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A novel microfluidic mixer-based approach for determining inactivation kinetics of *Escherichia coli* O157:H7 in chlorine solutions

Boce Zhang [a, b], Yaguang Luo [a, c, *], Bin Zhou [a, b], Qin Wang [b], Patricia D. Millner [a]

[a] The Environmental Microbial and Food Safety Lab, Agricultural Research Service, The United States Department of Agriculture, Beltsville, MD 20705, USA
[b] Department of Nutrition and Food Science, University of Maryland, 0112 Skinner Building, College Park, MD 20742, USA
[c] The Food Quality Lab, Agricultural Research Service, The United States Department of Agriculture, Beltsville, MD 20705, USA

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ABSTRACT

Determination of the minimum free chlorine concentration needed to prevent pathogen survival/cross-contamination during produce washing is essential for the development of science-based food safety regulations and practices. Although the trend of chlorine concentration-contact time on pathogen inactivation is generally understood, specific information on chlorine and the kinetics of pathogen inactivation at less than 1.00 s is urgently needed by the produce processing industry. However, conventional approaches to obtain this critical data have been unable to adequately measure very rapid responses. This paper reports our development, fabrication, and test of a novel microfluidic device, and its application to obtain the necessary data on pathogen inactivation by free chlorine in produce wash solution in times as short as 0.10 s. A novel microfluidic mixer with the capability to accurately determine the reaction time and control the chlorine concentration was designed with three inlets for bacterial, chlorine and dechlorinating solutions, and one outlet for effluent collection. The master mold was fabricated on a silicon wafer with microchannels via photopolymerization. Polydimethylsiloxane replicas with patterned microchannels were prototyped via soft lithography. The replicas were further assembled into the micromixer on glass via O_2 plasma treatment, and the inlets were connected to a syringe pump for solution delivery. To determine the kinetics of free chlorine on pathogen inactivation, chlorine solutions of varying concentrations were first pumped into the micromixer, together with the addition of bacterial suspension of *Escherichia coli* O157:H7 through a separate inlet. This was followed by injection of dechlorinating solution to stop the chlorine-pathogen reaction. The effluent was collected and the surviving bacteria cells were enumerated using a modified 'Most Probable Number' method. Free chlorine concentration was determined using a standard colorimetric method. The contact time was experimentally set by adjusting the solution flow rate, and was estimated by computational fluid dynamics modeling. Results showed that 1) pathogen inactivation was significantly affected by free chlorine concentration ($P < 0.0001$) and subsecond reaction time ($P < 0.0001$) and their interactions ($P < 0.0001$); and 2) the current industry practice of using 1.0 mg/L free chlorine will require more than 1.00 s total contact to achieve a 5-$\log_{10}$ reduction in an *E. coli* O157:H7 population, whereas a 10.0 mg/L free chlorine solution will achieve 5-$\log_{10}$ reduction in as little as 0.25 s. Information obtained from this study will provide critical insight on kinetics of bacterial inactivation for a broad range of sanitizers and produce wash operational conditions, thus facilitating the development and implementation of science-based food safety regulations and practices for improving food safety.

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1. Introduction

Since the 1993 *Escherichia coli* (*E. coli*) O157:H7 outbreak, when nearly 10,000 people were sickened, foodborne illness has become a major threat to the public health (CDC, 2011; Gould et al., 2013).

* Corresponding author. The Environmental Microbial and Food Safety Lab, Agricultural Research Service, The United States Department of Agriculture, Beltsville, MD 20705, USA. Tel.: +1 301 504 6186; fax: +1 301 504 5107.
E-mail address: yaguang.luo@ars.usda.gov (Y. Luo).

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Almost two decades later, microbial contamination remains one of the most serious challenges for assuring the safety of food supplies (FDA, 2012a; FDA, 2012b). In 2011, the Centers for Disease Control and Prevention (CDC) estimated that roughly 48 million people are sickened by food-borne pathogens each year, including 3000 cases ending in deaths. Among all food categories, fruits and vegetables have emerged as significant vehicles of foodborne bacterial pathogens because they are frequently consumed raw (CDC, 2011). Washing is an important step during fresh-cut produce processing as it removes the debris, soils, and produce latex released from the cut edges and maintains quality and shelf life of the final products, and can reduce 1–2 $\log_{10}$ CFU/g microbial loads. Sanitizers such as chlorine and peroxyacetic acid are often used during produce washing. Although the efficacy of those sanitizers on pathogen reduction is limited, the presence of sufficient sanitizer concentration in the fresh and fresh-cut produce wash water is critical for preventing pathogen survival in the wash water and its consequential transfer to the clean produce washed in the same tank/flume of water (Lopez-Galvez et al., 2009, 2010; Luo et al., 2011; Shen et al., 2013; Zhang et al., 2009).

Maintaining a high level of sanitizer in wash water is a practical challenge to the produce industry due to the rapid reaction of organic matter with sanitizers, especially the widely used hypochlorous acid (chlorine). Sanitizer, especially free chlorine, concentration usually declines rapidly during fresh produce wash operation directly as a result of its reaction with organic materials present in the wash water. It is important to note that as the wash process progresses the organic materials and the chlorine demand accumulates in the wash water (Zhou et al., 2014). Therefore, although chlorine can be replenished to certain degree by adding more concentrated sodium hypochlorite, large quantities of sodium hypochlorite will be needed to restore the required level. Thus it is critical to establish a minimum chlorine concentration that is effective for preventing pathogen cross-contamination, and also achievable by the industry. While a plethora of information is available regarding chlorine concentration on pathogen inactivation, specific information regarding the minimum chlorine concentration for preventing pathogen cross-contamination is scarce. We (Luo et al., 2011) reported that although no pathogen survival was noted in the wash water with at least 1.0 mg/L free chlorine and 30 s of leaf to chlorine exposure time, pathogen cross-contamination between adjacent leaf surfaces was observed during produce wash until the free chlorine level water reached 10 mg/L. Similar findings were also obtained during a pilot plant study (Luo et al., 2012). One major reason for this discrepancy may be related to reaction time as results on pathogen survival are often obtained with 30.00 s or longer exposure time, while pathogen cross-contamination could occur within a much shorter time frame. Therefore, it is important to determine the minimum chlorine concentration that results in the inactivation of pathogens quickly enough to avoid cross-contamination.

