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Inactivation dynamics of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in wash water during simulated chlorine depletion and replenishment processes

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ABSTRACT

Maintaining effective sanitizer concentration is of critical importance for preventing pathogen survival and transference during fresh-cut produce wash operation and for ensuring the safety of finished products. However, maintaining an adequate level of sanitizer in wash water can be challenging for processors due to the large organic load in the wash system. In this study, we investigated how the survival of human pathogens was affected by the dynamic changes in water quality during chlorine depletion and replenishment in simulated produce washing operations. Lettuce extract was added incrementally into water containing pre-set levels of free chlorine to simulate the chlorine depletion process, and sodium hypochlorite was added incrementally into water containing pre-set levels of lettuce extract to simulate chlorine replenishment. Key water quality parameters were closely monitored and the bactericidal activity of the wash water was evaluated using three-strain cocktails of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. In both chlorine depletion and replenishment processes, no pathogen survival was observed when wash water free chlorine level was maintained above 3.66 mg/L, irrespective of the initial free chlorine levels (10, 50, 100 and 200 mg/L) or organic loading (chemical oxidation demand levels of 0, 532, 1013 and 1705 mg/L). At this free chlorine concentration, the measured ORP was 843 mV and pH was 5.12 for the chlorine depletion process; the measured ORP was 714 mV and pH was 6.97 for the chlorine replenishment process. This study provides quantitative data needed by the fresh-cut produce industry and the regulatory agencies to establish critical operational control parameters to prevent pathogen survival and cross-contamination during fresh produce washing.

1. Introduction

Fresh and fresh-cut produce is susceptible to microbial contamination during wash operation (Lehto et al., 2011; Olaimat and Holley, 2012). Processing water is commonly re-circulated and reused for economic and environmental considerations. This wash water often contains soils, plant debris, and organic materials released from cut or damaged produce. Pathogens from contaminated produce can be dislodged from the plant surfaces and transferred to clean produce, thus resulting in cross-contamination. Therefore, maintaining effective sanitizer concentrations in wash water is critical for ensuring food safety of fresh and fresh-cut produce (Luo et al., 2012; Gil et al., 2009; Simons, 1997).

Currently, several types of sanitizers are commonly used in the produce industry, including chlorine, peroxyacetic acid, ozone, and organic acids (USFDA, 2013). Among those, chlorine is the most widely used sanitizer because of its low impact on the nutritional and aesthetic quality of the product, established ability to kill pathogens in suspension, and low cost (Adams et al., 1989; Zhuang et al., 1995). Although chlorine has limited efficacy on pathogen reduction from produce surface, its capacity in inactivating pathogens in suspension make it an effective antimicrobial agent for controlling wash water pathogens.

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Despite widespread use by the fresh-cut produce industry, chlorine's rapid reaction with organic materials and consequent loss of efficacy has been problematic (Nou and Luo, 2010). Although coarse debris is typically removed by mechanical screens, soluble organic matter still accumulates in the wash water over time, along with the formation of chloramines and organochloramines (Beuchat et al., 1998; Garg, 1990; Luo et al., 2012; Pirovani et al., 2001). During the washing process, only a limited amount of fresh water is added to the wash system to compensate for water loss during washing. Water re-circulation allows steady organic matter accumulation and accelerated free chlorine depletion.

To maintain a steady free chlorine level, concentrated sodium hypochlorite (NaClO) is frequently added to the wash system. In the absence of organic matter, free chlorine concentration is proportional to the NaClO added in a given system. However, in the presence of organic matter, chlorine dosing is a technical challenge as the complex chlorination breakpoint has to be considered (Zhou et al., 2014).

Efficacy of chlorine to inactivate pathogens is impacted by several factors, including chlorine concentration, pH, contact time, and pathogen strain (Virto et al., 2005). Chlorine's efficacy to inactivate pathogens is positively correlated with chlorine concentration and exposure time in the chlorine demand-free liquid system (Kouame and Haas, 1991). In general, higher than 5 log cfu/L pathogen reduction can be achieved with 0.5–1.0 mg/L free chlorine with 30 s or longer exposure (Luo et al., 2011; Shen et al., 2013; Van Haute et al., 2013). However, studies regarding the effect of chlorine on pathogen inactivation in the presence of organic matters are limited, and critical data gaps exist in many areas (Shen et al., 2013; Gomez-Lopez, 2014; Van Haute et al., 2013). Although chlorine is often considered a Critical Control Point (CCP) in the industry-wide Hazardous Analysis and Critical Control Point (HACCP) program, scientific validation for the critical control limit during produce wash operation is lacking.

