln\left[\frac{1+\exp(-\kappa h)}{1-\exp(-\kappa h)}\right] + \left(\psi_p^2 + \psi_c^2\right)\ln\left[1-\exp(-2\kappa h)\right]\right\}$$

where h = separation distance between bacteria and substrate; ψ_p and ψ_c = surface potentials of *E. coli* O157:H7 and spinach leave, respectively; a_p = radius of bacteria; ε_0 = permittivity of vacuum; ε = the dielectric constant of water.

The van der Waals attraction energy was calculated according to the equation (3):

$$V_{vdW} = \frac{A_{102}a_p}{6h}\left[1+\frac{14h}{\lambda}\right]^{-1}$$

where λ = characteristic wavelength of the dielectric and A_{102} = Hamaker constant.

The Hamaker constant is function of material properties, and the constant used was 6×10^{-21} J, and was calculated using the known Hamaker constants for water and bacteria, as well as the contact angle of the spinach leaf surface.

Resulting interaction energy profiles are shown in Figure S1.

Materials and Methods

Bleach concentrations were reported in parts per million (ppm), which is the typically relevant unit in food safety literature. For reference, the following are conversions to units of molarity: 4ppm = 0.054mM NaHClO (Bleach); 200ppm = 6.7mM NaHClO

Bacterial Attachment and Rinsing

E. coli O157:H7 attachment and detachment experiments were conducted in a parallel plate flow chamber (PP) [19, 48-51] (GlycoTech, Rockville, MA) positioned on an inverted fluorescent microscope (BX-52, Olympus). The inner dimension of the chamber is 6 cm × 1cm × 0.0762 cm and is composed of a Plexiglas® block that is mounted by a flexible silicone elastomer gasket and a microscope slide sealed by vacuum grease. The fluid stream enters the chamber from a capillary tube that is connected to a syringe, which is controlled by a syringe pump at a flow rate of 0.1 mL/min, corresponding to an average flow velocity of 0.79 m/h, and a Péclet number of 6.47×10^{-4}. The fluorescently labeled bacteria are imaged by a 40× long working distance objective (UPlanFl, Olympus) using a filter at excitation and emission wavelengths of 480 nm and 510 nm, respectively (Chroma Technology Corp., Brattleboro, VT).

Attachment was observed over a 30 minute period with images recorded with a digital camera (Demo Retiga EXI Monochrome, QImaging) every minute to determine the kinetics of cell adhesion. Enumeration of cells was determined by comparison of successive images. A suspension of cells (5×10^7 cells/mL) was utilized and the

concentration was determined with a counting chamber (Bürker-Türk chamberMarienfield Laboratory Glassware, Lauda-Konigshofen, Germany). Adhesion experiments were conducted in 1 to 100 mM KCl and 3.3 to 10mM artificial groundwater (AGW) at ambient temperature (22 - 25 °C). Surfaces prepared by the techniques described below were rinsed with DI water within the parallel plate flow cell prior to cell adhesion experiments.

The number of bacterial cells deposited was plotted versus time, and calculation of bacterial flux, *J*, was achieved by dividing the initial slope of the line by the microscope viewing area (230 μm × 170 μm). The mass transfer rate coefficient for the bacteria, k_{pp}, is calculated using the bacterial flux (number of cells per area per time), and the bulk cell concentration (number of cells/mL), C_0, via (4-6):

$$k = \frac{J}{C_0}$$

The mass transfer rate coefficients for these experiments are identified as k_{att} or k_{det}. Each experiment was performed in triplicate on each batch of generated surfaces. Statistical analysis was performed using the student t-test to identify significant differences between data sets where a 95% confidence level was confirmed (when $p < 0.05$).

