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The efficacy of chemical sanitizers on the reduction of *Salmonella* Typhimurium and *Escherichia coli* affected by bacterial cell history and water quality

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ABSTRACT

Washing fresh produce with potable water helps to remove microorganisms, providing about a 1- to 2-log reduction, but this process can also pose an opportunity for cross-contamination of bacteria in the washing tank. The objective of this study was to evaluate the efficacy of three chemical sanitizers, sodium hypochlorite, chlorine dioxide, and a silver-copper solution on the reduction of *S.* Typhimurium and extended-spectrum beta-lactamase (ESBL) *E.* coli as well as to evaluate the impact bacterial cell history and water quality had on sanitizer efficacy. This was investigated with three scenarios representing different contamination routes and history of cells: (i) on starved and non-starved cells in potable water, (ii) on starved and non-starved cells in lettuce wash water and on lettuce leaf punches, and (iii) on non-starved cells in organically loaded process wash water (PWW). Sodium hypochlorite (NaClO) and chlorine dioxide (ClO_2) were more effective in preventing cross-contamination in the potable water than the silver-copper solution. Starved and non-starved bacterial cells displayed minor differences in their susceptibility to sanitizing agents in the (i) potable water and (ii) lettuce wash water demonstrating that other conditions greater influenced sanitizer efficacy. Particularly, the organic load of the water, wash water temperature, and pathogen attachment and release from the produce were shown to affect a sanitizer's efficacy during washing. Furthermore, results emphasize that chemical sanitizer use should focus more on wash water disinfection, rather than produce decontamination, to prevent pathogenic cross-contamination during processing. Future research should investigate the feasibility of ClO_2 application during pilot-scale processing.

1. Introduction

Different studies have reported the increased consumption of fresh produce in the last decades (Olaimat & Holley, 2012; Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Consumers are increasingly interested in consuming healthy, convenient foods including fresh-cut or ready-to-eat produce (Jacxsens et al., 2010) as these may help them to conveniently reach dietary recommendations and may reduce the risk of certain cancers (Bradbury, Appleby, & Key, 2014). When microbiological food safety does not improve, consumers have a higher probability of consuming contaminated fresh produce. Fresh produce is reported to be vulnerable to pathogenic contamination such as from *Salmonella* spp. and pathogenic *Escherichia coli* (Callejón et al., 2015; EFSA Panel on Biological Hazards (BIOHAZ) Panel, 2013; Food and Agriculture Organization of the United Nations (FAO) & World Health Organization (WHO), 2008; Tirpanalan, Zunabovic, Domig, & Kneifel, 2011; Van Haute, Sampers, Holvoet, & Uyttendaele, 2013). In order to maintain the safety and quality of fresh and fresh-cut produce, prevention and control measures should be implemented along the supply chain (Gil et al., 2015).

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At the fresh(-cut) produce processor, washing aims to remove dirt and reduce microbial contaminants from the surface of the product. However, during washing, pathogens that may be incidentally present on the produce can be released into the washing water and thus, cross-contamination between clean and contaminated produce may occur. Since washing can be a potential cause of cross-contamination during processing, techniques that can improve processing, including those that can limit pathogenic cross-contamination, are proposed (Holvoet, Jacxsens, Sampers, & Uyttendaele, 2012; Luo, Ingram, & Khurana, 2014). Some processing practices include washing with or without sanitizing agents like chlorine (e.g., as sodium hypochlorite, calcium hypochlorite, or chlorine gas) to prevent dispersal and transmission of pathogens. Current insights show that the main expected effect of sanitizing treatments during produce washing is to reduce and control the microbial load of the water rather than produce decontamination (Van Haute et al., 2015; Van Haute, Tryland, Veys, & Sampers, 2015). Consequently, by maintaining the water quality throughout produce processing, the potential for cross-contamination during washing can be diminished (Gil, Selma, López-Gálvez, & Allende, 2009; Parish et al., 2003; Van Haute, Sampers, Jacxsens, & Uyttendaele, 2015).

Overall, there is a need to reduce cross-contamination events; this need is particularly apparent when sanitizers are not applied during washing, which is the current situation for some European Union countries (Holvoet et al., 2012). Certain process wash water (PWW) disinfectants have shown potential in pilot-scale studies (Davidson, Buchholz, & Ryser, 2013; Gil, Marín, Andujar, & Allende, 2016; Luo et al., 2012) to maintain the water quality during processing and prevent cross-contamination of pathogens, such as *Salmonella* spp. and *E. coli.* The objective of this study was to evaluate the efficacy of three chemical sanitizers, sodium hypochlorite, chlorine dioxide, and a silver-copper solution on the reduction of *Salmonella* Typhimurium and extended-spectrum beta-lactamase (ESBL) *E. coli*. This evaluation was investigated with three scenarios related to bacterial cell history: (i) on non-starved and "short-term" starved cells in potable water, (ii) on non-starved and "long-term" starved cells in lettuce wash water and on lettuce leaf punches, and (iii) on non-starved cells in organically loaded PWW. These three scenarios provided a novel (*i.e.* not published) perspective for evaluating the effect of strain history on efficacy, for example between starved and non-starved cultures, as well as in different water mediums. Furthermore, the extent to which bacterial cell release and (re-) attachment to fresh-cut produce occurs provides insight on recontamination dynamics of *S.* Typhimurium and *E. coli* as well as the impact of chemical sanitizers during fresh(-cut) washing.

2. Materials and methods

2.1. Bacterial strain and inoculum preparations

A *Salmonella enterica* subspecies *enterica* sevorar Typhimurium (*S.* Typhimurium) lettuce isolate 1638 and an *E. coli* ESBL-positive human isolate, reported as 0247_1 (van Hoek et al., 2015), were maintained at −80 °C in Luria Broth (LB; BD Difco™ Luria Broth Base, Miller) containing 25% (v/v) glycerol. Cultures were prepared by inoculation of a single colony isolate in one-fifth filled Erlenmeyer flasks with LB at 37 °C in a 200 rpm shaking air incubator and were grown for 18 (±1) h to obtain stationary phase cells. The cultures (c.a. 10^9 CFU/mL) were transferred into sterile Greiner tubes and centrifuged (1800 ×*g*) at 20 °C for 10 min. The supernatant was decanted, and the resulting pellet was resuspended in 20 mL of potable water and re-centrifuged at the conditions above. This washing step was repeated twice more. The pellet was then resuspended in potable water, and the overnight culture solution was separated into two equal parts. One part of the culture was diluted a hundred fold with potable water to obtain a final concentration of c.a. 10^7 CFU/mL. From this solution, 200 μL was added to 20 mL of potable water, resulting in a concentration of c.a. 10^5 CFU/mL. The other part of the culture was used to make "short-term" and "long-term" starved cultures. For short-term starvation experiments, cultures were incubated for 24 h at 5 °C before further dilution with potable water, as previously described, and then used in the inactivation experiments in potable water. For long-term starvation experiments, *S.* Typhimurium and *E. coli* were examined for 430 h at 5 °C in ultra-pure water. Cultures of both strains survived after 430 h with the difference between initial and final concentrations of ≤1 log CFU/mL (data not shown). Further analyses were not investigated. This long-term starved culture was used for subsequent experiments in lettuce wash water and on leaf punches. Fig. S1 provides an overview of the experiments with non-starved and starved cultures. For both cultures examined during these experiments, the cell history (*i.e.* non-starved and starved) was investigated.