The effect of chlorine concentration and contact time on pathogen inactivation has drawn increasing research interest. Several studies have investigated the contact time from 5 s to 120 min using different chlorine concentrations (0.1–100.0 mg/L) (Gil et al., 2009; Gómez-López et al., 2014; Lopez-Galvez et al., 2009, 2010; Luo et al., 2011; Uyttendaele et al., 2004; Zhang et al., 2009). One study found that low chlorine concentration (0.1–0.5 mg/L) cannot reach 5-$\log_{10}$ reduction in water even after 120 min of treatment (Gil et al., 2009; Uyttendaele et al., 2004). Most recently, Shen et al. (2013) determined that at least 2 mg/L is required to inactivate pathogens with at least 5 s exposure time. In another study, a minimum of 5.0 mg/L of chlorine was found to be effective to inactive *E. coli* O157:H7 under industrial conditions (Gómez-López et al., 2014). The differences in minimum chlorine concentration in these studies can be attributed to different contact times. However, as a result of manual methods used in these studies, the shortest contact time that could be controlled was 5 s, and the study results include many other factors that at the scale of the study could not be easily controlled, including time variations associated with thorough mixing of the chlorine with pathogen, time variation associated with the mixing of chlorine stop solutions, and the time variations caused by manual handlings. Therefore, an approach is needed to better control the reaction time and chlorine concentration, in order to obtain the critical time-dose information for pathogen inactivation. Therefore, developing other ways to control and manipulate liquids in short time frames (<1 s) without other confounding factors is highly desirable.

The stopped-flow technique (Stocker et al., 2008; Sasaki et al., 2012; Wessel et al., 2013) has been widely used to provide rapid mixing of two or more solutions to study millisecond (e.g., 10^{-4}–10^{-2} s) kinetics of irreversible reactions, including bacterial inactivation. However, this technique has limited capabilities for studying pathogen inactivation in food processing systems, where the detection and quantification of food-borne bacteria requires both high throughput and low limit of detection (e.g., 10 CFU/g of below). Stopped-flow technique can only handle small sample flow rate (Gomez-Henz and Perez-Bendito, 1991) of up to 0.50 mL/min or 0.0083 mL/s of the solution. The flow rate was insufficient for collecting enough sample size for pathogen enumeration in food systems, and the improvement of throughput capacity requires either a larger mixing chamber or a parallel multiple channel system, which can substantially increase the mixing time and the cost of the system respectively. In addition, to detect or quantify changes of bacterial survival rate, this technology has mostly relied on spectrometric or photometric detectors for qualitative and quantitative analysis (Wessel et al., 2013), which are not cost-effective and have a high limit of detection (at least 10^4 CFU/g). Therefore, it is important to develop a novel micromixer that will fulfill the requirements of studying pathogen inactivation in food processing systems.

On the other hand, microfluidic mixers (micromixer) have been widely used for biological and biotechnological applications that require very brief controlled reaction times (Stroock et al., 2002). A micromixer has two unique advantages compared to the traditional vortex-based or manual mixing method: solutions can be mixed effectively in very short mixing length when passing through a microscale fluid channel (Kim et al., 2005; Stroock et al., 2002); the mixing time (contact time) can be set by adjusting the flow rate of the steady pressure-driven flows (Boskovic et al., 2011; Stroock et al., 2002). Different mixing microchannel designs were developed to improve mixing efficiency. In this study, we adopted three commonly used designs, Y-injection, Dean's vortex (serpentine), and chaotic mixers (Kim et al., 2005; Stroock et al., 2002; Whitesides, 2006) in our microfluidic mixer (Fig. 1A). In such devices, sample mixing is achieved by enhancing the diffusion effect between the laminar flows of two different fluids by incorporating Y-injection and Dean's vortex mixers (Kim et al., 2005; Stroock et al., 2002). The modification and design of the chaotic mixer can enhance folding, stretching and breaking of the laminar flows, which improves their mixing efficiency (Whitesides, 2006). The diminutive scale of the flow channels in microfluidic systems increases the surface-to-volume ratio, and provides instant and adjustable contact time (<1.00 s) by adjusting flow rate, which is therefore advantageous for investigating cross-contamination and sanitization in wash water. Therefore, the main objectives of this study were to design and fabricate a microfluidic mixer and to use it to determine pathogen inactivation kinetics via chlorine solution in sub-second range.

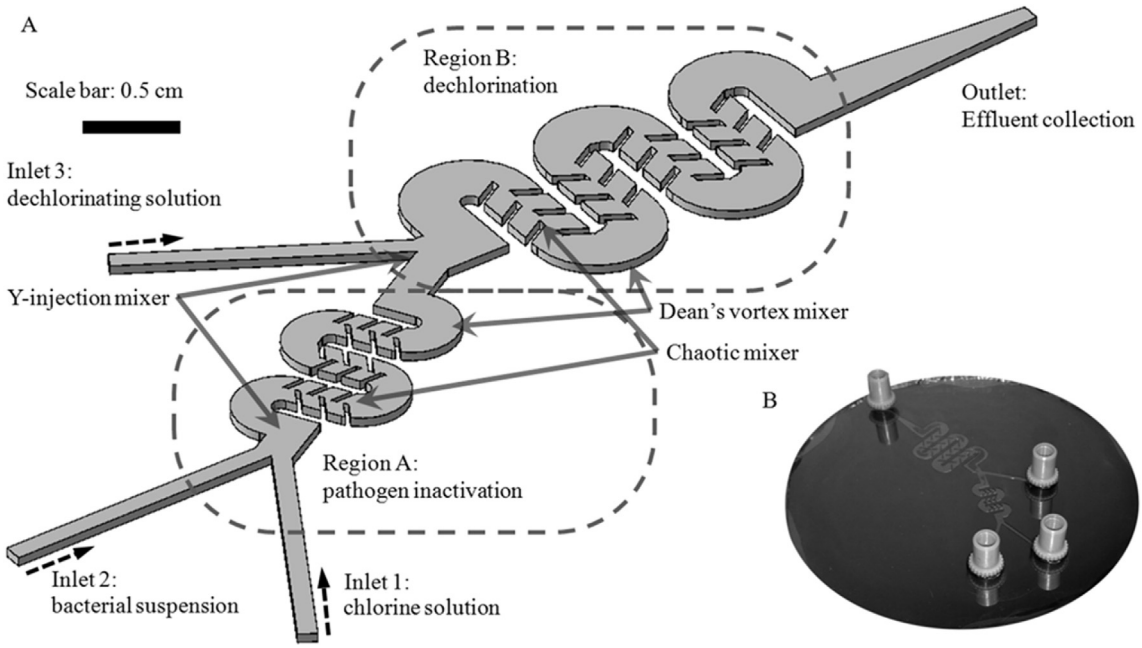

Fig. 1. Diagrams and image of the microfluidic mixer: A. Schematic view of a microfluidic mixer that simulates instant cross-contamination and sanitization scenarios. B. Image of sample microfluidic device used to simulate pathogen survival scenarios.