Bacterial Growth and Preparation

This bacterium was labeled with enhanced green fluorescent protein (EGFP) and gentamicin resistance for visualization, and is referred to as *E. coli* O157:H7/pGFP throughout the article (7). Cultures were grown in Luria-Bertani (LB) broth at 30°C in

the presences of gentamycin sulfate antibiotic (30 mg/L, OmniPur, Gibbstown, NJ) for 3.5 hours to reach mid-exponential phase (8, 9). Cells were harvested for experiments and characterization by centrifugation (accuSpin 3R centrifuge, Fisher Scientific, Pittsburgh, PA). Centrifugation for 15 minutes at 3689xg was alternated with rinsing the pelleted cells in salt solutions (KCl or AGW) twice to remove traces of growth medium. The KCl and AGW solutions were prepared with reagent-grade salt (Fisher Scientific) and DI Water (Millipore, Billerica, MA) at unadjusted pH (5.6-5.8).

Bacteria Characterization: Relative Hydrophobicity

To analyze the relative hydrophobicity of *E. coli O157:H7 g* cells, a semi-quantitative microbial adhesion to hydrocarbons (MATH) test was employed (10). The relative hydrophobicity of the organism in each of these solutions is reported as the percent of total cells that partition into the model hydrocarbon (dodecane) (11).

Test tubes were prepared with 4mL of the cell suspension (in the respective analyte salt solutions) and 1 mL of n-dodecane (laboratory grade, Fisher Scientific). Test tubes were vortexed (AutoTouch Mixer Model 231, Fisher Scientific) for 2 minutes, followed by a rest period of 15 minutes to allow phase separation. The final absorbance reading after the rest period was compared to the initial absorbance acquired after harvesting. The optical density of the cells in the aqueous phase was measured using a spectrophotometer at 546 nm (BioSpec-mini, Shimadzu Corp., Kyoto, Japan). The resulting partitioning of cells gives an indication of the polarity of the microbes and how

they may interact with surfaces in specific liquid environments (12, 13). The following equation was used,

$$\left(\frac{ODi-ODs}{ODi}\right)\times 100 = \text{Relative hydrophobicity (\%)}$$

where OD_i = initial optical density and OD_s = sample optical density.

Bacteria Characterization: Zeta Potential

Zeta potential was used as a measure of the electrokinetic properties of the microbe using a ZetaPALS analyzer (Brookhaven Instruments, NY) (14, 15). The zeta potential was calculated from the electrophoretic mobility (EPM) using the Smoluchowski equation (16. 17).

Surface Characterization

Scanning Electron Microscopy (FEI XL-30, Netherlands) samples were mounted with conductive adhesive on pin stubs (Ted Pella, Redding, CA) and sputter coated with Pt/Pd for 30 seconds. Atomic Force Microscopy (MFP-3D AFM, Asylum Research, Santa Barbara, CA) scans were taken over a minimum of three different regions using a Silicon AFM All-in-One short cantilever C (Ted Pella, Redding, CA). Spinach leaves were evaluated using contact mode immersed in water to reduce drying effects. Epicuticle layers were evaluated using contact mode in air. Roughness measurements were based on four scanned areas, each 10μm by 10μm). Static contact angles were measured using a Drop Shape Analyzer – DSA25 (KRÜSS, Hamburg, Germany). Using double-sided adhesive tape, spinach leaves were mounted onto a glass slide. Average

contact angles were obtained from measurements at three different points on three separate leaves. Static contact angles were calculated using the tangential curve fitting method at 10 seconds after the drop of water (3μL) was placed onto the surface.

Adhesion and Detachment

In industrial agriculture, post-harvest processing typically includes rinsing spinach leaves either in field or at an on-site facility to remove dirt and debris (18). To evaluate the potential for cross-contamination during such stages, epicuticle surfaces with attached *E. coli* O157:H7/pGFP were subsequently rinsed with DI water. Additionally, bacteria are known to deposit under a variety of field conditions, which may affect bacterial detachment during post-harvest rinsing. Therefore, the impact of initial attachment parameters (such as water chemistry and ionic strength) on bacterial detachment during DI water rinsing was investigated.