2.2. Preparation of treatment solutions

All solutions were freshly prepared before each experiment. Sodium hypochlorite (NaClO; Sigma-Aldrich, Schnelldorf, Germany) stock solutions were prepared by diluting the solution obtained from the supplier (4.00–4.99%) with potable water to achieve a final concentration of 10 mg/L active chlorine. Aqueous chlorine dioxide (ClO_2; Lifarma B.V., Baexem, The Netherlands) stock solutions were prepared according to manufacturer's instructions. In brief, one ClO_2 tablet was dissolved in 1 L potable water within a sealed container to obtain a 0.2% (w/v) solution (*i.e.* 2000 mg/L). Subsequently, 50 μL of the ClO_2 stock solution was diluted with potable water to obtain 20 mL final volume with a final concentration of 5 mg/L. For NaClO and ClO_2, concentrations were verified with a DULCOTEST® DT4B photometer (ProMinent Verder B.V., Vleuten, The Netherlands); values were within a 1–5% deviation from the set value. An antimicrobial solution consisting of an undiluted mixture of silver and copper was commercially supplied and prepared according to manufacturer's instructions (Modern Water, Cambridge, United Kingdom). The concentration of the silver-copper (Ag-Cu) solution was determined using inductively coupled plasma atomic emission spectroscopy/optical emission spectrometry (ICP-AES/OES; Vista MPX, Varian) to be 9.1–9.9 mg/L Ag and 1.2 mg/L Cu.

2.3. Lettuce cultivation conditions and pre-treatment of lettuce leaf punches

Iceberg lettuce (*Lactuca sativa* 'Dublin') was grown in a greenhouse at a day/night regime of 16 h at 20 °C/8 h at 16 °C in potting soil (Lentse Potgrond B.V., Katwijk, The Netherlands) without applied insecticides or fungicides. The outer leaves of three to five-week-old lettuce were sliced off and placed into plastic bags or boxes to prevent dehydration, and then, transported at room temperature before being processed for analysis within 2 h upon arrival.

Circular punches of the adixial side of the lettuce leaves were made with a sterilized apple borer (22 mm diameter) in the middle of the leaf excluding major veins. One punch per leaf, with a maximum of two punches (technical replicates) from the lettuce leaves, was taken. Leaf punches were placed onto Petri dishes and inoculated by pipetting 10 μL non-starved or long-term starved culture suspensions of *S.* Typhimurium and *E. coli* at densities of 10^4, 10^6, or 10^8 CFU/mL, reaching total inoculum cell numbers per

leaf punch of 10^2, 10^4, or 10^6 CFU, respectively. Liquid drops were placed in the middle of the leaf punch to avoid contact of the bacterial inocula with leaf wounds. Sterilized potable water was used as a control. Leaf punches with bacterial inoculum or water were then incubated for 15 s, 1, 2, 5, 10, 30 or 60 min at room temperature. Afterward, the lettuce leaf punches were removed using sterile forceps, and liquid drops were carefully removed with a sterile pipet, thereby avoiding contact of the pipet tip with the leaf punch. Any remaining liquid on the leaf punch surface was carefully removed by blotting with sterile filter paper.

2.4. Process washing water

PWW was obtained from an endive wash line of a commercial Dutch fresh-cut vegetable processor. This industrially-supplied PWW was transported under refrigerated conditions to the lab for further analysis. The pH (Beckman Φ34), total ammonia, nitrate, phosphate (SFA-$CaCl_2$, Skalar, SAN++), and total organic carbon (TOC; Shimadzu 5050A) of potable water and industrially-supplied PWW were determined (Table 1). Briefly, the relationship between chemical oxygen demand (COD) or TOC can be characterized and varies according to water composition (The Dow Chemical Company, 2015); for example, Dubber and Gray (2010) observed that TOC could be used for generic replacement of COD in final effluents (*i.e.* treated wastewater). Previous research modeling water quality has indicated TOC as a predictive parameter for disinfection by-products (DBPs) in (drinking) water (Sadiq & Rodriguez, 2004).

The industrially-supplied PWW was also analyzed for aerobic mesophilic plate counts and aerobic psychrotrophic plate counts on Tryptone Soy Agar (TSA; Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 25 °C for 3 d and 7 °C for 7 d, respectively. This wash water was aliquoted and stored at −20 °C until further use. For each additional experiment, a new tube was taken from the freezer. Experiments were performed within 2 months of storage. TOC of the wash water was re-measured after 9 months and had declined by only 5%. For experiments with NaClO on *E. coli* non-starved cultures, wash water was laboratory-made. The pH, total ammonia, nitrate, and phosphate were measured as described above; these were 7.06, 0.45 mg/L, 10.9 mg/L, and 9.83 mg/L, respectively. Laboratory-made wash water was prepared by hand cutting endive from a local supermarket and washing it in 1 L cold potable water. Cutting and washing were repeated twice, each time using the same water to achieve the highest organic carbon load concentration. The wash water was then diluted with cold potable water to obtain a TOC measurement equal to the industrially-supplied PWW.