2. Experimental section

2.1. Bacterial strains and culture preparation

A three strain cocktail of *Escherichia* coli O157:H7 (RM4406, ATCC 43895, and ATCC 700728) with nalidixic acid resistance was used in this study. The isolate RM4406 (lettuce outbreak isolate) was provided by Robert Mandrell (U.S. Department of Agriculture, Agricultural Research Service, Albany, CA, USA). Strains ATCC 43895 and 700728 originally were obtained from the American Type Culture Collection (Manassas, VA, USA), and were selected for nalidixic acid resistance. The strains were individually maintained frozen at −80 °C in nutrient broth supplemented with 20% glycerol. For the culture preparation, frozen suspensions from each strain were thawed and streaked onto tryptic soy agar (TSA, Neogen, Lansing, MI, USA), and the plates were incubated at 37 °C for 24 h. Single colonies from the TSA plate were transferred to 30 mL tryptic soy broth (TSB, Neogen, Lansing, MI, USA) containing 50 μg nalidixic acid per ml, and incubated for 20 h at 37 °C with shaking. Cells were harvested by centrifugation at 4300 *g* for 5 min, washed once in sterile phosphate-buffered saline (PBS), and then resuspended in 10 mL of PBS. Equal volumes of cell suspensions from each strain were mixed and diluted in PBS to achieve a cocktail of inoculums with approximately 10^7 CFU *E. coli* O157:H7 per ml.

2.2. Design, fabrication, and assembly of the micromixer

A micromixer was designed to incorporate three mixing designs with "Y" inlet junction, Dean's vortex mixer, and chaotic mixer (Fig. 1A) (Kim et al., 2005; Stroock et al., 2002) using AutoCAD software program. The device has three inlets for bacterial, chlorine, and dechlorinating solutions (Hach, Loveland, CO) respectively, and one outlet for effluent collection. These designs create two mixing regions, one for bacteria-chlorine (Region A, Fig. 1A) and the other for chlorine-dechlorinating solutions (Region B, Fig. 1A). The device was fabricated and assembled using modified microfabrication methods that are previously reported (Kim et al., 2005; Zhang et al., 2013). The master mold was fabricated on a silicon wafer with SU-8 microchannels via contact photolithography using a mask aligner according to the supplier's instruction. Polydimethylsiloxane (PDMS) replicas with patterned microchannels were prototyped via soft lithography by curing the PDMS mixture (base: curing agents = 10:1) on the mast mold at 125 °C for 20 min. Cured replicas were further assembled into the micromixer on a clean silicon wafer via O_2 plasma treatment (March Jupiter III, Westlake, OH, USA). Both inlet and outlet ports were created using Nanoports (IDEX Health and Science, Oak Harbor, WA, USA) to provide seamless connection with an external syringe pump, which allows adjustable flow rate.

Upon successful assembly of the device, the micromixer initially was evaluated for its effectiveness and accuracy in providing constant, stable input of solutions through the microchannel. The stability of bacterial concentration was tested by pumping the bacterial suspension (via Inlet 1, Fig. 1A) and sterile water (via Inlets 2 and 3) at different flow rates. The effluent was collected and the bacteria were enumerated using modified 'Most Probable Number' (MPN) procedures as described in the following section (Xia et al., 2012). The chlorine stability was tested similarly by pumping chlorine solution (via Inlet 2, Fig. 1A) with water, and the effluent was collected to quantify chlorine concentration using the DPD colorimetric method (Shen et al., 2013). In the follow up chlorine stability test and dechlorinating efficiency studies, both chlorine (via Inlet 2, Fig. 1A) and dechlorinating (via Inlet 3, Fig. 1A) solutions were pumped into the micromixer with water, and the effluent was collected to detect chlorine residual using the DPD method.

The mixing efficiency and mixing pattern were evaluated by modeling scalar mixing (mixing efficiency) using the Computational Fluid Dynamics software (Autodesk Simulation CFD, 2014). The chlorine diffusion coefficient in water used in modeling was 1.38×10^{-5} cm^2/s (Tang and Sandall, 1985). The solution has a low Reynolds number in this device, and the liquid was determined to be incompressible and the flow was characterized as laminar in the scalar mixing model (Groisman and Steinberg, 2001). The boundary

conditions (i.e. inlet volume flow rate, and outlet pressure) were adjusted to reflect the actual experimental value. The contact time between bacteria and chlorine solution was defined as the time required to pass through the mixing Region A (Fig. 1A). The contact time was controlled by adjusting the flow rate of the syringe pump. The scalar mixing simulation was averaged over the z-axis (i.e., 15 layers) from a steady three-dimensional computation involving five repeated cycles of 100 iterations each (Autodesk Simulation CFD, 2014). During each repeat cycle, a suitable level of convergence was achieved after 30 iterations with similar contour plots generated by all five repeat cycles. During the simulation, the two fluids were considered passive and nonreactive during the modeling; the micromixer was spatially discretized via the finite element method; and the discretized equations, including the Navier–Stokes equation using primitive variables, were solved using SIMPLE-R algorithm in the commercial CFD software. The contact time distribution was quantified using the trace function in the CFD software. All computations were performed using a spatial discretization with 15 identical uniformly spaced layers in the *z*-direction, each consisting of 604,398 grid points with a mean spacing of 0.0357 mm in the commercial CFD software.

2.3. Pathogen inactivation study

A series of chlorine solutions with free chlorine concentrations of 0.5, 1.0, 5.0, 10.0, 20.0, 50.0 mg/L were freshly prepared using sodium hypochlorite (Shen et al., 2013). The free chlorine concentration before and after the tests were verified using a chlorine photometer (CP-15, HF Scientific Inc., Ft. Myers, FL, USA). To determine the kinetics of chlorine on pathogen inactivation, chlorine solutions were pumped into the micromixer, together with the bacterial suspension of *E. coli* O157:H7 through a separate inlet. This was followed by injection of dechlorinating solution at the entry to Region B to stop the chlorine-pathogen reaction. To consider a dilution effect inside the micromixer (Region A, Fig. 1A), the chlorine concentration in the start solution was double the effective concentration in the microchannel. All concentrations discussed in the study are effective concentrations after considering the dilution factors. The dechlorinating solution containing 0.1% sodium pyruvate and dechlorinating reagent (containing 1–10% sodium thiosulfate and 90–99% sodium sulfate) was pumped via Inlet 3 (Fig. 1A) to stop the inactivation process. The contact time was controlled by adjusting the solution flow rate and was determined quantitatively via CFD modeling. Pathogen survival was quantified using a modified MPN method as detailed in our previous publications (Luo et al., 2012). The pathogen inactivation results were fitted to Watson (1908), Hom (1972), and Sellecket al. (1978) models using the nonlinear least square method in SigmaPlot software (Version 12.5, Systat Software Inc., San Jose, CA, USA).

2.4. Statistics

All experiments were repeated at least three times, and data were analyzed using SAS software (Version 9.3). *E. coli* O157:H7 populations were subjected to $\log_{10}$ transformation before statistical analysis. Data were analyzed as a two-way analysis of variance (ANOVA) with treatment and contact time as the main factors. Assumptions of normality and variance homogeneity were checked and the variance grouping technique was used to correct for variance heterogeneity. When effects were statistically significant, means comparisons were done using Tukey's range test with adjusted p-values to maintain experiment-wise error of $\leq$0.05.

