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

Nichola M. Kinsinger, Holly M. Mayton, Madeline R. Luth, and Sharon L. Walker

Department of Chemical and Environmental Engineering, University of California, Riverside, California, 92521, United States

Keywords: *Escherichia coli* O157:H7, foodborne pathogens, bacterial attachment, disinfection, produce, spinach

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 $88 \pm 4\%$ 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.

1. Introduction

Within the United States, food safety is frequently compromised via microbial contamination (Cuthbert, 2001; Olaimat & Holley, 2012) and sickens one in six Americans annually (CDC, 2014). Additionally, foodborne illness results in significant economic losses within the agricultural industry, estimated to be over \$75 billion per year (Scharff, 2012). Approximately 20% of single food commodity outbreaks from 2003-2008 were attributed to leafy green produce alone (CDC, 2014). Contamination of such minimally processed and ready-to-eat produce is of specific concern since it is frequently consumed uncooked or raw (FDA, 2008).

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 (Cooley et al., 2007). 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 (Stuart, 2008). *E. coli* O157:H7, a gram-negative rod-shaped bacterium (Fujisawa et al., 2001), is an enterohemorrhagic strain of the bacterium that causes acute diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (LeBlanc, 2003).

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 (Cuthbert, 2001; Olaimat & Holley, 2012). 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 (Han et al., 2000; Singh et al., 2002; Zhang et al., 2009; Escalona et al., 2010; Macarasin et al., 2012; Markland et al., 2013; Cálix-

Lara et al., 2014; O'Beirne et al., 2014) 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 (Busscher et al., 2010). 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 (Supporting Information (SI), Figure S2). With this experimental design, bacterial attachment can be imaged onto a variety of surfaces with real-time *in situ* analysis (Kline & Chen, 2008; Chen et al., 2009; Vanoyan et al., 2010; Orgad et al., 2011; Chowdhury & Walker, 2012; Cai et al., 2013) and track individual cells within field of view during rinsing and disinfection experiments.

E. coli O157:H7 is also known to seek stomas and damaged regions of leaves for protection, nutrients, and moisture, which may extend its survival within the field (Niemira & Cooke, 2010; Saldana et al., 2011). 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.

2. Material and Methods

2.1 Bacterial Cell Preparation and Characterization

E. coli O157:H7/pGFP was used as a model pathogen in this study (Cooley et al., 2007). *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 (Haznedaroglu et al., 2009). Cells were characterized for relative hydrophobicity using microbial adhesion to hydrocarbons (MATH) test and zeta potential (Rosenberg et al., 1980; Marcus et al., 2013). 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 (Kim et al., 2009; Kim et al., 2010). In the presence of UV light, the persistence of the cells' fluorescence could be assessed visually using the inverted fluorescent microscope.

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 (Ensikat et al., 2000). 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) (Ensikat et al., 2000; Jetter et al., 2000). 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 (Walker et al., 2002).

2.3 Bacterial Attachment and Rinsing

Bacterial attachment and detachment experiments were conducted in a parallel plate flow chamber (van Kooten et al., 1992; Patton et al., 1993; McClaine & Ford, 2002; Kline & Chen, 2008; Chen et al., 2009) (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 (UPlanFI, 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 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 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-Königshofen, Germany). Adhesion experiments were conducted in 1 to 100 mM KCl and 3.3 to 10 mM artificial groundwater (AGW) (Bolster et al., 1999) 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 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 (FDA, 2013), 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 1).

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 ($230 \mu\text{m} \times 170 \mu\text{m}$). Representative attachment and detachment curves are shown in Figure 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 (Chen et al., 2009; Chowdhury et al., 2012; Elimelech et al., 1998):

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

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 $k_{\text{det,DI}}$ 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 $k_{\text{det,bleach}}$ 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.

2.5 Modeling

2.5.1 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 (Derjaguin & Landau, 1948). 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 Supporting Information.

2.5.2 COMSOL Model Formulation

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.

3. Results and Discussion

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 2A). Waxes with a well-defined prism structure, approximately 500 nm, were also consistently observed but irregularly dispersed across the leaf surface (Figure 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 2B).

