CENTER for PRODUCE SAFETY

CPS 2013 RFP
Project Title
Effectiveness of a batch ozonated retail wash system for iceberg lettuce

Project Period
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Objectives

1. Characterize the microbiological and oxidative-demand status under a representative operational retail wash unit applied to heads of cored crisphead lettuce with ozonated water
2. Assess the total heterotrophic bacteria (HPC) and total coliform populations on cored crisphead lettuce from a wholesale distribution center over time to establish a short-term baseline of seasonal variation.
3. Evaluate the efficacy of ozonated water treatment on HPC and total coliforms, as well as generic *Escherichia coli*, *Listeria innocua* (a surrogate for *Listeria monocytogenes*), and an attenuated strain of *Salmonella*. Determine whether efficacy in prevention of cross-contamination is maintained during continual batch soak-washing.

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FINAL REPORT

Abstract

Recently, there has been increasing interest and commitment to maintain the continuity of food safety preventive controls across the produce supply and marketing chain. Many retail foodservice and supermarket outlets are currently processing various fresh produce items in-store with the goal of delivering the freshest possible product to consumers. In store handling and food preparation for retail display and sales involves different modes of water use. In quick-serve-restaurant (QSR) outlets and supermarket backroom preparation areas it is common to have batch immersion as one or the only step in produce washing. Distinct from efficacy evaluations in dump and flume systems, small-scale batch immersion and washing, which involves extended contact time of product in water, was identified as an application where ozonated water could be an effective practice. This Proof of Concept project focused on washing of cored head lettuce, one of the most common produce items handled in retail stores, as the first application for assessment of a continuous ozone injection system. The specific objectives were to 1. Characterize the microbiological and oxidative-demand status under a representative operational retail washing and crisping; 2. Assess the total heterotrophic bacteria (HPC) and total coliform populations on cored crisphead lettuce from a wholesale distribution center to establish a short-term baseline of seasonal variation; and 3. Evaluate the efficacy of ozonated water treatment on HPC and total coliforms, as well in preventing cross-contamination by inoculated generic *Escherichia coli*, *Listeria innocua* (a surrogate for *Listeria monocytogenes*), and an attenuated strain of *Salmonella enterica*. Focusing on Objective 3, in each of 3 replicates, 24 heads of lettuce obtained directly from a single distribution center were removed of wrapper leaves and cored the day of processing. After filling a stainless steel tank with 75 L of tap water, 22 heads were submerged, cored side up, and covered with 5400 g of crushed ice. During filling and icing, a specifically designed small-scale ozone generator (Model SW-1-01, DEL Ozone, San Luis Obispo, CA) was used to charge the recirculating chilled water and left on for the duration of the sampling interval. Lettuce and water samples were taken at 20, 26, 32, and 38 minutes, with and without the addition of ozone in the batch wash water. Water samples were analyzed at a commercial retail outlet (Objective 1) and during each experimental batch wash setup for physicochemical parameters including: temperature, pH, oxidation-reduction potential, chemical oxygen demand, electro-conductivity turbidity, and dissolved ozone. For batch rinsing of uninoculated head lettuce with water (no ozone), populations of total heterotrophs in the wash water increased significantly ($P < 0.05$) by 2.90 log CFU/100 ml after 40 min. After 30 and 40 min of processing with the pilot-scale system, water with continual ozone injection contained lower populations ($P < 0.05$) of heterotrophs (2.33 and 3.81 log CFU/100 ml lower, respectively) and coliforms (2.30 and 2.77 log CFU/100 ml lower) than during processing with the water control. As expected, for inoculated water, a greater log-reduction was observed in the ‘cleanest’ batch water with low COD, exceeding 5-log reduction of pathogen numbers in two minutes. Pathogen surrogates released from the surface of individual inoculated lettuce leaves accumulated in water without ozone treatment but not in water when continually injected with ozone during the soak-interval. The accomplishments to date support an expectation that this ozone system approach is transferrable to other applications at points of retail produce handling and food preparation. Though preliminary relative to a standard optimization and comparative research study, this project has developed data which better defines and determines the performance of a small-scale ozonated wash water system. These results serve as a foundation for future studies that enhance guidelines for food service companies to use as an additional step for improving the continuity of produce safety from farm to fork.