3. Results and discussion

3.1. Characterization of micromixer

The effectiveness of the microfluidic mixer in maintaining stable concentrations of chlorine and bacterial suspensions during pumping was validated. The stability of the solution delivery first was tested by separately pumping chlorine (via Inlet 2, Fig. 1A) with water, and then bacterial suspension (Inlet 1, Fig. 1A) with water, and collecting the effluent of each independent test for quantification. As shown in Fig. 2, both chlorine concentration (5.0 mg/L) and bacterial suspension (10^7 MPN/mL) were stable for the entire 20 min test period during continuous pumping at a flow rate of 0.16 mL/min, or the equivalent of a 0.75 s contact time. The deviation of chlorine concentration was no more than 3%. *E. coli* O157:H7 concentrations varied by less than 2.5% (Fig. 2) from the respective expected values. The expected concentrations detected from the outlet were calculated based on a three-fold dilution of the inlet concentration of chlorine (Inlet 1) or *E. coli* (Inlet 2) solution, as all inlets were maintained at the same flow rate of 0.16 mL/min throughout the characterization. The variations are randomly distributed above and below the expected values, which can be attributed to random downstream measurement error associated with spectroscopic N,N-diethyl-p-phenyldiamine (DPD) method for chlorine and MPN method for *E. coli* O157:H7. Color development time was the major cause for the experimental error in the DPD method, which requires at least five minutes to develop relatively stable and consistent absorbance measurements. The precision of the MPN method, however, was impaired by the limited sample volume that can be collected from a microfluidic mixer.

The mixing efficiency of Region A and Region B (Fig. 1) were also evaluated in separate experiments. The total pathogen inactivation (negative control) was evaluated by mixing chlorine (50.0 mg/L) and pathogen solution (10^8 MPN/mL) inside the micromixer without the addition of dechlorinating solution, resulting in no bacterial survival detected from the collected effluent at the flow rate and contact time of 1.20 mL/min and 0.10 s, respectively. The neutralizing power of dechlorinating reagent was demonstrated when pumping both chlorine and dechlorinating solutions into the micromixer resulted in no chlorine residue being detected in the collected effluent. These results are consistent with other reported micromixer studies with different solutions and flow rates (Mansur et al., 2008; Stroock et al., 2002).

The mixing efficiency and mixing pattern were studied using CFD modeling of scalar mixing (Vanka et al., 2004). During the modeling process, all solutions were considered as Newtonian fluids with non-slip boundary conditions. The solution has a low Reynolds number in this device, and laminar flow characteristics, due to the small scale (Groisman and Steinberg, 2001). Achieving efficient and rapid mixing is one of the challenging tasks in micromixers, because mixing under laminar flow conditions depends on slow diffusion of chemicals and bacterial cells (Kwon et al., 2008). Therefore, our study incorporated multiple passive micromixer designs that can be completed by simple modification of channel geometries into the following ones: “Y” injection, serpentine channels (Kim et al., 2005), and channels with patterned grooves (Whitesides, 2006). These modifications and designs enhance folding, stretching and breaking of the laminar flows, which improves the mixing efficiency during flow. Fig. 3 shows the averaged modeled scalar mixing of chlorine and bacterial solutions in the micromixer Region A (Fig. 1), which was averaged over the z-axis (i.e., 15 layers) from a steady three-dimensional computation involving five repeated cycles of 100 iterations each. During each repeat cycle, a suitable level of convergence was achieved after 30

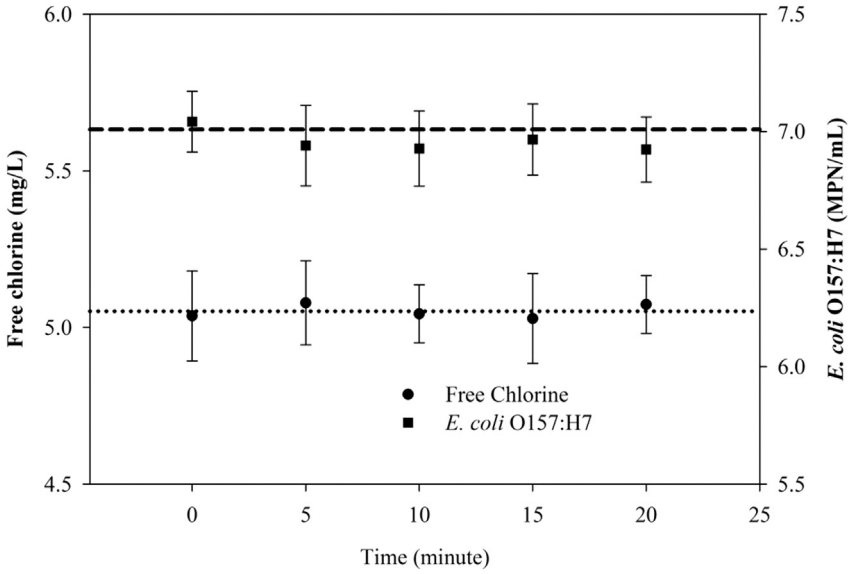

Fig. 2. Graph showing stability of chlorine and bacterial solutions concentration at 20 min; chlorine solution (left axis) and bacteria suspension (right axis). Lines indicate theoretical values for chlorine solution (dotted) and bacteria suspension (dashed). Flow rate was set for 0.75 s contact time.

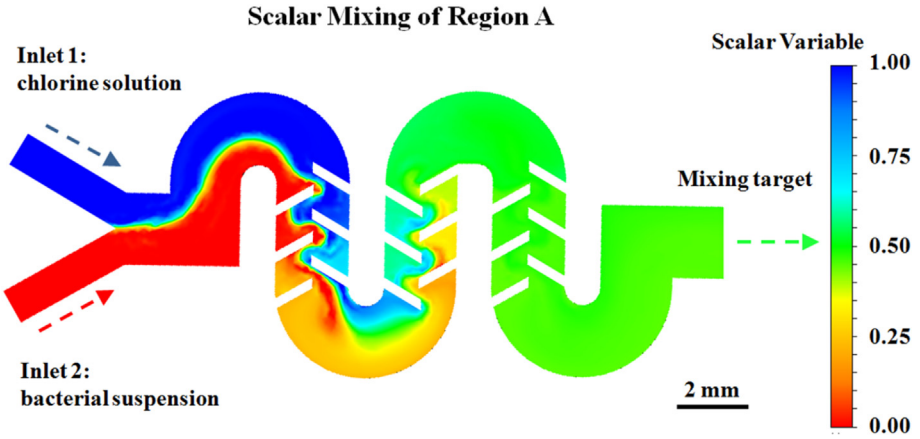

Fig. 3. Schematic view of the modeled scalar mixing of bacteria, chlorine, and dechlorinating solutions inside the micromixer at a flow rate equivalent to 0.75 s contact time.