High resolution AFM images (Figure 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 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 (Chen et al., 2009; Tang et al., 2009). On both surfaces, the prismatic structures observed in SEM were measured in AFM to be 482 ± 155 nm high (protruding from surface).

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 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.

Salt	Ionic Strength (mM)	Spinach Leaf ζ Potential (mV)	<i>E. coli</i> O157:H7/pGFP ζ Potential (mV)	Spinach Leaf Contact Angle ^b (°)	<i>E. coli</i> O157:H7/pGFP MATH ^c (%)
KCl	1	-8.98 ± 1.27	-2.71 ± 4.11	56 ± 10	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		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

Table 1. Spinach Leaf and *E. coli* O157:H7/pGFP properties.

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)

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 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 (Chen et al., 2009; Chen et al., 2010). 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 (Walker et al., 2005).

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 SI, Figure S2) 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 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 (Rijnaarts et al., 1999; Walker et al., 2005).

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+}) (Benjamin, 2002). 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 (Gregory, 2006; Elimelech et al., 1998). 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.

Cell surface appendages and organelles and their conformations have been observed to play a significant role in bacterial attachment to a variety of surfaces (Walker et al., 2004; Rossez et al., 2014). Surface appendages of *E. coli* O157:H7/pGFP are known to selectively adhere to specific surface moieties on hosts' cells for leaf colonization (Collinson et al., 1991; Saldana et al., 2011; Macarisin et al., 2012). 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 (McClaine & Ford, 2002; Chen & Walker, 2012; Kim et al., 2009). 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 (Khemakhem et al., 2005) 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 1 as contact angle and percent relative hydrophobicity values, respectively.

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 (McClaine & Ford, 2002; Vigeant et al., 2002). Irreversible attachment was defined as cells attaching and remaining in place indefinitely (McClaine & Ford, 2002; Vigeant et al., 2002). 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 ($k_{\text{det,DI}}$ 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 $k_{\text{det,DI}}$ 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 1). Nor was the zeta potential of the epicuticle layer impacted substantially. Hence, DLVO profiles (see SI, Figure S2) 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 water (Xu et al., 2005; Meinders & Busscher, 1993). 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 (Vadillo-Rodríguez et al., 2004; Xu et al., 2005) (not including metabolic processes of microorganisms and biofilm formation).

Additionally, water chemistry has been shown to impact the conformation of surface polymers and appendages (Kim et al, 2009; Chen & Walker, 2007). Divalent ions, such as Ca^{+2} , have been observed to form calcium bridges and form complexations with organics in various

systems (Vigeant et al., 2002; Chen & Elimelech, 2007; Saldana et al., 2011; Chowdhury et al., 2012; Rossez et al., 2014). 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.

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 $k_{\text{det,bleach}}$ 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 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 Supporting Information.

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 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 (Fukuzaki, 2006). At the concentrations investigated, the pH ranged between 5 and 7 where HOCl is the predominant species (Fukuzaki, 2006). 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 (Fukuzaki, 2006). Increasing detachment with increased bleach concentration is attributed to this oxidation and possible cleavage of surface proteins that were adhered to the epicuticle.

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 (Han et al., 2000; Niemira & Cooke, 2010; Saldana et al., 2011). 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 (SI, Figure S3) 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 $2.27 \pm 0.65 \mu\text{m}$; 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 (Chen et al., 2009; Tang et al., 2009).

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 (Niemira & Cooke, 2010; Saldana et al., 2011; Olaimat & Holley, 2012). A representative image of the model (evaluated at 50 ppm NaOCl, 60 seconds) is shown in Figure 5. Concentrations were evaluated over 60 seconds; a typical industry rinse time (FDA, 2013).

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 (US EPA, 1975) and for organic produce rinses (USDA, 1990)) to 200 ppm (maximum bleach concentration typically used in fresh produce processing (FDA, 2013)). These significantly reduced concentrations of bleach occurred in crevices (valleys) and stomas on the leaf surface. The inset of Figure 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 Supporting Information, 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.