The fresh-cut produce industry needs information on which operational parameters e.g., sanitizer concentration, pH, residence time, organic load, etc. are critical to measure and monitor, as well as the significance of the measurements in terms of the performance of the wash water as a microbial control system. Hence, research projects that can address these data gaps are vital (CPS, 2014; 2011; 2012, 2013). Recently, a study from Gómez-López et al. (2014) reported that the minimum free chlorine of 5 mg/L is required to keep water free from foodborne pathogens during produce wash using a dynamic produce wash system. However, the exact role of initial chlorine concentration and the level of organic matter, and especially their interaction on chlorine depletion and replenishment processes remain unknown. Furthermore, some studies shown that *Listeria monocytegenes* is more resistant to environmental stresses than *Escherichia coli* O157:H7, *Salmonella enterica*; yet their comparative survival patterns during chlorine depletion and replenishment processes are unknown. Thus, this study is designed to address these critical data gaps, and to identify the most critical factors that directly impact pathogen inactivation and survival during produce wash in the presence of organic load.

2. Materials and methods

2.1. Lettuce extract preparation

Iceberg lettuce was purchased from a local produce wholesale market, cut into pieces, pressed through a household juicer, and filtered through eight layers of cheesecloth. Filtered iceberg lettuce extract was aliquoted and stored at −20 °C for use throughout this study.

2.2. Bacterial strains and culture preparation

A three-strain cocktail of *E. coli* O157:H7 with nalidixic acid resistance, a three-strain cocktail of *S. enterica* transformed with plasmid pGT-KAN, and a three-strain cocktail of *Listeria monocytogenes* were used in this study (Table 1). A single colony for each strain was inoculated into 30 mL tryptic soy broth (TSB, Neogen, Lansing, MI, USA) with 50 mg/L nalidixic acid (for *E. coli*), 50 mg/L kanamycin (for *Salmonella*), or without antibiotics (for *L.* monocytogenes) and incubated for 20 h at 37 °C with shaking. Cells were harvested by centrifugation at 4,300 g for 5 min, washed once in sterile phosphate-buffered saline (PBS), and re-suspended in 10 mL of PBS. Equal volumes of cell suspensions from each strain were mixed and diluted in PBS to achieve a cocktail of inoculum with approximately 10^8 CFU/mL for each of the three species.

2.3. Simulation of chlorine depletion in wash water

Sodium hypochlorite (NaClO) (7.56%) was added to 3 L of water to obtain chlorinated water with targeted initial free chlorine concentrations of 10, 50, 100, and 200 mg/L. The pH was adjusted to 6.5 with 25% citric acid. Lettuce extract was incrementally added to the chlorinated water in 2 mL aliquots every 2 min to simulate the accumulating organic load in commercial fresh-cut lettuce wash water. Water samples were taken at varying intervals of lettuce extract addition, depending on the expected impact of the incremental increase in organic load on free chlorine concentration. Higher sampling frequencies were applied when free chlorine levels changed more precipitously. Water samples were taken one minute after lettuce extract addition and analyzed for chemical oxygen demand (COD), oxidation–reduction potential (ORP), pH, and free and total chlorine. Three aliquots (15 mL) of wash water were removed at each of the indicated sampling points for assessing bactericidal activities using the cocktails representing the three bacterial species. Bacteria cells in each cocktail were exposed to the water sample at a ratio of 1:100 for 30 s, followed by neutralizing the residual chlorine with 0.1% sodium thiosulfate, and enumerating the surviving populations using a modified most probable number (MPN) method (Section 2.5).

2.4. Simulation of chlorine replenishment in wash water

Wash water was simulated by adding lettuce extract to distilled water to reach a final concentration of 1.2%, 2.4%, and 3.6% with COD levels averaging at 532, 1013, and 1705 mg/L. NaClO was repeatedly added in 4 mg/L increments in 3 min intervals to the simulated wash water containing different levels of lettuce extract until the final chlorine concentration passed the chlorination breakpoint (the point where chlorine demand in the simulated wash water was fully satisfied and free chlorine started to increase proportionally to added NaClO). Two minutes after each chlorine addition, a water sample was taken and analyzed for ORP, pH, and free and total chlorine. Bactericidal activity was determined as described in section 2.3.

2.5. Water quality assays and bacterial enumeration

COD, ORP, pH, total and free chlorine in simulated wash water were determined as previously described (Zhou et al., 2014). For bacterial enumeration, a previously described mini-most probable number (MPN) method was adapted with modifications (Zhou

Table 1
Strains used in this study.

Strain	Isolation source and relevant characteristics	Sources[c]
E. coli O157:H7[a]		
RM1918	Bagged lettuce associated with illness outbreaks	WRRC
RM4406	Lettuce associated with illness outbreaks,	WRRC
RM5279	Bagged vegetables associated with illness outbreaks,	WRRC
S. enterica[b]		
Typhimurium SL1344	Laboratory strain, kan^R	WRRC
Thompson RM1987	Clinical, cilantro associated outbreak Kan^R,	WRRC
Newport FDA2757	Tomato linked to an outbreak, Kan^R	FDA
L. monocytogenes		
NRRL B59186	Clinical, cantaloupe associated outbreak, serotype 2b	NRRL
Scott A 45A54	Clinical, foodborne disease outbreak, serotype 4b	EMFSL
Scott A 45A65	Clinical, foodborne disease outbreak, serotype 4b	EMFSL