2.5. Pathogen releasing efficacy and (re-) attachment to lettuce leaf punches during washing

The methods used to evaluate the releasing efficiency of the pathogens from the lettuce leaf punches into the washing water and bacterial reattachment from contaminated wash water to uninoculated lettuce leaf punches was investigated (Fig. S2). First, to determine the releasing capacity of *S.* Typhimurium or *E. coli* cells to the water, leaf punches were inoculated with *S.* Typhimurium (6.8 log CFU/punch) or *E. coli* (6.0 log CFU/punch) and remained on the punch for 1 h at 20 °C. Directly afterward, the inocula were removed, and the punch was washed three times, in which each time the inoculated leaf punch was transferred to a fresh tube with 10 mL potable water (Fig. S2.A). Also, to determine if *S.* Typhimurium and *E. coli* cells that had released from contaminated leaf punches would re-adhere to uncontaminated leaf punches, fresh leaf punches were added to the wash water from the first wash rinse (*S.* Typhimurium 6.3 log CFU/mL; *E. coli* 5.5 log CFU/mL) and were incubated for 2 min at room temperature (Fig. S2.B). After the final wash, punches were transferred to BioReba (10 mL volume) bags (BioReba AG, Reinach, Switzerland) containing 1 mL of sterile Ringer's solution (BR0052; Oxoid, part of Thermo Fisher Scientific, Breda, The Netherlands), and were gently homogenized. Subsequently, tenfold serial dilutions in Ringer's solution were made from the leaf punch homogenates. Then, 100 μL of lettuce punch wash water and diluted leaf punch homogenates were pipetted onto Petri dishes of Xylose-Lysine-Desoxycholate agar (XLD; Oxoid Ltd., Basingstoke, United Kingdom) and Brilliance *E. coli*/coliform selective agar (BECSA; Oxoid Ltd., Basingstoke, United Kingdom) ± 1 mg/mL Cefotaxime sodium salt (Ct; Sigma-Aldrich, Zwijndrecht, The Netherlands), respectively for *S.* Typhimurium and *E. coli* recoveries. Afterward, liquid drops were spread over the agar surfaces to allow enumeration of individual CFUs of *S.* Typhimurium or *E. coli* following incubation for 18 h at 37 °C.

Table 1
Potable and industrially-supplied process wash water (PWW) characteristics ($n = 2$).

Parameter	Mean value ± SD	
	Potable water	Industrially-supplied PWW
pH at 20 ± 1 °C	8.23 ± 0.06	8.28 ± 0.24
NH_4 −N [mg/L]	0.00 ± 0.00	0.19 ± 0.04
NO_3 −N (+NO_2 −N)[mg/L]	0.04 ± 0.01	10.39 ± 0.58
PO_4 −N [mg/L]	0.01 ± 0.002	3.37 ± 0.05
TOC [mg/L]	2.30 ± 1.41	354 ± 15

2.6. Inactivation experiments in potable water

The inactivation efficacy of sanitizer treatment solutions NaClO (10 mg/L), ClO_2 (5 mg/L), and Ag-Cu (9.1–9.9 mg/L Ag, 1.2 mg/L Cu) on non-starved and short-term starved cultures of *S.* Typhimurium and *E. coli* in potable water were investigated (Fig. S1.A). During sanitizer treatments, the respective cultures were periodically swirled and continuously kept in ice water to maintain the temperature at 5 °C to simulate conditions at the industrial setting. At regular time intervals, 1 mL samples were taken and serially diluted into a peptone physiological salt solution (PPS; Tritium Microbiologie B.V., Eindhoven, The Netherlands). Following exposure to the sanitizer treatments, 100 μL of the appropriate dilutions were plated onto LB agar (LBA) plates containing 1.2% agar and incubated at 37 °C for 5 d with daily inspection of colonies. Colonies were inspected for up to 5 d to check if potentially damaged cells could eventually grow out. The number of culturable cells was determined between 0 and 20 min, to compare the efficacy of all chemicals sanitizers overtime for both cell types.

2.7. Sanitizing experiments in lettuce washing water and on lettuce leaf punches

The releasing efficiency of bacteria from inoculated lettuce leaf punches to the lettuce wash water, and the effects of the pathogens on the lettuce leaf punches were investigated (Fig. S1.B). The lettuce leaf punches, cut as described in section 2.3, were inoculated with either non-starved cells (c.a. 10^5 CFU/punch) or long-term starved cells (c.a. 10^6 CFU/punch) of *S.* Typhimurium or *E.* coli. Then, the punches were placed for 1 h at 20 °C in 50 mL sterilized Greiner tubes, after which the punches were taken out of the Greiner tubes, and the inoculum was removed from the surfaces as described in section 2.3. Directly afterward, the punches were treated for 2 min with 10 mL of potable water or 10 mL solutions of NaClO (10 mg/L), ClO_2 (5 mg/L), or Ag-Cu (9.1–9.9 mg/L Ag, 1.2 mg/L Cu) at 5 or 20 °C;

these punches were gently shaken to simulate processing conditions. Then, punches were washed in 50 mL potable water in order to remove residues of disinfecting agents before further analysis; neutralizing agents were not applied. After treatments, CFUs from the lettuce wash water and lettuce leaf punches were enumerated on XLD and BECSA + Ct, respectively for recovery of *S.* Typhimurium and *E. coli*.

2.8. Inactivation experiments in process wash water

The inactivation efficacy of sanitizer treatment solutions NaClO (10 mg/L) and ClO_2 (5 mg/L) were performed as described in section 2.6 yet with non-starved *S.* Typhimurium cultures in industrially-supplied PWW with TOCs of 354 and 177 mg/L, of which the latter was diluted with potable water. Similarly, the inactivation efficacy of ClO_2 on *E. coli* was determined. The inactivation efficacy of NaClO was also determined, yet with laboratory-made PWW with TOCs of 354 and 177 mg/L (Fig. S1.C). Cells were recovered on LBA media as outlined in section 2.6. The number of culturable cells was determined between 0 and 20 min, representing the inactivation efficiency of sanitizers in PWW.

Furthermore, the inactivation efficacy of NaClO and ClO_2 on non-starved *E. coli* cultures in PWW with a TOC of 354 mg/L, being laboratory-made and industrially-supplied, respectively, were recovered on the selective media BECSA + Ct to allow for the potential qualification of Ct resistant *E. coli*. After exposure to the sanitizing treatment, 100 μL of PPS diluted samples were plated on BECSA + Ct media and were incubated at 37 °C for up to 5 d with daily inspection of the colonies after 48 h.

2.9. Statistical analysis

For the sanitizing experiments in lettuce washing water and on lettuce leaf punches (Fig. S1.B), fractions of *S.* Typhimurium or *E. coli* CFUs, expressed as percentages of the inoculum densities, were calculated and used for statistical comparisons of average values with sanitizing agent type and incubation temperature as treatments and replicate experiments as separate blocks using analysis of variance (ANOVA; GenStat release 12.1, Hemel Hempstead, United Kingdom). These independent experiments were carried out in duplicate ($n = 2$), each time by using four leaf punches from two separate plants.