iterations with similar contour plots generated by all five repeat cycles. The purpose of the modeling was to simulate the mixing of two fluids, and use the scalar boundary to track the relative concentrations of the two fluids. The chlorine stock solution (Fig. 1, inlet 1) was diffusive and assigned a scalar boundary condition of 1, and the bacterial stock solution contained nondiffusive particles. The scalar variable (i.e., 0–1) was defined as relative chlorine concentration in the mixing channel, where 1 and 0 represent 100% and 0% of the original stock concentration respectively. The modeled result indicated that chlorine homogenously diffuses inside the chaotic mixer and mixes with the bacterial solution to achieve the contact time. Future improvement of the scalar modeling could include the reactive nature of the two fluids to better simulate real-time reactions between chlorine and bacteria. Therefore, the micromixer provides the means to conduct studies on pathogen inactivation kinetics in time periods as brief as 0.10 s. The CFD modeling also indicated that increased flow rates which result in decreased contact time, could increase mixing efficiency (Groisman and Steinberg, 2001; Vanka et al., 2004).

The micromixer was also evaluated for its correlation between flow rate and contact time. By changing flow rate at different inlets, the time needed for fluid elements to pass through the mixing region can be varied, which allows the contact time to be adjusted based on flow rate. The relationship between the initial flow rate required and the specific contact time distribution can also be determined and predicted by the trace function in the CFD modeling software. Fig. 4A shows the correlation between inlet flow rate and contact time distribution. The micromixer had channel dimensions of 1 mm (width) by 0.2 mm (height), both determined by profilometry (P-1 long scan profiler, Tencor, Milpitas, CA, USA), resulting in a cross-sectional area (A) of 0.2 mm^2. The travel distance (D) of the chaotic mixer was 10 mm. The contact time (t) can be adjusted by changing the flow rate (r), and is calculated as:

$$t = D \times A/r \quad (1)$$

Ideally, every fluid element entering mixing Region A should spend the same amount of time before it enters mixing Region B. However, with the folding and stretching of the laminar flow condition, different fluid elements took different amounts of time to pass through the mixing patterns. Fig. 4B shows the contact time

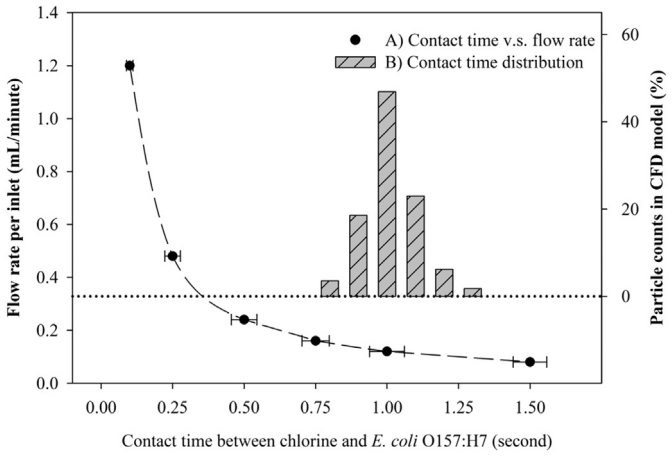

Fig. 4. Predicted values and distributions for parameters modeling using the CFD trace function; A) Relationship between flow rate and contact time in the micromixer; B) Contact time distribution at 0.12 mL/s, or equivalently 1.00 s contact time.

distribution at a flow rate of 0.12 mL/min, which is equivalent to 1.00 s contact time. In reality, not all fluid elements spend the same amount of time passing through the mixing regions in the micromixer. In the following pathogen inactivation study, the flow rate was adjusted to set the volume-averaged residence time based on Equation (1) at values between 0.10 and 1.50 s.

3.2. Pathogen inactivation study

The effect of chlorine concentration and exposure time on *E. coli* O157:H7 inactivation was tested using the micromixer after the device was evaluated. As shown in Fig. 5 and Table 1, the inactivation of *E. coli* O157:H7 was significantly affected by free chlorine concentration ($P < 0.0001$), exposure time ($P < 0.0001$), and their interactions ($P < 0.0001$). The chlorine concentration and contact time exhibited a negative relationship, i.e., more concentrated chlorine solution required less contact time to achieve a 5-$\log_{10}$ reduction. With an *E. coli* O157:H7 suspension solution containing 1×10^8 CFU/mL, 1.0 mg/L free chlorine solutions achieved 0.14-$\log_{10}$ reduction in 0.25 s, and 4.97-$\log_{10}$ reduction in 1.00 s. Increasing chlorine concentration significantly reduced exposure time required to achieve the same $\log_{10}$ reduction. With a free chlorine concentration of 10.0 mg/L, a 3.87-$\log_{10}$ reduction was achieved in 0.1 s, and a 5.77-$\log_{10}$ reduction in 0.25 s. Although no previous study has reported subsecond inactivation kinetics of *E. coli* O157:H7 with chlorine, the results are in line with our previous study (Shen et al., 2013) which was conducted with a longer reaction time. Pathogen inactivation with over 4.5-$\log_{10}$ reduction can be achieved by exposure to 0.5 mg/L free chlorine solution for over 30.00 s or 1.0 mg/L free chlorine for over 1.00 s.

Table 1
Effects of chlorine concentration and contact time on pathogen inactivation ($\log_{10}$ reduction).

Concentration		Time						
		0.00 s	0.10 s	0.25 s	0.50 s	0.75 s	1.00 s	1.50 s
0.5 mg/L	Reduction *log(MPN/mL)*	0.000	0.113	0.182	0.446	0.304	0.542	1.035
	Standard error *log(MPN/mL)*	0.708	0.373	0.403	0.169	0.143	0.057	0.277
1.0 mg/L	Reduction *log(MPN/mL)*	0.000	0.163	0.146	1.252	2.823	4.972	5.815
	Standard error *log(MPN/mL)*	0.708	0.214	0.312	0.333	0.227	0.037	0.109
5.0 mg/L	Reduction *log(MPN/mL)*	0.000	1.963	4.563	5.374	5.561	6.699	6.276
	Standard error *log(MPN/mL)*	0.708	0.497	0.429	0.358	0.293	0.153	0.042
10.0 mg/L	Reduction *log(MPN/mL)*	0.000	3.868	5.772	7.755	7.167	7.755	7.473
	Standard error *log(MPN/mL)*	0.132	0.296	0.369	0.260	0.588	0.287	0.282
20.0 mg/L	Reduction *log(MPN/mL)*	0.000	4.187	6.603	7.755	7.755	7.755	7.755
	Standard error *log(MPN/mL)*	0.132	0.189	0.583	0.000	0.000	0.000	0.000
50.0 mg/L	Reduction *log(MPN/mL)*	0.000	4.187	6.603	7.755	7.755	7.755	7.755
	Standard error *log(MPN/mL)*	0.132	0.100	0.000	0.235	0.000	0.000	0.000