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 (McClaine & Ford, 2002), 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. 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.

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 (FDA, 2013). 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 (Harris et al., 2003; FDA, 2015). 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.

Acknowledgements

This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2013-67012-21277. This work also supported in part by the National Science Foundation IGERT: WaterSENSE – Water Social, Engineering, and Natural Sciences Engagement Program (award number GE-1144635) and the Chancellor’s Research Fellowship at the University of California, Riverside. The authors are thankful for the help from Alexander Dudchenko for his aid in obtaining the AFM images used in this study and for the guidance from Ian Marcus in the revision process.

References

- Benjamin, M.M., 2002. *Water Chemistry*, McGraw-Hill Higher Education.
- Bolster, C.H.; Mills, A.L.; Hornberger, G.M.; Herman, J.S., 1999. Spatial distribution of deposited bacteria following miscible displacement experiments in intact cores. *Water Resour. Res.*, 35: 1797-1807.
- Busscher, H. J., Norde, W., Sharma, P.K., van der Mei, H.C., 2010. Interfacial re-arrangement in initial microbial adhesion to surfaces. *Current Opinion in Colloid & Interface Science*, 15(6): 510-517.
- Cai, P., Huang, Q., Walker, S.L., 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.
- Cálix-Lara, T.F., Rajendran, M., Talcott, S.T., Smith, S.B., Miller, R.K., Castillo, A., Sturino, J.M., Taylor, T.M., 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.
- Center for Science in the Public Interest, 2009. Outbreak alert. Analyzing foodborne outbreaks 1998 to 2007.
- Centers for Disease Control and Prevention, 2009. Surveillance for foodborne disease outbreaks - United States, 2006. *Morbidity and Mortality Weekly Report (MMWR)*, 58: 609-615.
- Centers for Disease Control and Prevention, 2010. Surveillance for foodborne disease outbreaks - United States, 2007. *Morbidity and Mortality Weekly Report (MMWR)*, 59: 973-979.
- Centers for Disease Control and Prevention, 2014. CDC and Food Safety. <http://www.cdc.gov/foodsafety/cdc-and-food-safety.html> (accessed 03.06.16)

- Chen, G., Bedi, R.S., Yan, S.Y., Walker, S.L., 2010. Initial colloid deposition on bare and zeolite-coated stainless steel and aluminum: Influence of surface roughness. *Langmuir*, 26(15): 12605-12613.
- Chen, G., Beving, D.E., Bedi, R.S., Yan, Y.S., Walker, S.L., 2009. Initial bacterial deposition on bare and zeolite-coated aluminum alloy and stainless steel. *Langmuir*, 25(3): 1620-1626.
- Chen, G. and Walker, S.L., 2007. Role of solution chemistry and ion valence on the adhesion kinetics of groundwater and marine bacteria. *Langmuir*, 23(13): 7162-7169.
- Chen, G. X. and Walker, S.L., 2012. Fecal Indicator Bacteria Transport and Deposition in Saturated and Unsaturated Porous Media. *Environmental Science & Technology*, 46(16): 8782-8790.
- Chen, K. L. 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.
- Chowdhury, I., Cwiertny, D.M., Walker, S.L., 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.
- Chowdhury, I. and Walker, S.L., 2012. Deposition mechanisms of TiO₂ nanoparticles in a parallel plate system. *Journal of Colloid and Interface Science*, 369(1): 16-22.
- Collinson, S. K., Emödy, L., Muller, K.H., Trust, T.J., Kay, W.W., 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. *Journal of Bacteriology*, 173(15): 4773-4781.
- Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M.T., Myers, C., Rose, C., Keys, C., Farrar, J., Mandrell, R.E., 2007. Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. *PLoS One*, 2(11): e1159.
- Cuthbert, J.A., 2001. Hepatitis A: Old and New. *Clinical Microbiology Reviews*, 14(1): 38-58.
- Derjaguin, B.V., 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.
- Elimelech, M.; Jia, X.; Gregory, J.; Williams, R., 1998. *Particle Deposition & Aggregation: Measurement, Modelling and Simulation*. Elsevier Science.
- Ensikat, H. J., 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.
- Escalona, V., Aguayo, E., Martinez-Hernandez, G.B., 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.
- Food and Drug Administration, 2008. Guidance for industry: Guide to minimize microbial food safety hazards for fresh-cut fruits and vegetables. *Guidance for Industry*. Food and Drug Administration: Silver Spring, MD, USA.
- Food and Drug Administration, 2013. Methods to reduce/eliminate pathogens from produce and fresh-cut produce. *Safe Practices for Food Processes*. Food and Drug Administration: Silver Spring, MD, USA.
- 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.