All rinsing studies were conducted following a rinse of background salt solution to remove cells loosely associated with the surface. In previous studies, reversible attachment has been defined as cells attaching for several seconds or swimming, tumbling, or walking along the surface. Irreversible attachment was defined as cells attaching and remaining in place indefinitely (4, 14). In contrast, we considered "reversibly attached" cells to be those that remained attached through the duration of the 30 minute attachment period and background salt rinse (Stages 1 and 2), and then subsequently released due to the introduction of a new solution (DI water rinse).

GFP Fading

When quantifying cells during rinse and disinfection experiments, all fluorescent, attached cells are assumed to be alive because they are continuing to produce GFP. To ensure that fluorescence fading did not impact the analysis, control experiments were conducted by rinsing with background salt solutions for extend time periods.

The fluorescence associated with the production of GFP was observed to fade following the detachment plateau (Figure S4). Control experiments were conducted to ensure that fading did not interfere with observations. Fading was not observed at ≤ 100ppb bleach, AGW, or KCl systems for the duration of the rinsing. The time required to fade was reduced with increasing bleach concentration from 23 ± 6 minutes at 200ppb to 5 ± 2 minutes at 1000ppb. While fading of the GFP fluorescence may indicate cell stress, it does not directly indicate a compromised membrane or cell death. Therefore, following the completion of the rinse, the chamber was rinsed with the background salt solution to remove the bleach solution. Live/Dead stain was then introduced to evaluate the state of attached cells. The percentage of live attached cells remaining on the epicuticle declined with increasing bleach concentration from 88 ± 4% remaining at 10ppb to 0% remaining at 1000ppb (Figure S4). There was insignificant difference in the percentage of live cells attached following 10ppb bleach and DI water rinses.

As observed in the DI water rinsing, bacterial detachment ceased after a segment of time. Below 100ppb bleach, the duration of detachment (Stage 3) was not statistically different from the DI rinsing system and reached the plateau after approximately 15 minutes (Figure S4).