3. Results

3.1. Pathogen releasing efficacy and (re-) attachment to lettuce leaf punches during washing

The attachment capacity of non-starved cultures of *S.* Typhimurium and *E. coli* to lettuce leaf punches, their release and medium recovery efficacy in the wash water, and reattachment to uninoculated lettuce punches were previously investigated. Results showed that the percentage of cells that adhered to the lettuce leaf punches, expressed as a fraction of inoculum, increased from <1% after 15 s to 6% after 60 min for *S.* Typhimurium, while for *E. coli* this was <1% after 15 s to 2% after 60 min (data not shown). For both strains, maximum attachment occurred after 30–60 min. In this study, the inoculation time for both strains was standardized to 60 min.

Previous experiments also indicated that the recovery of *S.* Typhimurium cells that adhered to lettuce leaf punches was observed at the lowest tested inoculum level of 10^2 cells, of which 15% of the inoculum cells was recovered. For *E. coli*, the recovery percentage was substantially lower, <1% of the inoculum cells, meaning fewer cells attached to the lettuce leaf punch at this inoculum level compared to *S.* Typhimurium cells (data not shown). The difference in inoculum density for both strains did not severely affect the percentage of bacterial cells that adhered to and were recovered from the lettuce leaves. Therefore, in this study, the inoculum cell number was standardized to 10^6 bacterial cells for subsequent experiments.

Within the lettuce leaf punch wash water (Fig. S2.A), transfer results demonstrated that *S.* Typhimurium cells from the non-starved culture were detected at 40%, 1%, and <1% of the original inoculum after the first, second, and third (final) wash, respectively. Cells that adhered to the lettuce leaf punches after the third rinsing step were detected at <1% of the original inoculum. Similarly, for the *E. coli* non-starved culture, cell counts in the lettuce washing water were 32%, 3%, and 1% of the original inoculum after the first, second, and third (final) wash, respectively. Cells that adhered to the lettuce leaf punches after the third rinsing step were detected at 2% of the original inoculum.

Furthermore, the ability of bacterial cells to reattach to uninoculated lettuce leaf punches were investigated (Fig. S2.B). Results indicated that *S.* Typhimurium cells that initially adhered to the lettuce leaves, but then were released during the first washing into the wash water, were still able to reattach to new, uninoculated lettuce punches (9% of the wash water inoculum), which was also observed by the decreased cell count in the washing water (30% of the wash water inoculum). Similarly, for *E. coli* cells, these values were 4% and 11%, respectively.

3.2. Inactivation efficiency of sanitizers in potable water

The inactivation efficiency of NaClO (10 mg/L), ClO_2 (5 mg/L), and Ag-Cu (9.1–9.9 mg/L Ag, 1.2 mg/L Cu) solutions, dissolved in potable water, against *S.* Typhimurium and *E. coli* were investigated (Fig. S1.A). Results for *S.* Typhimurium and *E. coli* exhibited a 4 log reduction after short contact times with NaClO (seconds) and ClO_2 (1 min) in potable water (Fig. 1). This result was observed for cells from the non-starved culture, which were grown in a rich medium (LB) at 37 °C and short-term starved cultures, which were grown in potable water at 5 °C for 24 h. Although results may be influenced by the non-use of neutralizers for the sanitizing agents, serial dilutions in PPS were made before plating. In a previous experiment, NaClO (1.5 mg/L) and ClO_2 (10 mg/L) were measured in potable water and industrially-supplied PWW (TOC = 354 mg/L) without pathogens. Results demonstrated a 20-fold and 10-fold reduction within 20 s between potable and industrially-supplied PWW, respectively for NaClO and ClO_2 (data not shown). Furthermore, in our study, the Ag-Cu solution resulted in a 4 log reduction in potable water within 10 min for non-starved and short-term starved cultures of *S.* Typhimurium, while for both *E. coli* culture types, a 4 log reduction occurred within 20 min. Overall, the cell history, being non-starved vs. starved, did not affect the inactivation rate of the sanitizers.

3.3. Sanitizer efficacy in lettuce washing water and on lettuce leaf punches

The efficacy of NaClO (10 mg/L), ClO_2 (5 mg/L), and Ag-Cu (9.1–9.9 mg/L Ag, 1.2 mg/L Cu) solutions in iceberg lettuce wash water on *S.* Typhimurium and *E. coli* non-starved and long-term starved cultures that had been released from contaminated lettuce leaf punches into the water was investigated at two temperatures: 5 and 20 °C (Fig. S1.B). Although results may be influenced by the non-use of neutralizers for the sanitizing agents, punches had been washed to remove agents before analysis.

3.3.1. Treatments in lettuce wash water

Results for the control treatments (*i.e.* with no sanitizer)