The determination of minimum effective free chlorine concentration required to prevent pathogen survival and cross-contamination during produce wash is a major challenge facing the produce industry and regulatory agencies. The US industry-wide hazard analysis and critical control points (HACCP) program has set 1.0 mg/L free chlorine as the critical control limit (CCP) previously. Recent studies have shown that this chlorine concentration is insufficient in preventing pathogen cross-contamination

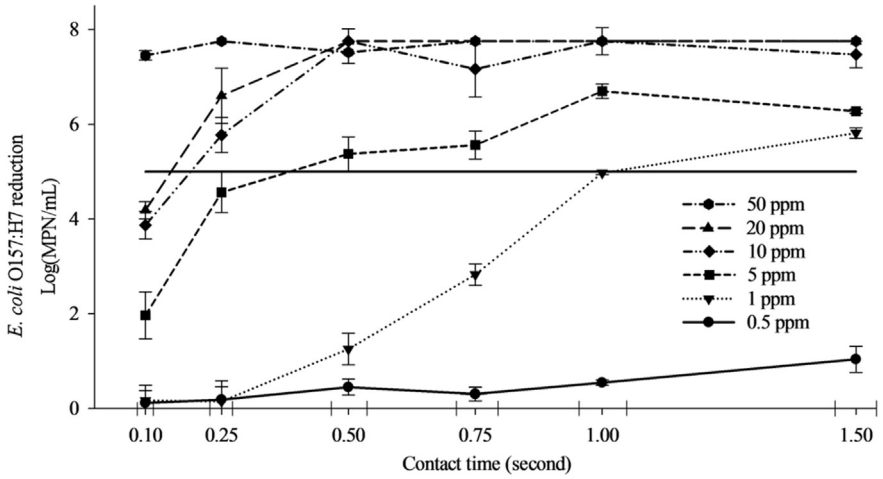

Fig. 5. Effects of chlorine concentration and contact time on pathogen inactivation ($\log_{10}$ reduction). The solid line without data points indicates sufficient inactivation (5-$\log_{10}$ reduction) to prevent cross-contamination.

(Luo et al., 2011). However, the effective minimum free chlorine concentration to prevent cross-contamination has not yet been determined and validated. During water-mediated cross-contamination, pathogens are first dislodged from the surface of the contaminated produce, survive in wash water, and then transfer to the originally uncontaminated produce as they move along with wash water. Therefore, the effective minimum free chlorine concentration required to prevent pathogen cross-contamination must be a concentration that can inactivate pathogens instantaneously. In this study, we determined that a free chlorine concentration of 1.0 mg/L will achieve a 5-$\log_{10}$ reduction in 1.00 s, 5.0 mg/L in 0.50 s, and 10.0 in 0.25 s. Chlorine concentration at 0.5 mg/L, 20.0 mg/L and 50.0 mg/L were included in the study, but the accurate determination of exposure time required to achieve a 5-$\log_{10}$ reduction requires additional modification of the micromixer to accommodate such low and high chlorine concentrations. The results obtained with 1.0, 5.0, and 10 mg/L in this study provide a reasonably good explanation for the observation that cross-contamination occurs at 1.0 mg/L, but not at 10.0 mg/L, as 1.0 mg/L requires more than 1.00 s to achieve a 5-$\log_{10}$ reduction in pathogen while 10.0 mg/L kills pathogens much faster (0.25 s for a 5-$\log_{10}$ reduction). The results were analyzed with the contour graph (Fig. 6), with the predicted $\log_{10}$ reduction (1–5 $\log_{10}$ CFU/mL) at different chlorine concentrations and contact times.

3.3. Kinetic models of pathogen inactivation

Kinetic modeling was also applied to simplify the complicated disinfection phenomena of produce wash system. Three models that are commonly used for studying bacterial disinfection kinetics with chemical-based disinfectant, include the Chick–Watson Model (Haas and Karra, 1984; Watson, 1908), the Hom Model (Haas and Karra, 1984; Hom, 1972), and the Selleck Model (Lee and Nam, 2002; Selleck et al., 1978).

Watson (1908) developed an empirical logarithmic equation to relate the inactivation constant (k) to disinfectant concentration (C) and reaction time (t):

$$\log\left(\frac{N}{N_0}\right) = -kC^n t \tag{2}$$

where

N = number of pathogen cells per unit volume,
N_0 = number of pathogen cells initially at time zero,
k = strain- and condition-specific inactivation constant,
C = free chlorine concentration,
n = coefficient of dilution,
t = reaction (contact) time.

From the experimental data in Fig. 5 and Table 1, k and n were determined as:

$$\log\left(\frac{N}{N_0}\right) = -3.48C^{0.24}t \tag{3}$$

The Hom model is a generalized empirical equation of the Chick–Watson model considering chlorine disinfection as a pseudo first-order reaction (Hom, 1972):

$$\log\left(\frac{N}{N_0}\right) = -kC^n t^m \tag{4}$$

where m is a reaction rate constant and other factors are as described above for Equation (2). The constants were modeled using experimental data as:

$$\log\left(\frac{N}{N_0}\right) = -3.18C^{0.28}t^{0.18} \tag{5}$$

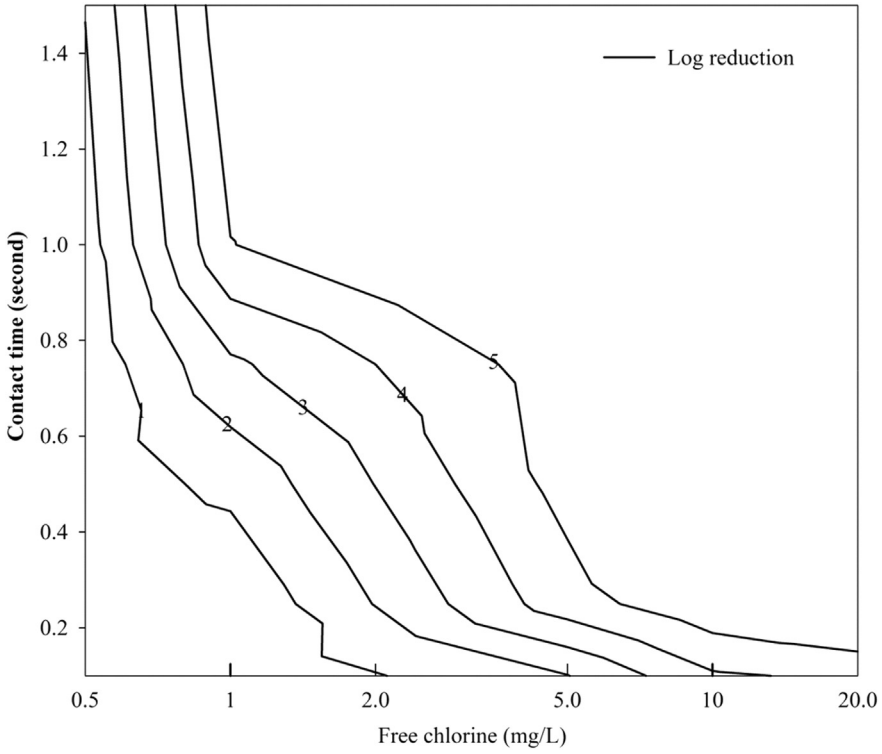

Fig. 6. Contour graph of log reductions (1–5 $\log_{10}$) of *E. coli* O157:H7 at different chlorine concentrations and contact times.