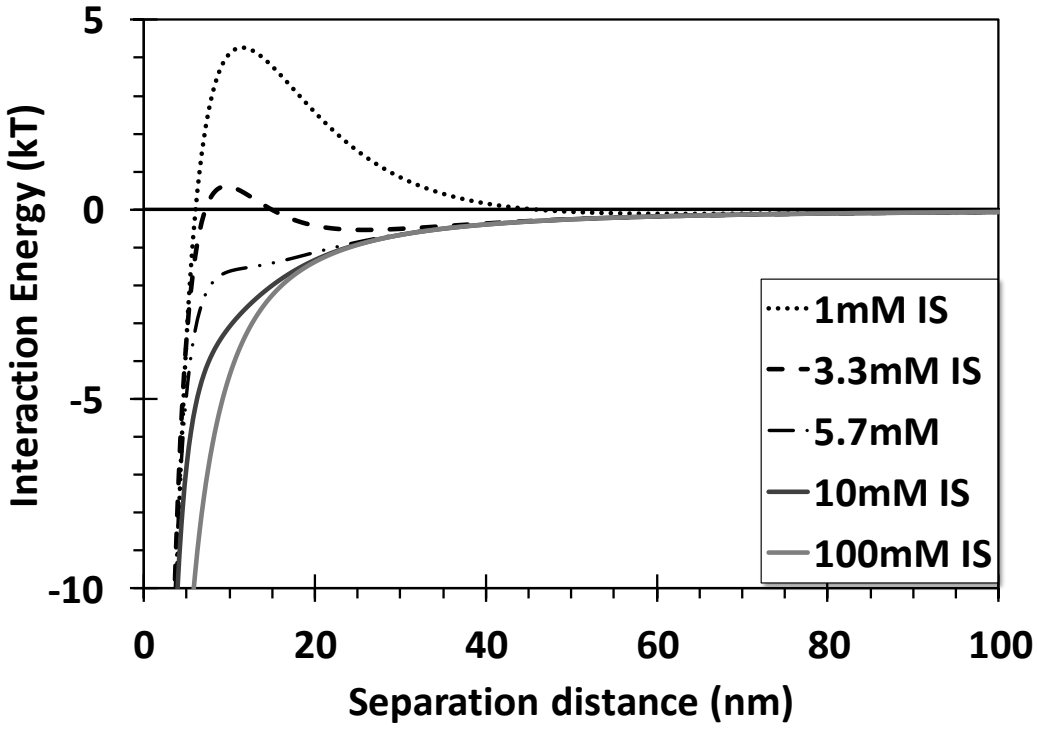

Figure S1. DLVO sphere-plate interaction energy profiles as a function of ionic strength representing potential electrostatic interactions between the spinach leaf epicuticle and *E. coli* O157:H7/pGFP.

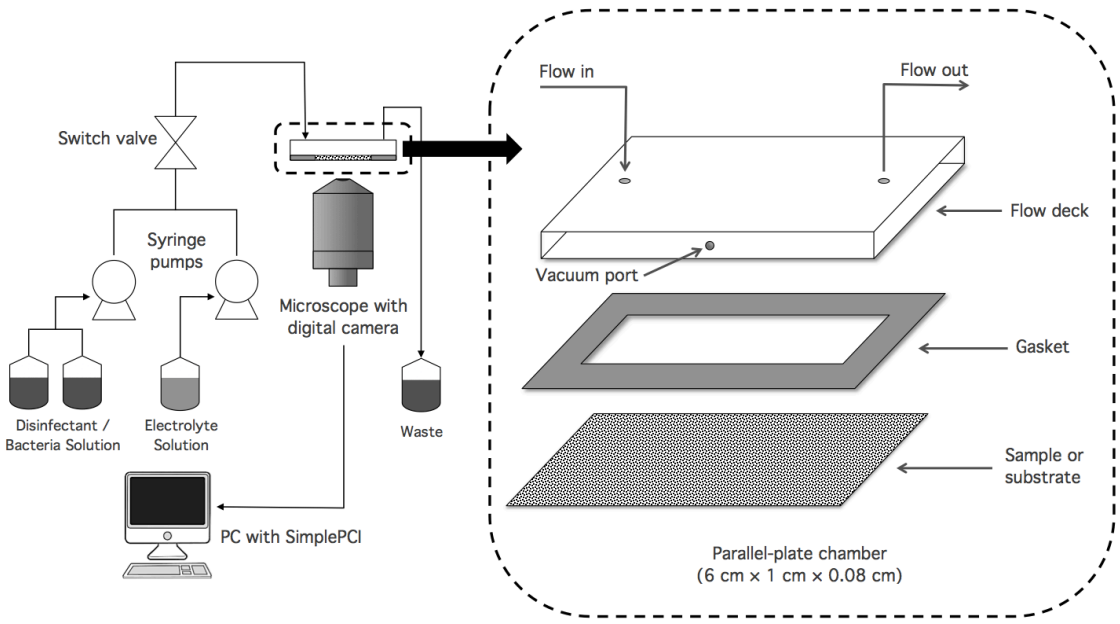

Figure S2. Schematic of parallel plate flow chamber (PP) used to conduct attachment and detachment experiments.