[a] For all *E. coli* O157:H7 strains, stable nalidixic acid resistant mutants were selected and used in this study.
[b] All *S. enterica* strains were transformed using plasmid pGT-kan, conferring kanamycin resistance.
[c] EMFSL referred to Environmental Microbiology and Food Safety Lab, USDA ARS; FDA referred to U S Food and Drug Administration; NRRL referred to Northern Regional Research Laboratory (ARS Culture Collection), USDA ARS; WRRC referred to Western Regional Research Center, USDA ARS.

et al., 2014). Briefly, aliquots (0.3 mL) of neutralized bacteria-wash water solutions were added to 2.7 mL of TSB containing 0.1% sodium pyruvate, with antibiotics (50 mg/L of nalidixic acid for *E. coli* O157:H7, or 50 mg/L of kanamycin for *Salmonella*, respectively), or to 2.7 mL of *Listeria* enrichment broth with selective supplements (Thermal Scientific, Odessa, TX) for *L. monocytogenes*. After 10-fold serial dilutions in the same media and incubation at 37 °C for 18 h, aliquots (3 μl) of the enriched culture from each well were arrayed onto MacConkey agar containing 50 mg/L of nalidixic acid, TSA containing 50 mg/L of kanamycin, and PALCAM (Thermal Scientific) for *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes*, respectively. Following incubation at 37 °C overnight, the numbers of wells for each dilution that gave rise to pale colonies characteristic of *E. coli* O157:H7, green fluorescent colonies characteristic of *Salmonella*, and dark colonies characteristic of *L. monocytogenes* on respective selective plates were recorded and used for calculating the MPN of human pathogens in each water sample.

2.6. Experimental design and data analyses

All experiments were run in triplicates. Water-quality measurements for ORP, pH, and total and free chlorine were analyzed as a function of lettuce extract concentration and cumulative NaClO addition. Data related to human pathogen populations were log-transformed and analyzed for statistical significance among treatments using the general linear model of SAS 9.3 (SAS Institute, Cary, NC, USA). When the effects were statistically significant, mean comparisons were determined with Sidak adjusted P values to maintain the experiment–wise error (a) at 0.05. A correlation matrix for all measured parameters, including water quality attributes and the survival potential of each of the tested human pathogens was constructed using the CORR procedure of SAS, which provided a value for the Spearman correlation (r_s) and the associated P value.

3. Results and discussion

3.1. Water quality changes during chlorine depletion process

Fresh-cut produce processing consumes large amounts of fresh water. In commercial operations, organic matter from plant tissue, debris, and soil disperse quickly in wash water upon entering the washing tank, leading to rapid water quality deterioration and chlorine depletion. In this study, the chlorine depletion process in wash water was simulated by incremental increases of organic matters in the form of lettuce extract. The changes in wash water quality and its association with the dynamics of pathogen survival in wash water during this process is closely monitored. As shown in Fig. 1A, free chlorine concentration decreased immediately upon the introduction of organic matter, and eventually depleted following the continued introduction of organic matter. This is in agreement with observations in previous studies (Luo et al., 2012; Van Haute et al., 2013; Zhou et al., 2014). However, in the high initial chlorine treatment groups (100 and 200 mg/L), when plotting the data against logarithmic scale, a two-phased decline in chlorine concentration was observed (Fig. 1a), i.e. a rapid decline followed by a slow decline. While free chlorine depleted rapidly in the low initial chlorine treatment, free chlorine leveled off at around 0.5 mg/L, following the continued introduction of organic materials. This result is likely attributable to the conversion between chloramines and chlorine. Studies have shown that organic chloramines can be hydrolyzed to release free chlorine (Phenylarsine Oxide or N, N-diethyl-p-phenylenediamine), and, subsequently, interfere with the measurement of free chlorine using amperometric titration or DPD method (Jensen and Johnson, 1990a,b). As wash water is loaded continuously with organics, the formation of large amounts of organic chloramines could falsely augment free chlorine measurements using DPD method, and thus result in overestimation of available free chlorine.

Total chlorine also declines following the introduction of organic materials, however at a much slower rate than free chlorine (Fig. 1B), since total chlorine includes both free chlorine and chloramines. In addition, the residual total chlorine approaches a stable level, which is proportional to the initial free chlorine level. This also indicates that chloramines accumulate during the addition of organic materials.

At the beginning of the test, the pH of all chlorinated water was adjusted to 6.5 with citric acid. As the concentration of lettuce extract (shown as COD) increased, the organic materials were oxidized to combined chloramines, with the formation of protons (White, 1999). This explains the steady declining pH (Fig. 1C). The decrease in pH of wash water with high initial concentration of NaClO was slower with the addition of organic material (Fig. 1C). Therefore, the actual measured pH level of the wash water is a function of the amount of lettuce extract and sodium hypochlorite added.

ORP values increased initially following the introduction of organic material (Fig. 1D). Since ORP is a function of pH and free chlorine, the slight increase in ORP is mostly likely attributable to the sharp decline in pH and slight decline in free chlorine initially. However, with the continued organic load input and decline in free chlorine concentration, there were sharp declines in ORP values.