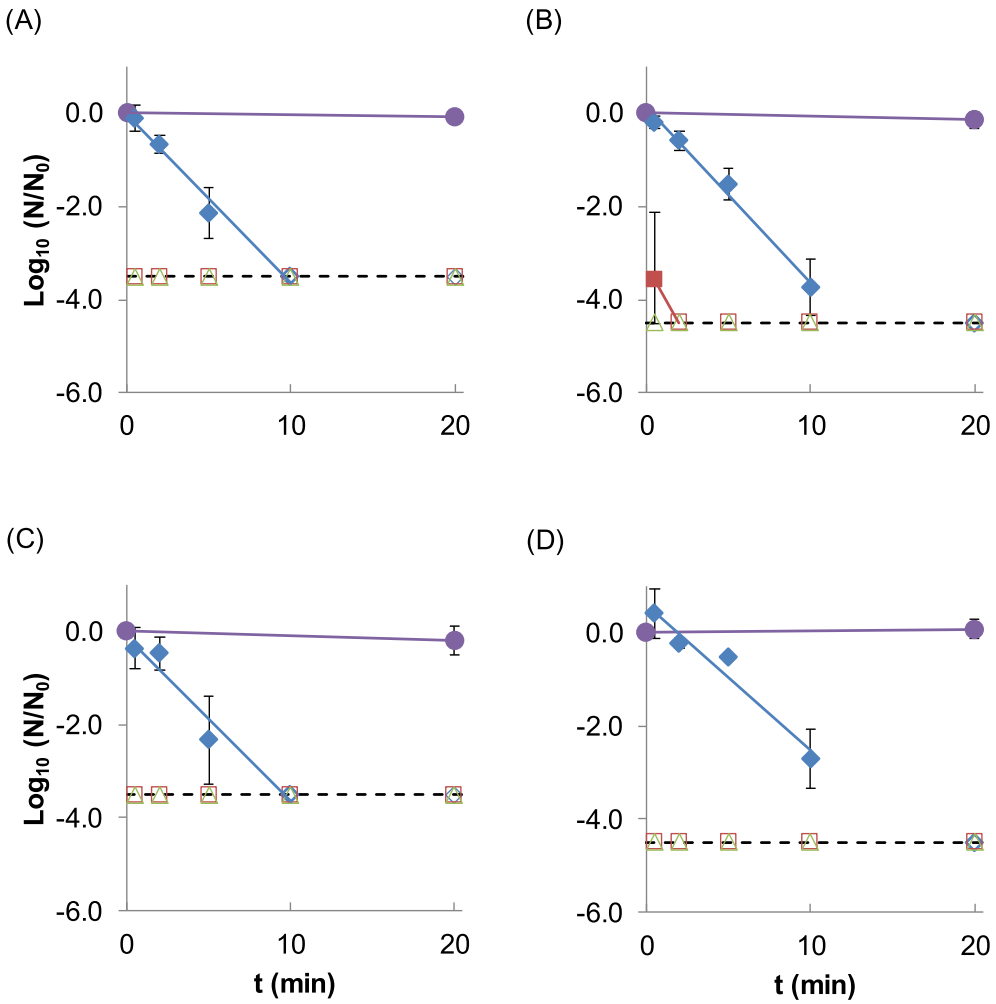

Fig. 1. Inactivation of *Salmonella enterica* Typhimurium 1638 (A, C) and *Escherichia coli* isolate 0247_1 (B, D) with 10 mg/L NaClO (▲), 5 mg/L ClO_2 (■), Ag-Cu with 9.1–9.9 mg/L Ag and 1.2 mg/L Cu (◆), and no sanitizer (●) in 5 °C potable water: (A, B) cells from the non-starved culture at 37 °C, (C, D) cells adjusted to 5 °C for 24 h representing "short-term" starved cultures. Data represent the average of triplicate experiments and error bars represent standard deviation. —, Limit of Detection.

indicated that *S.* Typhimurium non-starved cells that had released from the lettuce leaf punches into the washing water were detectable, irrespective of the applied incubation temperature. *S.* Typhimurium cells were 3.7 and 4.3 log CFU/punch, respectively, during washing with no sanitizers at 5 and 20 °C (Fig. 2A). This result contrasts the results for the sanitizing treatments with NaClO, ClO_2, and Ag-Cu, which demonstrated that *S.* Typhimurium non-starved cells were not recovered (Fig. 2A). Therefore, application of all three sanitizing agents resulted in complete eradication of culturable cells in the lettuce wash water. In brief, *S.* Typhimurium cell survival in the wash water was not statistically significant at 5 and 20 °C ($p > 0.05$, $n = 2$).

E. coli cells that released into the lettuce washing water during control treatments were 2.6 and 1.3 log CFU/punch, respectively, during washing at 5 and 20 °C (Fig. 2B). In contrast to *S.* Typhimurium cells, fewer non-starved *E. coli* cells were present in the water at 5 °C compared to 20 °C (Fig. 2B). For non-starved *E. coli*, there was statistically a highly significant difference in averaged values of the control treated wash water between incubation temperatures ($p < 0.001$, $n = 2$) meaning that temperature had an effect on colony recoverability in the wash water and/or cell release from the leaf surface (Fig. 2B); this phenomenon was not observed with *S.* Typhimurium (Fig. 2A). Similar to *S.* Typhimurium, for *E. coli*, treatment of all sanitizing agents resulted in complete eradication of culturable cells in the lettuce wash water.

3.3.2. Treatments on lettuce leaf punches

Results on the lettuce leaf punches showed that non-starved *S.* Typhimurium and *E. coli* cells remained attached during control treatments irrespective of the applied incubation temperature. The control treatments represent the maximum number of attached CFUs. *S.* Typhimurium on the lettuce leaf punches during control treatments was 2.4 and 2.3 log CFU/punch, respectively for 5 and 20 °C (Fig. 2C), while for *E. coli* this was 2.6 log and 2.8 log CFU/punch, respectively (Fig. 2D). *S.* Typhimurium cells from the lettuce leaf punches were also recovered following treatments with all sanitizing agents at both 5 and 20 °C. However, the washing temperature during treatments affected survival; fewer cell numbers were reduced when wash water was maintained at 20 °C than at 5 °C for all treatments ($p = 0.001$, $n = 2$). Similarly, *E. coli* cells were recovered from the leaf punches following all treatments, *i.e.* after the control treatment without sanitizer as well as for the sanitizing agents at both incubation temperatures (Fig. 2D). On average, the *E. coli* cells from the lettuce leaf punches (Fig. 2D) had a higher reduction than *S.* Typhimurium cells from lettuce leaf punches