Background

Many retail foodservice and supermarket outlets are currently processing various fresh produce items in-store with the goal of delivering the freshest possible product to consumers. In store handling and food preparation for retail display and sales involves different modes of water use including wash and repack, core:wash:minimally-process, wash and separate (individual leaf separation of head lettuce or romaine lettuce hearts), soak and peel, crisping (water uptake for increased turgor) of leafy greens and culinary herbs, wash of fruit and fruit vegetables for pre-cut and pre-sliced, and other diverse and related practices. In quick-serve-restaurant (QSR) outlets and supermarket backroom preparation areas it is common to have batch immersion as one or the only step in produce washing. Individual business units or entire retail store systems may treat their water supply with some approved antimicrobial during batch washing or soaking. In some areas it is common, though not uniform or standardized, to treat municipal water at these facilities with a filtration-UV system due to seasonal issues with microbiological challenges to water quality. While effective on source water, this treatment provides no residual activity to prevent in-use cross-contamination. Though not a wash step *per se*, retail produce display misting systems are commonly supplied by the same treated or non-treated water.

Recently, there has been increasing interest and commitment to maintain the continuity of food safety preventive controls across the produce supply and marketing chain. In discussion with individual retailers, that have a long history and experience with in-store produce washing prior to fresh processing preparation, a desire was expressed to have specific measureable performance expectations for key produce handling practices. There are ample examples which demonstrate that washing lettuce and other produce items during food preparation, without the use of any added sanitizers, may allow for cross-contamination to occur within and between batches or lots and potentially result in consumer illnesses and deaths. These companies are seeking practical and timely baseline data to make operational decisions to further improve and standardize product safety through the use of antimicrobial sanitizers during these processing and food preparation operations. During discussion, though there are several possible effective options, various internal customer preference feedback evaluations identified ozone as being highly desirable for several of the retail wash applications mentioned above. Distinct from our efficacy evaluation in dump and flume systems, small-scale batch immersion and washing, which involves extended contact time of product in water, emerged as a core practice and key opportunity where ozonated water could be an effective practice.

Leafy greens and culinary herbs continue to be subject to surveillance-based recalls and implicated in outbreaks of foodborne illness throughout the United States and globally. Recent incidents and evolving regulatory standards keep the concern for consumer protection and brand protection at the forefront of foodservice companies to improve their preventive safety performance. Within the past few years, ozone has gained renewed attention as a sanitizer for fresh produce, with studies examining its efficacy on diverse products, including lettuce, parsley, spinach, and watercress (*1*-6). Ozone has shown to be effective on a range of microorganisms, with reductions between 2-3 log values observed for aerobic mesophiles, coliforms, and fungi (*2*), as well as *Escherichia coli* (*4*) and *Listeria monocytogenes* (*5*), and slightly lower reductions (~1.5 log) for *Salmonella* Typhimurium (*6*). Effectiveness in wash water systems using ozone are strongly impacted by the amount of dissolved and suspended solids in both batch and recirculated water due to its high reactivity with oxidizable constituents. This reality has often limited the adoption of ozone as a primary antimicrobial processing aide in large-scale dump and flume wash systems. While it seems that short product contact time, typically occurring during large-scale, high-throughput processing, greatly limits bacterial reductions, the work done by Alexopoulos et al. (2013)