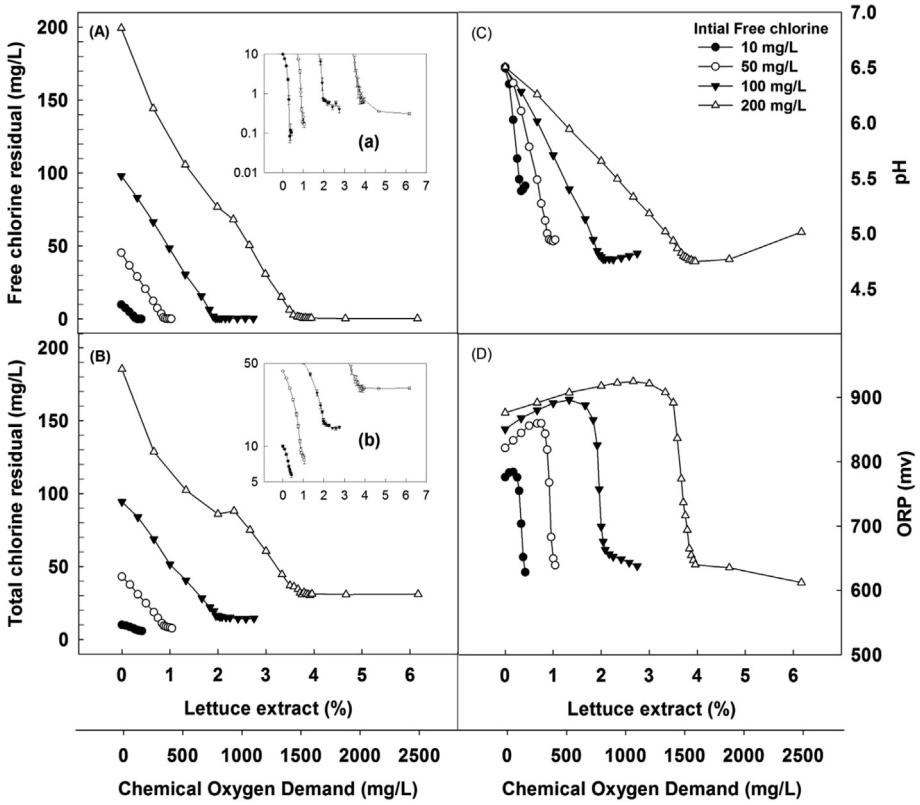

Fig. 1. Profiles of total and free chlorine, pH, and ORP of wash water during the simulated chlorine depletion process at different initial free chlorine levels. (A) Measured free chlorine; (B)Total chlorine; (C) pH; and (D) Oxidation–Reduction Potential (ORP). Both cumulative addition of lettuce extract and corresponding COD values are shown in the x-axis. Note that water sampling frequencies varied for wash water with different initial free chlorine levels and at different stages of free chlorine depletion.

3.2. Pathogen survival dynamics during chlorine depletion process

E. coli O157:H7, *S. enterica* and *L. monocytogenes* are foodborne pathogens frequently associated with fresh produce (Centers for Disease Control and Prevention, 2013; Griffin and Tauxe, 1991; Scallan et al., 2011). The FDA established a zero tolerance policy for these pathogens in fresh and fresh-cut produce. In this study, the survival of these three types of human pathogens over the chlorine depletion process was investigated. During chlorine decline, following the introduction of organic materials, wash water bactericidal activity remained strong initially with no pathogen survival in the wash solutions (Fig. 2A–C). However, with continued organic loading and free chlorine loss, survival was observed in the wash solution. Among all four initial chlorine treatments, the higher the initial free chlorine level, the more organic loading the wash water handled before pathogen survival was observed. Pathogen survival was observed first with the lowest initial chlorine, occurring after the accumulated addition of 2.91 mL/L lettuce extract, or the COD level reached 169 mg/L. On the other hand, the treatment containing the highest initial chlorine level did not shown any pathogen survival until after the accumulated addition of 39.58 mL/L of lettuce extract or the COD reached 1658 mg/L. Plotting the pathogen survival against the free chlorine measured in the system (Fig. 2D–F and Table 2) revealed that no pathogen survival was detected when free chlorine was maintained at 3.66 mg/L or above, regardless of initial chlorine concentration and organic loading. These results also indicate that under high organic loads, the free chlorine concentration needed to ensure water free of pathogens needs to be kept higher than 3.66 mg/L. Similar findings were reported by Gómez-López et al. (2014). In addition, the simulated wash water with higher organic load has a lower critical free chlorine level than other treatments (Table 2), probably attributable to the microbial inactivation of chloramines. The monochloramine can inactivate microorganisms by damaging cell membranes and breaking DNA (Ingols, 1958; Rose et al., 2007; Shih and Lederberg, 1976). Organic chloramines also can be used as disinfectants in water treatment (Amiri et al., 2010; Donnermair and Blatchley, 2003; Swango et al., 1987). Furthermore, free chlorine and chloramines have a synergic effect on inactivating bacterial cells (Kouame and Haas, 1991). Table 2 summarizes the wash water conditions when pathogen survival was first observed during chlorine depletion. *S. enterica* and *L. monocytogenes* have been reported as being more resistant to chlorine inactivation than *E. coli* O157:H7 (Shen et al., 2013; Van Haute et al., 2013). In our current study, a series of free chlorine residues was captured for the analysis of inactivation of three human pathogens. The results indicated that no significant difference in survival patterns was observed among those three major food-borne pathogens ($P < 0.01$) over the dynamic change of free chlorine residue. This suggests that bacterial strain is not likely to be a significant factor for bacterial inactivation in a free chlorine based produce washing system.