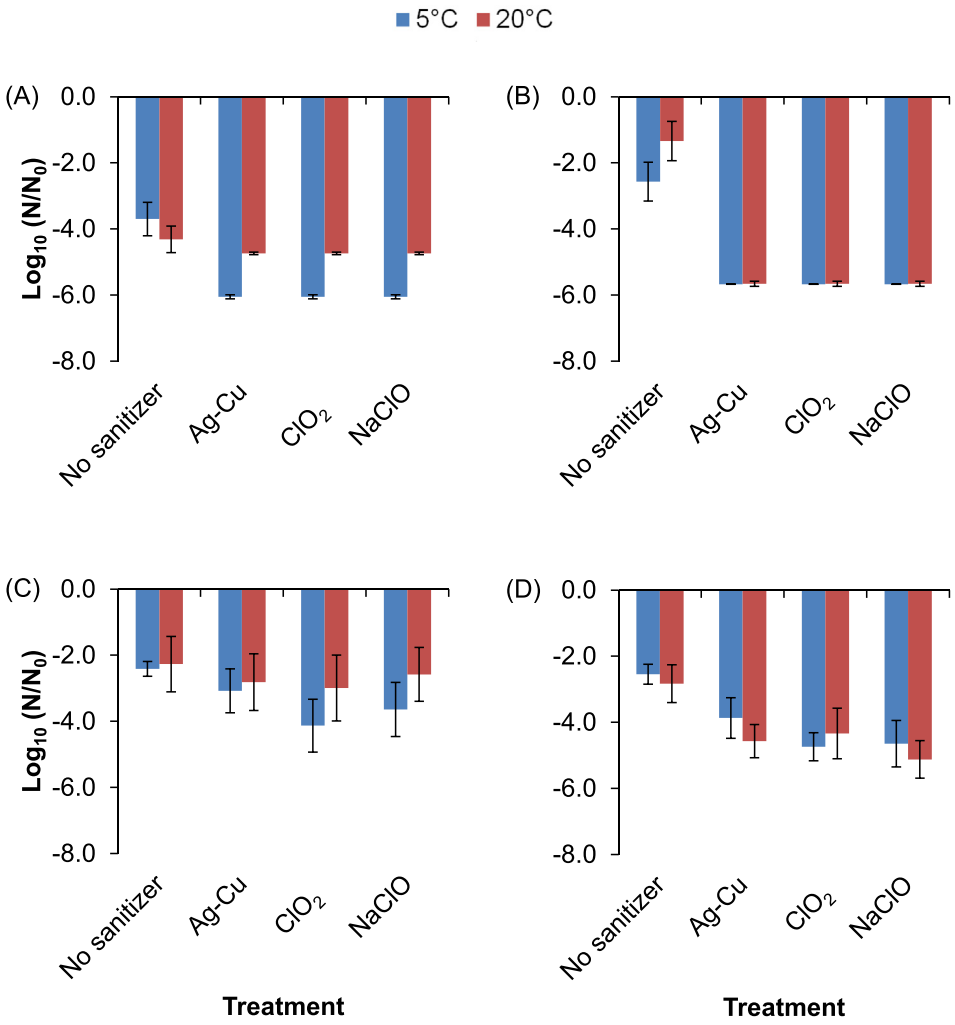

Fig. 2. *Salmonella enterica* Typhimurium 1638 (A, C) and *Escherichia coli* isolate 0247_1 (B, D) cell release after a two minute treatment at 5 °C and at 20 °C with no sanitizer, Ag-Cu at 9.1–9.9 mg/L Ag and 1.2 mg/L Cu, 5 mg/L ClO_2, and 10 mg/L NaClO: non-starved culture at 37 °C in (A, B) iceberg lettuce punch wash water, and on (C, D) iceberg lettuce leaf punches. The $\log_{10}(N/N_o)$ of 0 indicates the number of attached bacterial cells to the leaf punches. Data represent the average of four experiments and error bars represent standard deviation.

(Fig. 2C) demonstrating that the non-starved *E. coli* cells survive to a lesser extent on the lettuce leaf punch surface than the non-starved *S.* Typhimurium cells.

3.3.3. Survival of "long-term" starved cells

In order to compare the potential effect that the cell history may have on the efficacy and survival from these treatments, long-term starved cells were examined in the iceberg lettuce wash water and on lettuce leaf punches. *S.* Typhimurium and *E. coli* long-term starved cells were recovered following the control treatment without sanitizer at both 5 and 20 °C wash water, yet were undetectable in the lettuce wash water after treatment with the sanitizing agents (data not shown). When comparing the *S.* Typhimurium non-starved culture from the lettuce leaf punches washed in 20 °C water (Fig. 2C) with the long-term starved cells, the starved cells displayed no significant differences ($p > 0.05$, $n = 2$) during all treatments indicating that starvation did not lead to any detectable changes in survival against the sanitizing agents. Similarly, there was no significant difference ($p > 0.05$, $n = 2$) visualized during cell treatment on non-starved versus long-termed starved cultures for *E. coli* indicating that starvation did not result in a detectable, changed resistance towards sanitizing agents.

3.4. Inactivation efficiency of sanitizers in process wash water

NaClO (10 mg/L) and ClO_2 (5 mg/L) were tested in organically loaded water with non-starved cultures of *S.* Typhimurium and *E. coli*. Results for *S.* Typhimurium in the industrially-supplied PWW (Fig. 3A, C) demonstrated incomplete inactivation for both sanitizers when the PWW had a TOC of 354 mg/L (Fig. 3A). When the PWW had a TOC of 177 mg/L (Fig. 3C), ClO_2 inactivated *S.* Typhimurium more than NaClO. Results for *E. coli* in industrially-supplied PWW (for 5 mg/L ClO_2) and laboratory-made PWW (for 10 mg/L NaClO) were observed (Fig. 3B, D). For ClO2, there was <1 log reduction at a TOC of 354 mg/L (Fig. 3B), while at a TOC of 177 mg/L there was a 4.5 log reduction (Fig. 3D). NaClO had little to no inactivation in laboratory-made PWWs with TOCs of 354 and 177 mg/L. Similar experiments with *E. coli* at a TOC of 354 mg/L were cultured on BECSA + Ct and showed at least a 3 log reduction with ClO_2 (5 mg/L) in industrially-supplied PWW (data not shown). Although results may be influenced by the non-use of neutralizers