- Fukuzaki, S., 2006. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. *Biocontrol Science*, 11(4): 147-157.
- Gorny, J.R., Giclas, H., Gombas, D., Means, K., 2006. *Commodity specific food safety guidelines for the lettuce and leafy greens supply chain*. International Fresh-cut Produce Association: Alexandria, VA, USA.
- Han, S.D., Yang, H., Wang, L., Kim, J.W., 2000. Preparation and properties of vanadium-doped SnO₂ nanocrystallites. *Sensors and Actuators B-Chemical*, 66(1-3): 112-115.
- Han, Y., Sherman, D.M., Linton, R.H., Nielson, S.S., Nelson, P.E., 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.
- Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H., Busta, F.F., 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.
- Haznedaroglu, B.Z., Bolster, C.H., Walker, S.L., 2008. The role of starvation on *Escherichia coli* adhesion and transport in saturated porous media. *Water Research*, 42(6-7): 1547-1554.
- Haznedaroglu, B.Z., Kim, H.N., Bradford, S.A., Walker, S.L., 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.
- 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.
- Khemakhem, W.; Ammar, E.; Bakrouf, A., Effect of environmental conditions on hydrophobicity of marine bacteria adapted to textile effluent treatment. *World Journal of Microbiology and Biotechnology* **2005**, 21, 1623-1631
- Kim, H.N., Hong, Y., Lee, I., Bradford, S.A., Walker, S.L., 2009. Surface characteristics and adhesion behavior of *Escherichia coli* O157:H7: Role of extracellular macromolecules. *Biomacromolecules*, 10(9): 2556-2564.
- Kim, H.N., Walker, S.L., Bradford, S.A., 2010. Macromolecule mediated transport and retention of *Escherichia coli* O157:H7 in saturated porous media. *Water Research*, 44(4): 1082-1093.
- Kline, T.R., Chen, G., Walker, S.L., 2008. Colloidal deposition on remotely controlled charged micropatterned surfaces in a parallel-plate flow chamber. *Langmuir*, 24(17): 9381-9385.
- Koivunen, J. and Heinonen-Tanski, H., 2005. Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Water Research*, 39(8): 1519-1526.
- LeBlanc, J.J., 2003. Implication of virulence factors in *Escherichia coli* O157:H7 pathogenesis. *Critical Reviews in Microbiology*, 29(4): 277-296.
- Macarisin, D., Patel, J., Bauchan, G., Giron, J.A., Sharma, V.K., 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.
- Marcus, I.M., Bolster, C.H., Cook, K.L., Opot, S.R., Walker, S.L., 2012. Impact of growth conditions on transport behavior of *E. coli*. *Journal of Environmental Monitoring*, 14(3): 984-991.