3.3. Water quality changes during chlorine replenishment process

Addition of NaClO to process water is a common practice within the fresh produce industry to restore chlorine level in order to avoid cross-contamination (Gómez-López et al., 2014; Van Haute et al., 2013). In this study, the chlorine replenishment in wash water was simulated by incremental addition of NaClO to water containing varying levels of lettuce extract. The change in free chlorine level was monitored in wash water with varying organic load in

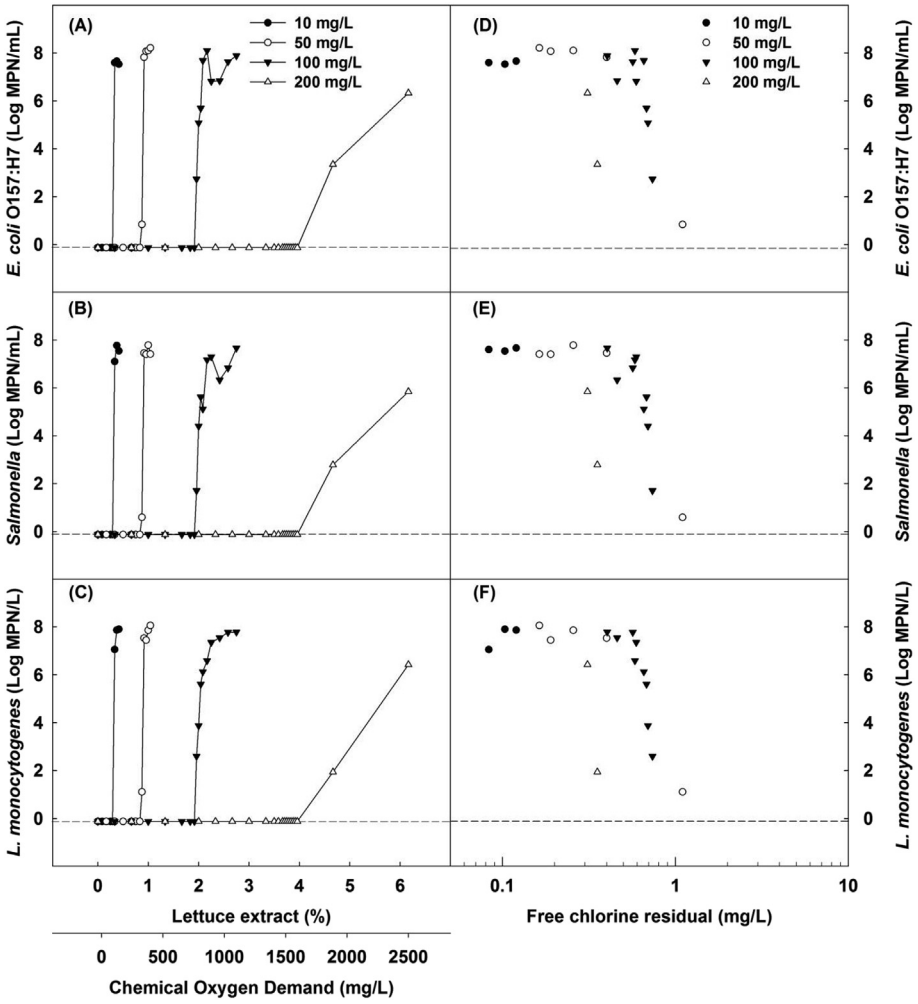

Fig. 2. Survival of three tested human pathogens during the simulated chlorine depletion process at different initial free chlorine levels. Graphs on the left show the survival populations of the three pathogens as a function of cumulative added lettuce extract (also expressed as chemical oxygen demand). Graphs on the right are scatter plots showing the measured free chlorine concentrations where survival of each pathogen was detected. (A and D) *E. coli* O157:H7; (B and E) *S. enteric*; (C and F) *L. monocytogenes*. The detection limit = 0.75 MPN/mL.

response to accumulating NaClO. As shown in Fig. 3A, the changes in free chlorine followed the typical pattern for breakpoint chlorination (Zhou et al., 2014) during the addition of NaClO. In the absence of organic materials, free chlorine increased immediately following the addition of NaClO. However, the increase in free chlorine concentration was significantly delayed in the presence of organic materials ($P < 0.01$). Increased organic loading resulted in longer lag periods before the chlorination breakpoint, when the chlorine demand in the wash water was fully met, and the free chlorine level increased linearly with the addition of NaClO. It was also observed that under conditions of high organic load (greater than 1000 mg/L), free chlorine concentration bounced around 1.00 mg/L, before the process approached the breakpoint.