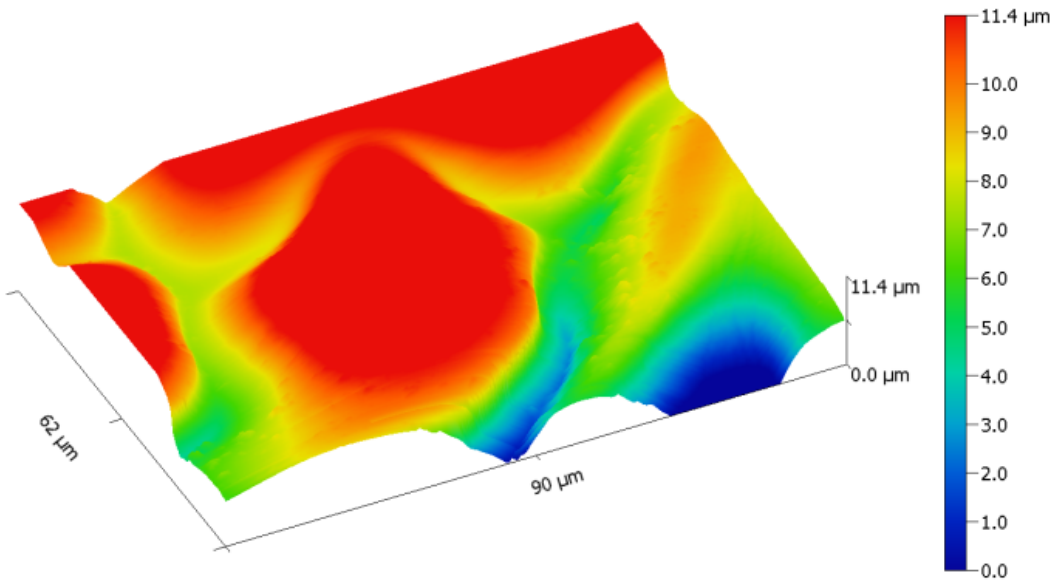

Figure S3. AFM image of spinach leaf (immersed in water)

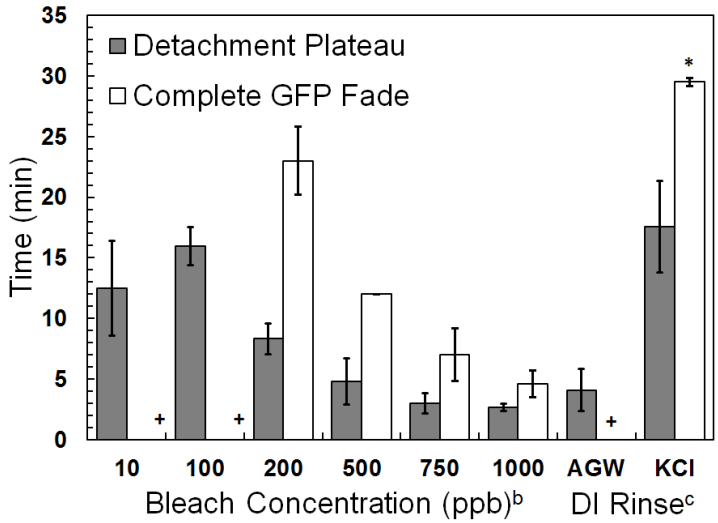

Figure S4. Rinsing time required to reach the detachment plateau and time required for green fluorescing protein (GFP) to completely fade. Rinsing time is from introduction of rinse solution (following the background salt solution rinse) and detachment ceases (Stage 3).

* Fading was only observed at the lower ionic strength of 3.3mM KCl and therefore data is representative of that condition only