indicates that longer contact times (15-30 min) may support higher reductions (2-3 log) in bacterial populations native to leafy greens. Based on the results from these studies, ozone shows promise as a sanitizer for leafy greens for controlling populations of microorganisms that could be harmful to consumers. In small batch wash and soak systems, the potential for ozone to be an effective treatment for the produce handling procedures, outlined above, suggested the research hypothesis that underpins this study. To begin this short-duration Proof of Concept project, washing of cored head lettuce, one of the most common produce items handled in retail stores, was selected as the first application for assessment. Considering the correlation between ozone efficacy and contact time, on-site observations of the process used for washing whole heads of lettuce – which includes a 20-30 min soaking period and up to 40 min total water contact time during batch food preparation– supported the expectation that food safety continuity from the supplier and for consumer protection may benefit from the use of ozone. Therefore, the goal of this project was to determine the efficacy of a recirculating ozone injection system during simulated processing of iceberg lettuce on surface-adhered and planktonic bacteria. Efficacy was examined with both naturally-occurring bacteria on iceberg lettuce and inoculated human pathogens or surrogates, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria innocua*. Standard wash water parameters during in-store retail preparation of lettuce were also characterized, in addition to seasonal variations in bacterial loads on lettuce and the impact of the specific ozone exposure on lettuce short-term shelf-life.

Hypothesis

Ozonated water when applied to cored heads of lettuce in a batch wash system can prevent the accumulation of microbial loading by at least 3 log in HPC (total heterotrophic bacteria) and total coliform populations in a batch wash system. Recirculating ozonated water can achieve at least a 3 log reduction during batch washing and prevent cross-contamination by select inoculated bacterial pathogens.

Research Methods and Results

1.1 Characterizing standard wash water parameters

Water samples were collected by the Suslow Lab during standard daily lettuce preparation by an in-store staff in Davis, CA to characterize batch wash water normally used during a typical foodservice outlet operation. These samplings occurred on four different occasions throughout the project duration. The timing of sample collection during processing varied slightly; on one occasion, the sample was taken after all lettuce had been removed from the sink, while other samples were obtained while the heads of lettuce were in-place at the end of the pre-processing soak interval. Various wash water parameters where characterized (details in section 1.6), and all samples were analyzed for total heterotrophic bacteria and total coliforms (1.5.1).

1.2 Seasonal variation in bacterial populations on lettuce

In each of six samplings done throughout the study, iceberg-type (head) lettuce was sampled directly as received from a wholesale supplier to preliminarily identify seasonal variations in naturally-occurring bacterial populations. Cases of lettuce obtained from a single primary grower/supplier, directly from a distribution center, were stored at 5˚C until processing for enumeration of target indigenous bacteria. Heads of lettuce (10 per sampling) were stripped of wrapper (outermost) leaves, as is the retail practice, and cored with a standard sterile stainless steel device. Multiple layers of spatially distinct leaves (inner and outer) were carefully removed and processed to quantify total heterotrophic bacteria and total coliform populations (1.5.2).

1.3 Evaluating ozone efficacy using a pilot-scale system

In each of 3 replicates, 24 heads of lettuce obtained directly from a single distribution center were removed of wrapper leaves and cored the day of processing. After filling a stainless steel tank with 75 L of tap water, 22 heads were submerged, cored side up, and covered with 5400 g of crushed ice. During filling and icing, a specifically designed small-scale ozone generator (Model SW-1-01, DEL Ozone, San Luis Obispo, CA) was used to charge the recirculating chilled water and left on for the duration of the experiment. Following an example food preparation protocol for head lettuce; after 20 min of soaking in water with or without ozone, 2 heads of lettuce were removed from the wash water every two minutes, draining as much excess liquid from the heads as possible back into the sink, as was observed for in-store processing. Lettuce samples taken at 20, 26, 32, and 38 minutes, after the start of processing were enumerated for native bacteria, as detailed in section 1.5.2. Water samples were taken at the start of processing and every ten minutes thereafter to assess various wash water parameters (1.6) and perform microbial analysis (1.5.1). After all heads of lettuce were removed, the sink was drained and equipment surfaces were similarly quantified for bacteria native to head lettuce (1.5.3).