Total chlorine increased immediately with the addition of NaClO, on the other hand, irrespective of the organic load (Fig. 3B). However, the coefficient between total chlorine and cumulative NaClO input was far less than 1, suggesting that there existed complex reactions between chlorine and organic components of lettuce extract: some generate chloramines, and others consume NaClO. Toivonen and Lu (2013) found that the organic materials released from cut fruit and vegetable tissue have different potential to quench free chlorine from wash water. In addition, Deborde and von Gunten (2008) have reported that there are three kinds of reactions: oxidation reactions, addition reactions to unsaturated bonds, and electrophilic substitution reactions at nucleophilic sites, depending on the chemical structure and oxidative capacity of free

Table 2
Major water quality parameters associated with pathogen elimination in water during chlorine depletion process.[a]

Initial chlorine (mg/L)	LE[b] added (mL/L)	COD (mg/L)	Free chlorine-measured (mg/L)	pH	ORP (mv)
10	2.92	169	0.71	5.49	755
50	8.33	390	3.66	5.12	843
100	19.17	907	1.86	4.84	826
200	39.58	1658	0.69	4.75	640

[a] All experiments were done in 3 L washing system with three replications. The values associated with free chlorine measured are obtained at the lowest free chlorine level when the system was pathogen-free. All other values reported are the system parameters at this free chlorine level.
[b] LE represents lettuce extract.

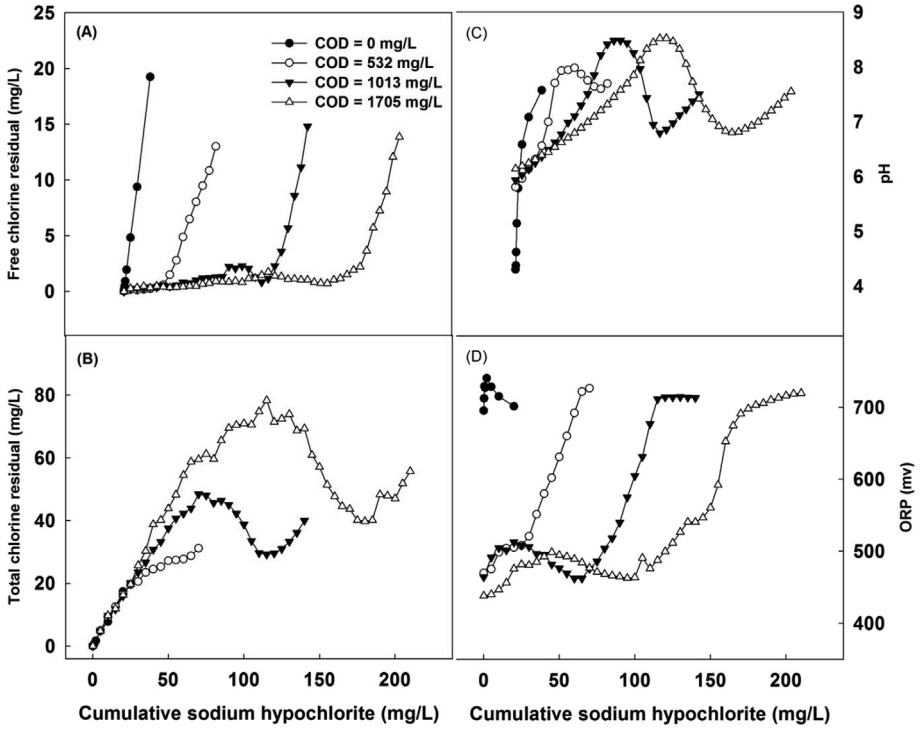

Fig. 3. Profiles of total and free chlorine, pH, and ORP value of wash water during the simulated chlorine replenishment process at different levels of organic load. (A) Measured Free chlorine, (B) Total chlorine; (C) pH; and (D) Oxidation–Reduction Potential (ORP).

chlorine and the different components of organic materials. Fig. 3C shows the pH changes in wash water with varying organic load as a function of chlorine addition. During the cumulative addition of NaClO to wash water with varying concentrations of lettuce extract, the change in pH follows a three-stage progression that includes a steady increase, followed by a steep decline, and another sharp increase. The bottom of the second stage coincided with the chlorination breakpoint. This confirms our earlier findings (Zhou et al., 2014).

The changes in ORP during chlorine replenishment are shown in Fig. 3D. ORP initially increases gradually, followed by a sharp increase until it reaches a plateau. When the chlorine replenishment passes the breakpoints, the ORP of wash water for all levels of organic load was over 650 mV, which has been reported as the minimum threshold for typical anti-bacterial activity (Steininger, 1985). It should be noted that during chlorine replenishment, the ORP reading past the breakpoint was much lower than the ORP reading at the corresponding point (in terms of free chlorine and COD levels) during the simulated chlorine depletion (Fig. 1D). This discrepancy was due to the water pH in these cases. While pH was not controlled in the simulated chlorine replenishment, pH in the wash water used for simulating free chlorine depletion was initially adjusted to 6.5, resulting in a lower pH when the lettuce extract was introduced.