The Selleck model was originally developed to predict chlorine inactivation of bacteria in wastewater. The model was empirical and can be adjusted to different sanitization systems:

$$\log\left(\frac{N}{N_0}\right) = -n\log\left(1+\frac{Ct}{k}\right) \quad (6)$$

The experimental data also was applied to compute values of empirical coefficient k and n, which yields:

$$\log\left(\frac{N}{N_0}\right) = -0.678\log\left(1+\frac{Ct}{0.25}\right) \quad (7)$$

The pathogen inactivation kinetics of the three selected models were compared statistically with the original experimental data. Fig. 7 shows the effect of chlorine concentration at a specific contact time (0.25 s). Both the Watson (P = 0.860) and Hom (P = 0.841) models reflected the pathogen inactivation scenarios with short contact times, and the computed parameters are globally fitted to contact times between 0.10 and 1.00 s. The nonlinear regression was performed using the 'dynamic curve fitting' option in the software, which automates the search for initial parameter values that lead to convergence and continues to improve until the differences between the residual sum of squares no longer decreases significantly. The Selleck model was used to predict pathogen survival in chlorine over a substantially longer contact time (e.g. 3 h) than was used in the studies reported here (Lee and Nam, 2002). Thus it is not surprising that the model didn't fit the results from this short time-course kinetic study (P < 0.0001). The variance in the experimental data is consistent with the heterogeneity of inherent chlorine resistance in a population of bacterial cells. That heterogeneity, as well as spatiotemporal variation of chlorine in the microchannel (Fig. 3), contribute to and are consistent with, the differences between the experimental data and the computational simulations (Cerf, 1977; Haas and Karra, 1984; Lee and Nam, 2002).

In a chlorine-based disinfection process, the decimal reduction time (D-value) is defined as the time required at a specified free chlorine concentration to kill 90% (one $\log_{10}$ reduction) of the organisms being studied. The D-value here was calculated based on the empirical models developed in this study, namely the Watson model (Equation (3)) and the Hom model (Equation (5)). Fig. 8 shows the relationship between chlorine concentration and D-value. For the widely used 1 mg/L free chlorine concentration in HACCP programs, the D-value is 28.06 ms. By increasing chlorine concentration to 5.0, 10.0, and 20.0 mg/mL, the D-value reduces to 5.72, 2.87, and 1.43 ms respectively.

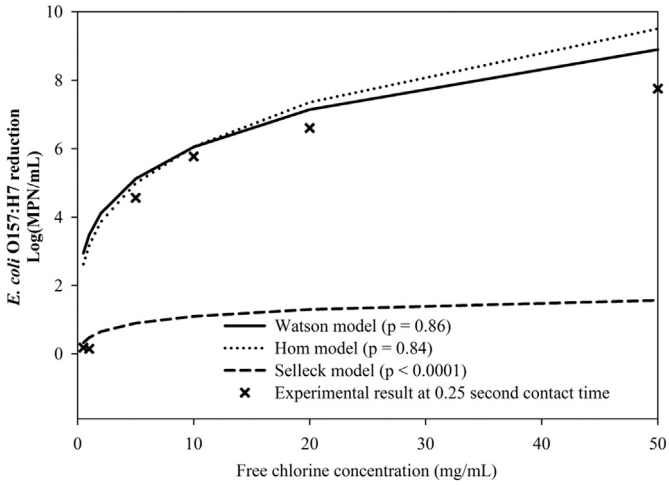

Fig. 7. Comparison of experimental results to three selected bacterial inactivation models: Watson, Hom, and Selleck, with associated goodness-of-fit probability values.

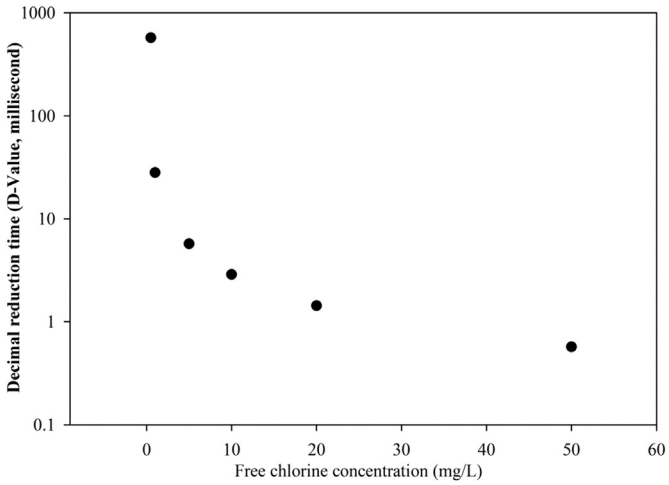

Fig. 8. Decimal reduction time (D-value) required at certain free chlorine concentration to kill 90% (1-$\log_{10}$ reduction) of *E. coli* O157:H7 in water.

4. Conclusions

A micromixer useful for assessing pathogen inactivation kinetics at less than 1.00 s was designed, fabricated, and tested. This device also was used to determine the time and dose-dependent response of pathogen inactivation via free chlorine. Test results indicate that 1) *E. coli* O157:H7 inactivation is significantly affected by free chlorine concentration (P < 0.0001), contact time (P < 0.0001), and their interactions (P < 0.0001); 2) A 5-$\log_{10}$ reduction of *E. coli* O157:H7 requires exposing *E. coli* O157:H7 cells to a solution containing 1.0 mg/L free chlorine for at least 1.00 s, or a solution containing 10.0 mg/L free chlorine for 0.25 s. These findings provide critical information regarding the determination of minimum free chlorine concentration required to prevent pathogen survival and cross-contamination during fresh produce wash operations. This study also provides an innovative tool for developing better processes for the produce industry. Future evaluations that build on results from this current study may need to incorporate the disinfection of process water containing varying organic loads, sanitizers, and pathogen strains that may have become adapted to chlorine. Further work should also include both chlorine -adapted and generic strains, and the validation should account for the difference between resistance and non-resistance strains. Ongoing research is fine-tuning the microfluidic mixer for improved accuracy and precision on solute concentration and contact time, and for accommodating a broader range of sanitizer concentrations and exposure time.

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References

Boskovic, D., Lobbecke, S., Gross, A., Kohler, M., 2011. Residence time distribution studies in microfluidic mixing structures. Chem. Eng. Technol. 34, 361–370.