To evaluate ozone efficacy, within the constraints of this small batch system, on human pathogens, the same processing setup described above was used, but the outer leaves of 24 heads of lettuce were inoculated to contain ~4.5 log CFU/g each of *E. coli* O157:H7 (PTVS 155), *Salmonella* Typhimurium (x3985), and *L. innocua* (TVS 451) (1.4). Lettuce and water samples were similarly obtained during processing for microbial analysis (1.5.2 and 1.5.1, respectively). In one experiment the sink was filled with lettuce and in a second assessment method, the inoculated leaves were immersed in water adjusted with strained lettuce homogenate to the approximate mean Chemical Oxygen Demand (COD) of the water measured during in-store surveys. The purpose of this variation in procedure was to specifically test cross-contamination potential and facilitate recovery of the inoculated leaves across the experiment timeline. These tests were conducted in a non-draining basin due to biosafety requirements for terminal treatment and proper disposal of wash water.

1.1 Visual shelf-life of lettuce washed with ozonated water

As a preliminary assessment, 5 heads of lettuce were removed of wrapper leaves and cored immediately prior to processing. Lettuce was placed core-side up in 20 L of tap water and covered with 3600 g of crushed ice for 20 minutes with or without the addition of ozone. ~ 8 x 10 cm squares were cut from each head of lettuce and stored at 5˚C. Samples were evaluated for visual shelf-life quality (pinking and decay) daily for 5 days.

1.2 Inoculation

Forty-eight hours prior to inoculation, pure, isolated colonies of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. innocua* were suspended in 100 µl of sterile Butterfield's phosphate buffer (Whatman, Florham Park, NJ) (BPB) and spread-plated on Trypticase Soy Agar (Difco, Becton, Dickinson & Co., Sparks, MD) amended with rifampicin (Fisher Scientific Inc., Waltham, MA) (80 mg L^{-1}) (TSA+rif) for 24 h of incubation at 37°C. Following incubation, lawns were separately harvested and suspended in sterile BPB to obtain an OD_{600} of 0.750, corresponding to ~9 log CFU/ml. Cultures were combined and further diluted in sterile BPB to obtain an inoculum containing ~6 log CFU/ml of each strain.

Twenty-four heads of lettuce were removed of wrapper leaves, cored, and placed cored-side down for inoculation. The outer leaves of each head were sprayed in a biohazard hood with ~1.5 ml of inoculum for 18h storage at 5°C. Immediately prior to processing, two unwashed heads of inoculated lettuce were quantified for populations of the three strains of bacteria. In the second study, methods were essentially the same with the following minor modifications; the sink was filled with 75L water and blended lettuce juices (553 g, strained through cheesecloth) to get COD of approximately 70 mg O2/L. 22 inoculated leaves were immersed in the water and covered with 5400 g ice, with or without ozone injection initiated at this time-point. After 20 min, 2 inoculated leaves were removed every 2 min (sample leaves at t=20, 26, 32, 38 min) as previously. Water samples were taken at t = 0, 10, 20, 30, 40 - for processing parameters and microbial analysis as above.

1.3 Microbial analysis

1.3.1 Water samples

Immediately upon collection in 100-ml sterile containers, all water samples were neutralized with sodium thiosulfate to inhibit the antibacterial activity of ozone and background chloramine/chlorine levels present in the tap water (0.36 mg/L). For experiments involving native lettuce microbiota, water samples were appropriately diluted and surface-plated for 48 h of incubation at 29˚C on Plate Count Agar (Difco) amended with pentachloronitrobenzene (PCNB; Amvac Chemical Corp., Newport Beach, CA) (5 mg ml^{-1}) to minimize fungal growth from potentially adhering soil. The enumeration agar was also amended with sodium pyruvate (Fisher Scientific Inc.) (1 g L^{-1}) to assist in resuscitation of sub-lethally injured cells (PCA+PCNB+pyr) for quantification of total heterotrophs. Water samples were additionally processed with Quanti-Trays (IDEXX Laboratories, Westbrook, ME) for 24 h of incubation at 37˚C to quantify total coliforms.