- Markland, S., Shortlidge, K.L., Hoover, D.G., Yaron, S., Patel, J., Singh, A., Sharma, M., Kniel, K.E., 2013. Survival of pathogenic *Escherichia coli* on basil, lettuce, and spinach. *Zoonoses and Public Health*, 60(8): 563-571.
- McClaine, J.W. and Ford, R.M., 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.
- Niemira, B.A. and Cooke, P.H., 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.
- 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.
- Olaimat, A.N. and Holley, R.A., 2012. Factors influencing the microbial safety of fresh produce: a review. *Food Microbiology*, 32(1): 1-19.
- Orgad, O., Oren, Y., Walker, S.L., Herzberg, M., 2011. The role of alginate in *Pseudomonas aeruginosa* EPS adherence, viscoelastic properties and cell attachment. *Biofouling*, 27(7): 787-798.
- Patton, J.T., Mentor, D.G., Benson, D.M., Nicolson, G.L., McIntire, L.V., 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.
- Rijnaarts, H.H.M., Norde, W., Lyklema, J., Zehnder, A.J.B., 1999. DLVO and steric contributions to bacterial deposition in media of different ionic strengths. *Colloids and Surfaces B: Biointerfaces*, 14(1-4): 179-195.
- Rogers, S. and Haines, J., 2005. Detecting and mitigating the environmental impact of fecal pathogens originating from confined animal feeding operations: review. U.S. Environmental Protection Agency, National Risk Management Research Laboratory. Cincinnati, OH, USA.
- Rossez, Y., Holmes, A., Wolfson, E.B., Gally, D.L., Mahajan, A., Pedersen, H.L., Willats, W.G., Toth, I.K., Holden, N.J., 2014. Flagella interact with ionic plant lipids to mediate adherence of pathogenic *Escherichia coli* to fresh produce plants. *Environmental Microbiology*, 16(7): 2181-2195.
- Saldana, Z., Sanchez, E., Xicohtencatl-Cortes, J., Puente, J.L., Giron, J.A., 2011. Surface structures involved in plant stomata and leaf colonization by shiga-toxigenic *Escherichia coli* O157:H7. *Front Microbiology*, 2: 119.
- Scharff, R.L., 2012. Economic burden from health losses due to foodborne illness in the United States. *Journal of Food Protection*, 75(1): 123-131.
- Singh, N., Singh, R., Bhunia, A.K., Strohshine, R.L., 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.
- Stuart, D., 2008. The illusion of control: Industrialized agriculture, nature, and food safety. *Agriculture and Human Values*, 25(2): 177-181.
- Tang, H., Cao, T., Liang, X., Wang, A., Salley, S.O., McAllister, J., Ng, K.Y., 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.

- U.S. Environmental Protection Agency, 1975. National Primary Drinking Water Regulations: Maximum Residual Disinfectant Levels. *Title 40*. U.S. EPA, Code of Federal Regulations. **141.65**.
- U.S. Department of Agriculture, 1990. Organic Foods Production Act. *Title 21*. Code of Federal Regulations.
- Vadillo-Rodríguez, V., Busscher, H.J., Norde, W., de Vries, J., van der Mei, H.C., 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.
- van Kooten, T.G., Schakenraad, J.M., van der Mei, H.C., Busscher, H.J., 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.
- Vanoyan, N., Walker, S.L., Gillor, O., Herzberg, M., 2010. Reduced bacterial beposition and attachment by quorum-sensing inhibitor 4-Nitro-pyridine-N-oxide: The role of physicochemical effects. *Langmuir*, 26(14): 12089-12094.
- Vigeant, M.A.S., Ford, R.M., Wagner, M., Tamm, L.K., 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.
- Walker, S.L., Bhattacharjee, S., Hoek, E.M.V., Elimelech, M., 2002. A novel asymmetric clamping cell for measuring streaming potential of flat furfaces. *Langmuir*, 18(6): 2193-2198.
- Walker, S.L., Hill, J.E., Redman, J.A., Elimelech, M., 2005. Influence of growth phase on adhesion kinetics of *Escherichia coli* D21g. *Applied and Environmental Microbiology*, 71(6): 3093-3099.
- Walker, S.L., Redman, J.A., Elimelech, M., 2004. Role of cell surface lipopolysaccharides in *Escherichia coli* K12 adhesion and transport. *Langmuir*, 20(18): 7736-7746.
- Xu, L.C., Vadillo-Rodríguez, V., Logan, B.E., 2005. Residence time, loading force, pH, and ionic strength affect adhesion forces between colloids and biopolymer-coated surfaces. *Langmuir*, 21(16): 7491-7500.
- Zhang, G., Ma, L., Phelan, V.H., Doyle, M.P., 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.

655
656

657
658

659
660

661
662

663
664

665
666

667
668