3.4. Pathogen survival dynamics during chlorine replenishment process

Water recirculation could increase the potential risk of foodborne illness by readily distributing contaminants to non-contaminated produce. It has been documented that *E. coli* O157:H7 strains can survive in the holding tank if wash water is not sanitized effectively, be circulated back to the washing tank and cross-contaminate newly loaded produce during the washing process (Gómez-López, 2014). Fig. 4A–C depict the survival of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*, respectively, during the course of chlorine replenishment as impacted by the wash water organic load. In the absence of organic load, bacterial cell populations decline rapidly following the addition of NaClO to the wash water, quickly reaching undetectable level with the continued addition of NaClO. In contrast, for wash water with varying levels of organic load, there is a period of time during which all added NaClO is reacting with organic matter and no free chlorine is available to inactivate bacteria. Thus the bacterial cell population declined more gradually and survived through the addition of a number of aliquots of NaClO, which resulted in a significantly less steep slope in Fig. 4A–C ($P < 0.01$). The length of the survival period was positively and the slope was negatively correlated with the organic load.

Fig. 4D–F show scatter plots of the pathogen survival patterns over the measured free chlorine concentration. Similar to that observed during the chlorine depletion process, pathogen survival was highly correlated to the free chlorine concentration, regardless of the initial organic load. All pathogens were completed eliminated when the free chlorine exceeded 3.58 mg/L (Fig. 4D–F and Table 3), which corresponds to an ORP value of 740 mv (Fig. 3A and D). This suggests that the impact of organic load on pathogen inactivation is indirect, and its impact on chlorine concentration is direct; the key factor controlling the pathogen survival and inactivation is the available or residual chlorine concentration.

It is worth noting that the maximal free chlorine level permissive to pathogen survival during chlorine depletion (mean value = 1.73 mg/L for four tested initial free chlorine levels) was lower than that during the chlorine replenishment (mean value = 2.37 mg/L for four tested organic loads). In addition, at the point just prior to pathogen survival during chlorine depletion, there was a lower pH (mean value = 5.05) and thus higher ORP (mean value = 766 mv) (Table 2). On the other hand, during chlorine replenishment, just after the pathogen became undetectable, there was a higher pH (mean value = 6.75) and lower ORP (mean

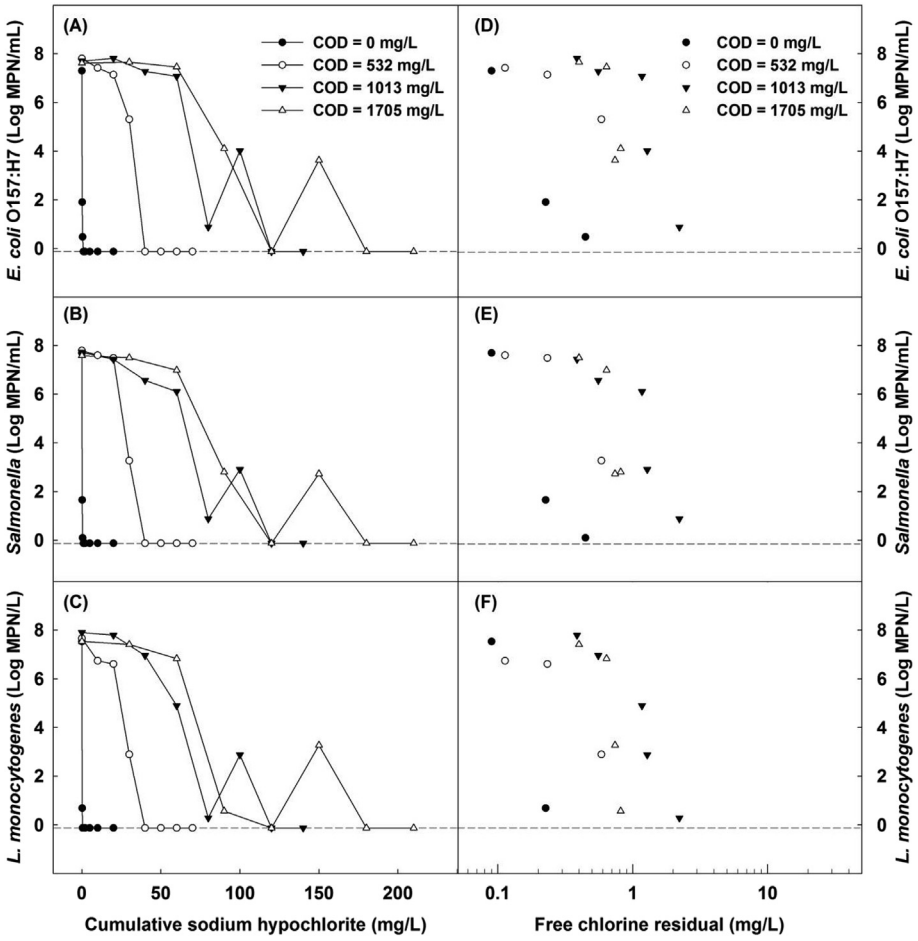

Fig. 4. Survival of human pathogen strains during the simulated chlorine replenishment process at different levels of organic load. Graphs on the left show the survival populations of the three pathogens as a function of cumulative chlorine addition. Graphs on the right are scatter plots showing the measured free chlorine concentrations where pathogen survival was detected. (A and D) *E. coli* O157:H7; (B and E) *S. enteric*; (C and F) *L. monocytogenes*. The detection limit = 0.75 MPN/mL.

value = 681 mv) (Table 3). Overall, no pathogen survival was observed when the free chlorine level was maintained above 3.66 mg/L or an ORP above 850 mv. It is further worth noting that chlorine efficacy to inactivate pathogens is dose-time dependant. In this study, data presented are obtained with 30 s reaction time. Higher chlorine concentration may be needed if a pathogen inactivation time less than 30 s is desired.