For experiments involving attenuated pathogens or a surrogate, neutralized water samples were filtered through 0.45 µm membranes using ISO-GRID/NEO-GRID Membrane Filter Systems (Neogen Corp., Lansing, MI) and enumerated for *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. innocua* after incubation at 37˚C on CHROMagar O157, CHROMagar *Salmonella* Plus, and CHROMagar *Listeria* (CHROMagar, Paris, France), all amended with rifampicin and pyruvate (O157+pyr+rif, Sal+pyr+rif, and Lis+pyr+rif). 100-ml water samples were additionally enriched in Buffered Peptone Water (Difco) containing rifampicin for 24 h at 37˚C (for *E. coli* O157:H7 and *Salmonella* Typhimurium) and *Listeria* Enrichment Broth (EMD Millipore, Darmstadt, Germany) containing rifampicin for 48 h at 37˚C (for *L. innocua*). 100-µl enrichment samples were spot-plated on respective media and incubated appropriately to determine presence of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. innocua*.

1.3.2 Lettuce samples

To determine a baseline of seasonal variation in bacterial populations on heads of iceberg lettuce, exterior and interior leaves (25 g each) from each head of lettuce were homogenized with 100 ml Difco™ Neutralizing Buffer (NB) (Difco) in a sterile Whirl-Pak™ bag (Nasco, Fort Atkinson, WI) by stomaching at 260 RPM for 1 min. Inner leaves were obtained by cutting vertically through the head of lettuce and sampling the leaves residing ~2.5 cm (1 in) below the outer layer. The main purpose of this research activity was to establish a typical bacterial distribution profile during the project timeline. Developing quantitative data on the spatial distribution in head lettuce provided us with the background to determine the potential for re-distribution (cross-contamination) during batch soaking with and without ozone treatment. Samples were appropriately diluted and surface-plated on CHROMagar ECC amended with pyruvate (ECC+pyr) for 24 h of incubation at 37˚C, and PCA+PCNB+pyr for 48 h of incubation at 29˚C for enumeration of total coliforms and total heterotrophic bacteria, respectively.

To quantify native microbiota when using the pilot-scale wash system, 25-g subsamples of exterior leaves from each head of the two-head sample were composited; the same was done with interior leaves. 50-g lettuce samples were then homogenized with 200 ml NB by stomaching at 260 RPM for 1 min. Samples were appropriately diluted and surface-plated on PCA+PCNB+pyr and ECC+pyr for enumeration of total heterotrophic bacteria and total coliforms, respectively. Lettuce samples were identically prepared during experiments with inoculated pathogens, but were surface-plated on O157+rif+pyr, Sal+rif+pyr, and Lis+rif+pyr for enumeration of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. innocua*, respectively.

1.3.3 Equipment surface samples

After processing and subsequent draining of the sink, three 100-cm² surfaces from the sink (two from walls, one next to the drain) were swabbed as described by Vorst et al. (7) with a one-ply, 11.4 x 21.5 cm Kim-wipe (Kimberly-Clark Corp., Roswell, Ga) moistened with 1 ml NB. Samples were homogenized with 45 ml of NB in a sterile Whirl-Pak™ bag at 260 RPM for 1 min, after which they were appropriately diluted and surface-plated on PCA+PCNB+pyr for 48 h of incubation at 29˚C, and ECC+pyr for 24 h of incubation at 37˚C for enumeration of total heterotrophic bacteria and total coliforms, respectively.