CDC, 2011. In: Prevention, C.F.D.C.A. (Ed.), CDC Estimates of Foodborne Illness in the United States. http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html.
Cerf, O., 1977. Tailing of survival curves of bacterial-spores. J. Appl. Bacteriol. 42, 1–19.
FDA, 2012a. Current good manufacturing practice and hazard analysis and risk-based preventive controls for human food. Fed. Regist. 78, 3646–3824.
FDA, 2012b. Standards for the growing, harvesting, packing, and holding of produce for human consumption. Fed. Regis. 78, 3504–3646.
Gil, M.I., Selma, M.V., Lopez-Galvez, F., Allende, A., 2009. Fresh-cut product sanitation and wash water disinfection: problems and solutions. Int. J. Food Microbiol. 134, 37–45.
Gomez-Henz, A., Perez-Bendito, D., 1991. The stopped-flow technique in analytical chemistry. Anal. Chim. Acta 242, 147–177.
Gómez-López, V.M., Lannoo, A., Gil, M.I., Allende, A., 2014. Minimum free chlorine residual level required for the inactivation of *Escherichia coli* O157:H7 and trihalomethane generation during dynamic washing of fresh-cut spinach. Food Control 42, 132–138.
Gould, L.H., Walsh, K.A., Vieira, A.R., Herman, K., Williams, I.T., Hall, A.J., Cole, D., 2013. Surveillance for foodborne disease outbreaks – United States, 1998–2008. MMWR Surveill. Summ. 62, 1–34.
Groisman, A., Steinberg, V., 2001. Efficient mixing at low Reynolds numbers using polymer additives. Nature 410, 905–908.
Haas, C.N., Karra, S.B., 1984. Kinetics of microbial inactivation by chlorine. 1. Review of results in demand-free systems. Water Res. 18, 1443–1449.
Hom, L.W., 1972. Kinetics of chlorine disinfection in an ecosystem. J. Sanit. Eng. Div. ASCE 98, 183.
Kim, D.S., Lee, S.H., Kwon, T.H., Ahn, C.H., 2005. A serpentine laminating micromixer combining splitting/recombination and advection. Lab-on-a-Chip 5, 739–747.
Kwon, S.K., Kou, S., Kim, H.N., Chen, X., Hwang, H., Nam, S.W., Kim, S.H., Swamy, K.M.K., Park, S., Yoon, J., 2008. Sensing cyanide ion via fluorescent change and its application to the microfluidic system. Tetrahedron Lett. 49, 4102–4105.
Lee, Y.J., Nam, S.H., 2002. Reflection on kinetic models to the chlorine disinfection for drinking water production. J. Microbiol. 40, 119–124.
Lopez-Galvez, F., Allende, A., Selma, M.V., Gil, M.I., 2009. Prevention of *Escherichia coli* cross-contamination by different commercial sanitizers during washing of fresh-cut lettuce. Int. J. Food Microbiol. 133, 167–171.
Lopez-Galvez, F., Gil, M.I., Truchado, P., Selma, M.V., Allende, A., 2010. Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. Food Microbiol. 27, 199–204.
Luo, Y., Nou, X., Millner, P., Zhou, B., Shen, C., Yang, Y., Wu, Y., Wang, Q., Feng, H., Shelton, D., 2012. A pilot plant scale evaluation of a new process aid for enhancing chlorine efficacy against pathogen survival and cross-contamination during produce wash. Int. J. Food Microbiol. 158, 133–139.
Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M., Conway, W., 2011. Determination of free chlorine concentrations needed to prevent *Escherichia coli* O157:H7 cross-contamination during fresh-cut produce wash. J. Food Prot. 74, 352–358.
Mansur, E.A., Ye, M., Wang, Y., Dai, Y., 2008. A state-of-the-art review of mixing in microfluidic mixers. Chin. J. Chem. Eng. 16, 503–516.
Sasaki, M.K., Vicente, S., Grassi, V., Zagatto, E., 2012. Differential reaction-rate methods in flow analysis. Open Anal. Chem. J. 6, 28–38.
Selleck, R.E., Saunier, B.M., Collins, H.F., 1978. Kinetics of bacterial deactivation with chlorine. J. Environ. Eng. Div. ASCE 104, 1197–1212.
Shen, C., Luo, Y., Nou, X., Wang, Q., Millner, P., 2013. Dynamic effects of free chlorine concentration, organic load, and exposure time on the inactivation of *Salmonella, Escherichia coli* O157:H7, and non-O157 shiga toxin-producing *E. coli*. J. Food Prot. 76, 386–393.
Stocker, R., Seymour, J.R., Samadani, A., Hunt, D.E., Polz, M.F., 2008. Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches. Proc. Natl. Acad. Sci. 105, 4209–4214.
Stroock, A.D., Dertinger, S.K.W., Ajdari, A., Mezic, I., Stone, H.A., Whitesides, G.M., 2002. Chaotic mixer for microchannels. Science 295, 647–651.
Tang, A., Sandall, O.C., 1985. Diffusion-coefficient of chlorine in water at 25–60 degrees-C. Chem. Eng. Data 30, 189–191.
Uyttendaele, M., Neyts, K., Vanderswalmen, H., Notebaert, E., Debevere, J., 2004. Control of *Aeromonas* on minimally processed vegetables by decontamination with lactic acid, chlorinated water, or thyme essential oil solution. Int. J. Food Microbiol. 90, 263–271.
Vanka, S.P., Luo, G., Winkler, C.M., 2004. Numerical study of scalar mixing in curved channels at low Reynolds numbers. AICHE J. 50, 2359–2368.
Watson, H.E., 1908. A note on the variation of the rate of disinfection with change in the concentration of the disinfectant. J. Hyg. 8, 536–542.
Wessel, A., Hmelo, L., Parsek, M., Whiteley, M., 2013. Going local: technologies for exploring bacterial microenvironments. Nat. Rev. Microbiol. 11 (5), 337–348.
Whitesides, G.M., 2006. The origins and the future of microfluidics. Nature 442, 368–373.
Xia, X., Luo, Y., Yang, Y., Vinyard, B., Schneider, K., Meng, J., 2012. Effects of tomato variety, temperature differential, and post-stem removal time on internalization of Salmonella enterica serovar Thompson in tomatoes. J. Food Prot. 75, 297–303.
Zhang, B., Feldman, A., Wang, Q., 2013. A novel insight in rapid allergen detection in food systems: from threshold dose to real-world concentration. Sensors Actuat. B Chem. 186, 597–602.
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. J. Food Prot. 2, 1392–1397.
Zhou, B., Luo, Y., Nou, X., Millner, P., 2014. Development of an algorithm for feed-forward chlorine dosing of lettuce wash operations and correlation of chlorine profile with *Escherichia coli* O157:H7 inactivation. J. Food Prot. 77, 558–566.