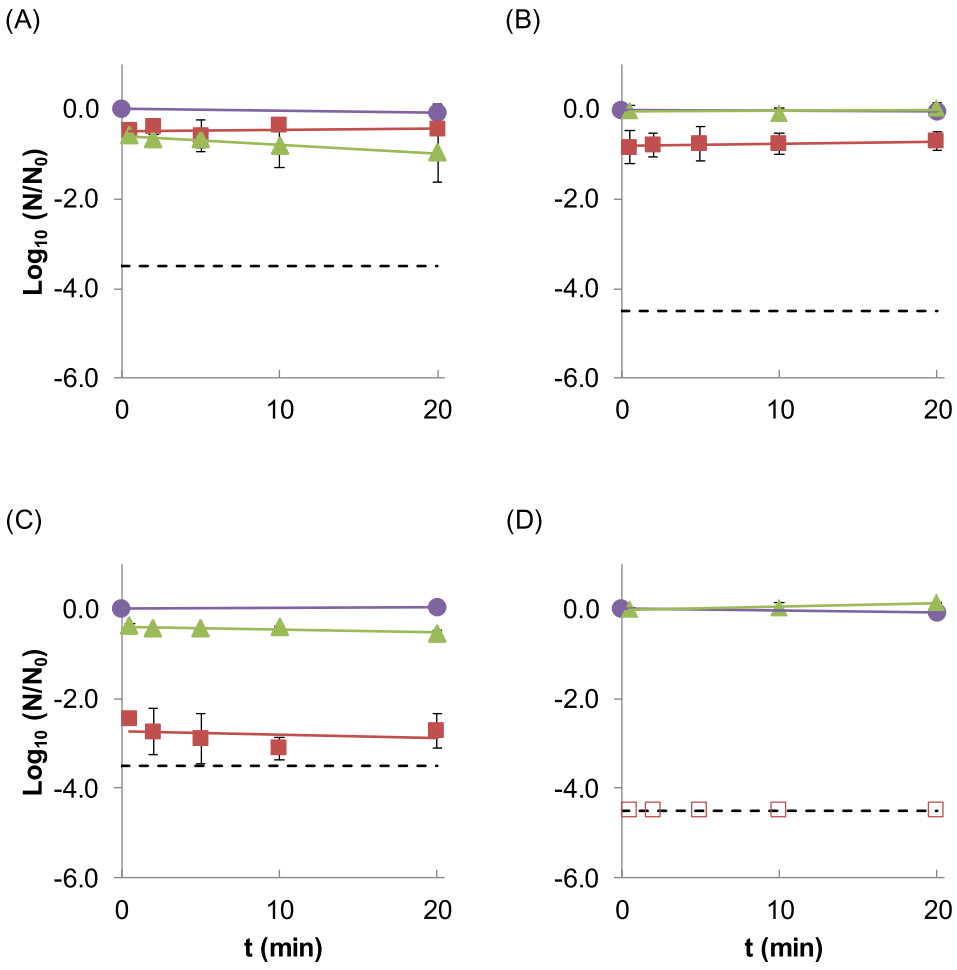

Fig. 3. Inactivation of *Salmonella enterica* Typhimurium 1638 (A, C) and *Escherichia coli* isolate 0247_1 (B, D) cells from the non-starved culture at 37 °C on LBA media with 10 mg/L NaClO (▲), 5 mg/L ClO_2 (■), and no sanitizer (●) in process washing water: (A, B) TOC = 354 mg/L, (C, D) TOC = 177 mg/L. Data represent the average of duplicate experiments and error bars represent standard deviation. –, Limit of Detection.

for sanitizing agents, serial dilutions in PPS were made before plating. Overall, water quality, including the organic load measured as TOC, and the media used to culture *E. coli* were relevant variables.

4. Discussion

4.1. Pathogen releasing efficacy and (re-) attachment to lettuce leaf punches during washing

Our results indicated that bacterial cell attachment to the iceberg lettuce leaf punches occurred within 15 s, while maximum attachment occurred after 30–60 min. These results are consistent with data obtained by Patel and Sharma (2010), which reported that *Salmonella enterica* serovars quickly attached to both intact and cut produce surfaces with attachment increasing over time. Furthermore, Takeuchi, Matute, Hassan, and Frank (2000) reported that *E. coli* O157:H7 preferentially attached to iceberg lettuce cut-edges rather than the surface, possibly a result of its non-hydrophobic surface, while *S.* Typhimurium attached equally at cut edges and surfaces. This non-preferential attachment mechanism for *S.* Typhimurium may be a reason for the difference in cell attachment numbers over time, as well as the lower attachment of *E. coli* cells on the lettuce leaf punch surface at the 10^2 CFU inoculum level compared to *S.* Typhimurium cells at the 10^2 CFU inoculum level. Overall, *S.* Typhimurium and *E. coli* attachment capabilities are shown to differ, with higher attachment rates after 60 min for *S.* Typhimurium than for *E. coli,* yet with minimal differences in the adherence and recovery based on higher inoculum densities (10^6 CFU).

Bacterial cell (re-)attachment to and release from produce can affect the degree of contamination during produce washing. In our study, although bacteria were added to the middle of the leaf punch, avoiding contact with the cut-edges, during re-attachment, the bacteria may have diffused to the edges. Cell attachment can be characterized by a two-stage process: (i) initial cell attachment, which can occur within seconds, is based on a weak, unspecific, and reversible binding; and (ii) irreversible or "firm" attachment, which is multi-mechanistic as it can be influenced by several factors including bacterial strain features, produce, and type of processing. Due to the presence of stronger forces (e.g., covalent and hydrogen bonds, strong hydrophobic interactions), bacterial cells cannot easily be removed; to overcome this, even stronger forces such as from physical methods or chemical sanitizers would need to be employed to remove or inactivate the pathogens (Goulter, Gentle, & Dykes, 2009; Van der Linden et al., 2014; Yaron & Romling, 2014). The results of our study indicated that bacterial adherence for *S.* Typhimurium and *E. coli* to the lettuce was firm even after multiple washings. According to these data, we can infer that after bacterial cell attachment to the leaves, bacteria were more difficult to

inactivate with sanitizers. These results may be related to produce surface properties such as the hydrophobic cuticle, abrasions in tissues, or preferred binding sites; such sites make pathogen inactivation, through the use of sanitizers, difficult as sanitizers cannot access the 'protected' sites where pathogens may be harboring (Beuchat, 2004). Overall, the number of bacterial cells that attached to the lettuce leaf punches may be related to the preferred binding sites as well as a microorganism's ability to attach weakly or firmly. These findings can help us to understand further the importance of preventing pathogenic cross-contamination and, consequently, the principal aim to maintain the PWW quality during produce washing.

4.2. Survival of non-starved and starved cells

4.2.1. Inactivation efficiency of sanitizers in potable water

Drinking water disinfection with chlorine-based disinfectants has long been employed to control waterborne diseases, namely to eliminate harmful pathogens in the drinking water and to provide safe and potable water. Our results with NaClO and ClO_2 for both non-starved and short-term starved cultures demonstrated a >4 log reduction in potable water given short contact times. Nevertheless, health related concerns from the use of chlorine, due to the formation of chlorine DBPs such as trihalomethanes, haloacetic acids, and aldehydes, has prompts the need for alternatives. ClO_2 is one potential alternative for chlorine-based disinfection since it does not form organohalogen DBPs (Gopal, Tripathy, Bersillon, & Dubey, 2007) and is less affected by the presence of organic matter than chlorine (Van Haute, Sampers, et al., 2015). However, the formation of chlorate residues could be problematic, yet studies on chloroxyanion accumulation in the PWW and presence on fresh-cut produce when ClO_2 is used as a sanitizer during processing are limited (López-Gálvez et al., 2010; Van Haute, Tryland, Escudero, Vanneste, & Sampers, 2017) and are not the main objectives of this study.

Furthermore, our study investigated a silver-copper sanitizer. Metals such as silver and copper have been employed in their ionic form, both unaided and in combination with other sanitizers, to control pathogenic microorganisms present in water for human consumption as well as for recreational purposes, in cooling towers and large water distribution systems such as those employed by hospitals, and for municipal waste water management (Huang et al., 2008; Luna-Pabello, Rios, Jimenez, & de Velasquez, 2009; Silvestry-Rodriguez, Sicairos-Ruelas, Gerba, & Bright, 2007). In our study, Ag-Cu demonstrated a 4 log reduction of *S.* Typhimurium and *E. coli* in potable water after longer contact times in comparison to NaClO and ClO_2. Also, after 10 min of *E. coli* exposure to Ag-Cu, there was about a 1 log difference between non-starved and short-term starved cultures. Accordingly, the short-term starved *E. coli* culture may survive better towards the Ag-Cu solution meaning *S.* Typhimurium cultures are more susceptible compared to *E. coli* cultures.