1.4 Wash water parameters

During each experimental batch wash setup and after collection of water samples, various physicochemical parameters were assessed: temperature, pH, oxidation-reduction potential, chemical oxygen demand (COD; Hach® DRB 200 and COD2 Mercury-free 20-1500 mg/L range, Loveland, CO), electro-conductivity, and turbidity. During pilot-scale processing, water samples were additionally assessed for ozone concentrations using AccuVac Ozone Mid-Range Test Kits (Hach®, Loveland, CO; Hach® Colorimeter O3 Program #73). **Note –** In parallel with testing dissolved ozone by the AccuVac Indigo method, a Dissolved Ozone sensor was deployed in the recirculating wash water basin. Despite repeated calibration, the low concentration of O3 at the point of available placement, restricted to the depth of submersion of the sensor probe, significantly affected the reliability and accuracy of real-time dose measurement. Therefore, only colorimeter measurements (AccuVac) are reported and recommended for routine verification in a system with a similar design for small-scale washing or hydration.

1.5 Statistical analysis

Bacterial counts from triplicate experiments were converted to log scale and subjected to an ANOVA using JMP 9.0 (SAS Institute Inc., Cary, NC). For all tests, a P value of < 0.05 was considered significant. Tukey HSD test was used to identify significant differences in bacterial populations and wash water parameters.

RESULTS AND DISCUSSION

When assessing seasonal variation in bacterial populations on head lettuce, outer lettuce leaves contained similar populations of total heterotrophs and total coliforms during the first 4 sampling dates (5.50 and 4.17 log CFU/g on average, respectively) with a significant ($P < 0.05$) decline between January 6 and January 16, 2014 (Table 1). Changes in populations over such a short *Proof of Concept* study period (~2 months) suggest that even greater variations on incoming lettuce may occur throughout the year, likely depending largely on weather patterns, product source/location, harvest handling, and, potentially, cold chain management to the point of food preparation. This is substantiated based on our earlier collaborative seasonal study with Romaine lettuce (8). Fluctuations in populations may result in greater build-up of undesirable levels of bacteria within the washing system during processing if dose standards are not adequately defined, potentially allowing for cross-contamination of other products.

Weather- and source-depending, incoming lettuce is likely to contain varying minor levels of adhering soil and other inorganic and organic constituents that may be released into wash water during batch washing. This is one likely reason for the fluctuations in COD levels obtained from water samples during in-store preparation of lettuce (Table 2). As with all oxidizers to a varying degree, high levels of organic constituents released from cut surfaces can impact ozone stability and therefore efficacy in microbial control; in preliminary studies it was established that not only was ozone depleted in the presence of high organic matter (liquid extract from blended lettuce solids), but that the time necessary for >6 log reduction of *Salmonella* Typhimurium increased dramatically when compared to clean water or water containing chopped lettuce and, especially, a significant amount of filtered lettuce juice (Table 3 and 4).

During pilot-scale processing of uninoculated head lettuce with water (no ozone), populations of total heterotrophs in the wash water increased significantly ($P < 0.05$) by 2.90 log CFU/100 ml after 40 min of processing. TPC and TC populations in lab-cored head lettuce were consistently lower than those observed from the retail outlet water samples (Table 2), likely due to increased handling of product and greater release of bacteria into the water during in-store preparation. After 30 and 40 min of processing with the pilot-scale system, water with continual ozone injection contained lower populations ($P < 0.05$) of heterotrophs (2.33 and 3.81 log CFU/100 ml lower, respectively) and coliforms (2.30 and 2.77 log CFU/100 ml lower) than during processing with the water control (Table 5 and Figure 1). This outcome supports the application of ozone as a water process aide option; when used during small-scale processing of iceberg lettuce, ozone prevented the build-up of microbial load in the batch water and it is possible this may prevent cross-contamination of product. However, the applied level of ozonated water in this system was no more effective than the water control at measurably reducing populations of native bacteria on lettuce surfaces (Table 6). This appears to be a consequence of the tight adherence of these bacteria to the lettuce as, proportionally very little of the total number present is released to the water in this handling system. Similarly, equipment surfaces contained similar ($P > 0.05$) populations of total heterotrophs and total coliforms (averages of 3.93 and 2.13 log CFU/100 cm^2, respectively) regardless of ozone use, in the absence of continual injection after removal of lettuce.