3.5. Correlations of wash water bactericidal activity and other water quality tributes

The survival of food-borne human pathogens is correlated to multiple variables that impact the quality of produce wash water. Spearman's correlation analyses were performed to determine the relationship between microbial populations and water quality parameters (Table 4). For free chlorine depletion and replenishment in wash water, there were strong, negative, and monotonic correlations between surviving microbial populations and free chlorine ($P < 0.01$), and between surviving microbial populations and ORP ($P < 0.01$). However, the negative monotonic correlations between microbial populations and total chlorine levels were strong for the free chlorine depletion process ($P < 0.01$), and weak for chlorine replenishment in simulated wash water ($P < 0.01$). Regardless of the initial free chlorine (in the free chlorine depletion process) and COD (in the chlorine replenishment process) levels, both free chlorine levels (Rs = −0.76 to −0.85) and ORP (Rs = −0.60 to −0.65) are strong indicators of the survival potential by bacterial pathogens in wash water. In order to be effective however, ORP must be controlled tightly over a narrow range. In current industry practices, ORP values vary with the tolerance of pH meter and controllers. If the tolerance is 0.5, the range of ORP measurements could be 100 mv (Steininger, 1985). Therefore, it is difficult to set

Table 3
Major water quality parameters associated with pathogen elimination in water during chlorine replenishment process.[a]

Organic load (mg/L COD)	NaClO added (mL/L)	Free chlorine-calculated (mg/L)	Free chlorine-measured (mg/L)	pH	ORP (mV)
0	0.013	1	0.91	5.14	727
532	0.507	40	2.78	7.94	580
1013	1.520	120	3.58	6.97	714
1705	2.280	180	2.2	6.93	703

[a] All experiments were done in 3 L washing system with three replicates. The values associated with free chlorine measured are obtained at the lowest free chlorine when the system was pathogen-free. NaClO added, pH, and ORP values are the system parameters at this selected free chlorine level. Free chlorine calculated values are obtained based on the calculation of total NaClO used over the 3 L volume in an organic load free system.

Table 4
Spearman correlations between surviving pathogen populations and water quality parameters during simulated free chlorine depletion and replenishments processes.

Bacterium	Spearman coefficient (r_s)			
	Total chlorine	Free chlorine	pH	ORP
Free chlorine depletion				
E. coli O157:H7	−0.60	−0.76	−0.40	−0.64
S. enteriaca	−0.60	−0.76	−0.39	−0.64
L. monocytogenes	−0.61	−0.76	−0.39	−0.65
Free chlorine replenishment				
E. coli O157:H7	−0.28	−0.84	−0.39	−0.65
S. enteriaca	−0.33	−0.85	−0.43	−0.60
L. monocytogenes	−0.30	−0.83	−0.39	−0.63

and maintain a target ORP level during produce washing operations.

4. Conclusion

This study examined in-depth the inactivation dynamics of bacterial pathogens during chlorine depletion and replenishment processes with respect to varying initial chlorine concentration and organic load. Even though initial chlorine concentration and organic matter load are two important process factors during fresh-cut produce wash operation, their impact on pathogen survival and inactivation is indirect. Initial chlorine concentration primarily affects the capacity of the chlorinated wash water against decline in concentration as a result of its reaction with organic matter, whereas the organic load affects the rate of chlorine depletion or the total amount of concentrated sodium hypochlorite required to restore the desired chlorine concentration. During both chlorine depletion and replenishment processes, the free chlorine concentration that led to no pathogen survival fluctuated from 0.71 to 3.66 mg/L, with no particular patterns exerted by either initial free chlorine concentration or organic load. This suggests that sanitizer strength as determined by available or residual free chlorine concentration is the key factor controlling pathogen survival and inactivation in the wash system, irrespective of initial chlorine concentration and organic load. Considering all variables tested and assuming a 30 s exposure time, a minimum of 3.66 mg/L free chlorine (at pH 5.12–6.97), which represents a pathogen reduction of greater than 6 log cycles, is required at all times to ensure that the wash water is free of pathogens. However, a higher chlorine concentration is expected if the reaction time is shorter than 30 s. These findings contribute to a better understanding of the complex relationship among wash water quality parameters and their association with pathogen survival and inactivation. This information can be used to help industry identify standard operational parameters to better control and monitor fresh-cut produce wash processes with improved pathogen control.

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