Overall in our study, no substantial differences were observed in the potable water between non-starved and short-term starved cultures for all tested sanitizers. Given the longer inactivation kinetics required for the Ag-Cu solution, it is not suitable for application to the PWW since PWW disinfection requires fast inactivation kinetics (Banach, Sampers, Haute, & van der Fels-Klerx, 2015).

4.2.2. Sanitizer efficacy in lettuce washing water and on lettuce leaf punches

Our results indicated that *S.* Typhimurium and *E. coli* cells that had released from the lettuce leaf punches during the control washing could be detected, and cells remain attached to the produce during control treatments, both irrespective of the applied incubation temperature. Furthermore, sanitizing treatments eradicated cultural cells in the lettuce wash water. Regarding lettuce leaf punches, the washing temperature during treatments affected survival; fewer cells were reduced when wash water was maintained at 20 °C than at 5 °C for all treatments ($p = 0.001$, $n = 2$). Nevertheless, overall cells were able to survive treatments when adhered to the lettuce leaf surface. These results concur with previous research, which has indicated that bacterial adhesion to produce during washing was less extensive at lower temperatures and with short exposure times (Patel & Sharma, 2010; Reina, Fleming, & Breidt, 2002). For example, for chopped and unchopped parsley, the efficacy of washing disinfectants against *S.* Typhimurium during different temperature-time conditions (5 °C/4 h; 5 °C/24 h; 30 °C/4 h) indicated the highest log reduction for unchopped parsley in a chlorine wash at low temperatures (5 °C) and with a shorter exposure (4 h) (Faour-Klingbeil, Kuri, & Todd, 2016). An important implication of our results is that pathogen reduction in the wash water can be influenced by processing parameters such as the use of chemical sanitizers in the wash water. Furthermore, produce decontamination may be less effective due to irreversible bacterial attachment mechanisms and/or protective mechanisms of the plant, and focus should, therefore, be aimed at preventing bacterial attachment at all steps along the chain.

Previous research has described the difficulties in culturing bacteria, like *E. coli* O157:H7, in water as when bacteria are exposed to sub-lethal stresses such as temperature, pH, nutrient changes, sanitizer exposure, etc., they may enter a viable but non-culturable (VNBC) state making isolation problematic (Li, Mendis, Trigui, Oliver, & Faucher, 2014; Liu, Gilchrist, Zhang, & Li, 2008; Oliver, 2005; Oliver, Dagher, & Linden, 2005; Sata, Osawa, Asai, & Yamai, 1999; Wang & Doyle, 1998). Thus, treatments with sanitizers may lead to non-culturability, while cells may still be alive. In our study, the long-term starved cells of both strains were recovered from the leaf punches after treatments with control and sanitizing agents at both 5 and 20 °C washing water temperatures. These results are consistent with Van der Linden et al. (2014), which reported that *E. coli* O157:H7 stressed cells, from temperature and fewer nutrients, attached similarly to the lettuce leaves as did unstressed, freshly grown cells. Similarly, Al-Nabulsi et al. (2014) described no apparent differences between stressed and unstressed *E. coli* O157:H7 cell attachment and survival on lettuce leaves after disinfection. Overall, the long-term starved cells of both strains from our study demonstrated minor detectable differences in cell survival after treatment with sanitizers in both the iceberg lettuce wash water and on lettuce leaf punches.

4.3. Inactivation efficiency of sanitizers in process wash water

Our results indicated that the efficacy of NaClO and ClO_2 is dependent on water quality, measured as the TOC of the PWW. Similarly, Van Haute et al. (2013) observed that in high organically loaded water, chlorine was quickly inactivated. Moreover, during pilot-scale experiments, Davidson et al. (2013) observed that chlorine-based sanitizers may help prevent cross-contamination in water with lower organic loads. In our study, ClO_2 was observed to prevent potential cross-contamination when the TOC of the PWW was 177 mg/L, yet not at 354 mg/L. However, our results were observed on a non-selective media, yet when *E. coli* was cultured on selective media, at least a 3 log reduction was seen with the industrially-supplied PWW (TOC = 354 mg/L). These variations may occur because ClO_2 treatment caused damage, but not inactivation. Overall, given the fluctuating environment of the PWW, the sanitizer dose needs to be properly controlled throughout

processing to prevent cross-contamination.

Moreover, in our study, we observed inactivation differences between non-starved *S.* Typhimurium and *E. coli* with ClO_2 (5 mg/L) in PWW with a TOC of 177 mg/L. Consequently, sanitizer efficacy is also shown to be influenced by the pathogen type. This result concurs with Lopez-Velasco, Tomas-Callejas, Sbodio, Artes-Hernandez, and Suslow (2012) who observed *S. enterica* serovar variability to ClO_2 dose and tolerance thereof in water. Therefore, along with the organic load of the PWW, pathogen and sub-type variability are parameters that should not be overlooked as they can be relevant factors for sanitizer selection and application (*in situ*).

5. Conclusions

Sodium hypochlorite (10 mg/L) and chlorine dioxide (5 mg/L) were similarly effective in inactivating *S.* Typhimurium, and ESBL *E. coli* in (i) potable water and (ii) lettuce wash water. The silver-copper solution was comparatively less effective in inactivating *S.* Typhimurium and ESBL *E. coli* in (i) potable water, yet sanitizer differences, for the tested chemical sanitizers, in (ii) lettuce wash water were less apparent. The difference between non-starved and starved cell survival in (i) potable water and (ii) lettuce wash water and on lettuce leaf punches was minimal and did not affect the inactivation rate of sanitizers. Sanitizer application in (iii) process wash water was shown to be dependent on the water quality (*i.e.* organic load of the water) and the pathogen type. In brief, pathogen inactivation was shown to be dependent on the organic load of the water, water temperature, and pathogen attachment and release from the produce. Furthermore, experiments with multiple washings demonstrated the firm attachment of pathogenic cells, once attached to the produce, and thus, stress the relevance of preventing microbial attachment along the fresh(-cut) produce chain. Future research should investigate the feasibility of chlorine dioxide application, given minimum effective concentrations, as a process wash water disinfectant during pilot-scale processing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2017.05.044.

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