+ Fading was not observed

References

1. Derjaguin, B. V.; Landau, L., Acta Physicochim U.S.S.R. 1941, 14, 300. (30) Verwey, E. J.; Overbeek, J. T. G., Theory of the Stability of Lyophobic Colloids. Elsevier: New York, N.Y., 1948.
2. Hogg, R.I.; Healey, T.W.; Fuerstenau, D.W. Mutual coagulation of colloidal 173 dispersions. Trans. Faraday Soc. 1966, 62, 1638-1651. 174
3. Gregory, J. Approximate expression for retarded Van der waals interaction. J. 175 Colloid interface sci. 1981, 83, 138-145
4. Chen, G.; Beving, D. E.; Bedi, R. S.; Yan, Y. S.; Walker, S. L., Initial Bacterial Deposition on Bare and Zeolite-Coated Aluminum Alloy and Stainless Steel. *Langmuir* **2009,** *25*, (3), 1620-1626.
5. Chowdhury, I.; Walker, S. L., Deposition mechanisms of TiO2 nanoparticles in a parallel plate system. *J. Colloid Interface Sci.* **2012,** *369*, (1), 16-22.
6. Elimelech, M.; Jia, X.; Gregory, J.; Williams, R., *Particle Deposition & Aggregation: Measurement, Modelling and Simulation*. Elsevier Science: 1998.
7. McClaine, J. W.; Ford, R. M., Reversal of Flagellar Rotation Is Important in Initial Attachment of *Escherichia coli* to Glass in a Dynamic System with High- and Low-Ionic-Strength Buffers. *Appl. Environ. Microbiol.* **2002,** *68*, (3), 1280-1289.
8. Patton, J. T.; Mentor, D. G.; Benson, D. M.; Nicolson, G. L.; McIntire, L. V., Computerized analysis of tumor cells flowing in a parallel plate chamber to

determine their adhesion stabilization lag time. *Cell Motil. Cytoskeleton* **1993,** *26*, (1), 88-98.

9. van Kooten, T. G.; Schakenraad, J. M.; Van der Mei, H. C.; Busscher, H. J., Development and use of a parallel-plate flow chamber for studying cellular adhesion to solid surfaces. *J. Biomed. Mater. Res.* **1992,** *26*, (6), 725-38.

10. Rosenberg, M.; Gutnick, D.; Rosenberg, E., Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* **1980,** *9*, (1), 29-33.

11. Marcus, I. M.; Wilder, H. A.; Quazi, S. J.; Walker, S. L., Linking Microbial Community Structure to Function in Representative Simulated Systems. *Appl. Environ. Microbiol.* **2013,** *79*, (8), 2552-2559.

12. Gutman, J.; Walker, S. L.; Freger, V.; Herzberg, M., Bacterial Attachment and Viscoelasticity: Physicochemical and Motility Effects Analyzed Using Quartz Crystal Microbalance with Dissipation (QCM-D). *Environmental Science & Technology* **2012,** *47*, (1), 398-404.

13. Ensikat, H. J.; Neinhuis, C.; Barthlott, W., Direct Access to Plant Epicuticular Wax Crystals by a New Mechanical Isolation Method. *International journal of plant sciences* **2000,** *161*, (1), 143-148.

14. Haznedaroglu, B. Z.; Kim, H. N.; Bradford, S. A.; Walker, S. L., Relative Transport Behavior of *Escherichia coli* O157:H7 and *Salmonella* enterica Serovar Pullorum in Packed Bed Column Systems: Influence of Solution Chemistry and Cell Concentration. *Environ. Sci. Technol.* **2009,** *43*, (6), 1838-1844.

15. Jetter, R.; Schäffer, S.; Riederer, M., Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from Prunus laurocerasus L. *Plant Cell Environ.* **2000,** *23*, (6), 619-628.

16. Walker, S. L.; Bhattacharjee, S.; Hoek, E. M. V.; Elimelech, M., A Novel Asymmetric Clamping Cell for Measuring Streaming Potential of Flat Surfaces. *Langmuir* **2002,** *18*, (6), 2193-2198

17. Vigeant, M. A. S.; Ford, R. M.; Wagner, M.; Tamm, L. K., Reversible and Irreversible Adhesion of Motile *Escherichia coli* Cells Analyzed by Total Internal Reflection Aqueous Fluorescence Microscopy. *Appl. Environ. Microbiol.* **2002,** *68*, (6), 2794-2801.

18. FDA, U. F. D. A., Methods to reduce/eliminate pathogens from produce and fresh-cut produce, 2013. In 2013