Prior to pilot-scale studies using inoculated iceberg lettuce, it was established that the *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. innocua* strains used here were not inhibitory to each other and could therefore be co-inoculated without impacting populations during storage overnight or during recovery on appropriate selective media. Surface-adhered pathogens behaved similarly to native bacteria during processing of head lettuce inoculated to contain 4.42, 4.43, and 4.61 log CFU/g of *E.* coli O157:H7, *Salmonella* Typhimurium, and *L. innocua* on the outer leaves; no measurable reductions in populations on lettuce samples were observed when low doses of continual ozone injection was employed (Table 7). However, immersion and hold times of lettuce in the water control or ozonated water did not result in detectable transference or carryover of bacteria from outer leaves to inner leaves, which contained similarly low populations ($P > 0.05$) (0.65 - 0.72 log CFU/g of each pathogen) throughout processing. As a consequence of the experimental design with low inoculum levels, ozonated water was no more effective than the water control at reducing pathogen populations in wash water samples based on qualitative detection by enrichment; the only significant difference occurred after 30 min of immersion for *L. innocua* populations (1.05 log MPN/ml lower with ozone) (Table 8). The variation in ozone efficacy between planktonic bacteria native to head lettuce and human pathogens could be due to differences in their initial populations in the wash water; populations of human pathogens were likely initially too close to our limit of detection to allow significant reductions to be discerned. When water samples were enriched to determine pathogen presence below the limit of detection (-1.52 log MPN/ml), fewer positive enrichments were generally found in samples exposed to ozone (Table 8).

During pilot-scale experiments with both native microbiota and inoculated human pathogens, the only parameter affected by ozone was ORP, with values significantly higher ($P < 0.05$) after 10-40 min in the presence of ozone (Tables 9 and 10). Differences in ORP values obtained from retail foodservice outlet water samples and water samples from pilot-scale experiments could be due to slight delays (~30 min) in obtaining ORP readings from off-site samples, or the close proximity of the ORP probe to heads of lettuce during pilot-scale processing. Ozone concentrations varied slightly throughout processing, ranging from 0.07 to 0.63 mg O_3 L^{-1} and may have been limited in measurement accuracies by the necessary sampling location relative to water supply and return orifices.

In a variation of the above study, inoculated outer leaves were soaked in water with and without ozone which had been amended to adjust the COD to ~70 mg O2/L (the unit of measure for oxidizer-demand) using blended and filtered lettuce extracts. All water and lettuce samples were taken identically to previous tests (water samples every 10 min, lettuce samples after 20, 26, 32, 38 min). Populations on lettuce leaves did not change during washing, within the limits to enumerate small changes in released/detached bacteria, in either batch water (just water, or water + ozone). After 40 min, wash water containing ozone resulted in ~2-2.5 log MPN/ml less of each pathogen compared to washing with water alone in this high organic burden challenge (Table 11 and 12; Fig 3 a, b, and c).

When assessing the impact of ozone on the shelf-life of iceberg lettuce, it was found that regardless of ozone presence in the wash water, only slight pinking/browning was observed at cut surfaces on leaf samples (Figure 2). In a preliminary assessment, crispness of leaves seemed impacted to a greater extent; after 3 days of storage, leaves washed in water without ozone were considerably less firm and crisp than those washed with ozonated water and were tearing easily after 5 days of storage. However, no discernable differences in color or crispness were observed on leaves within the standard storage time observed or reported by retail establishments (< 32 h).

Outcomes and Accomplishments

Within the purpose and scope of a rapid *Proof of Concept* opportunity, we feel the foundation hypothesis has been demonstrated to be supported by the data developed during this project period. Maintaining low concentrations of ozone dissolved in a closed-loop chilled re-circulating batch lettuce wash system prevents the build-up of bacteria in the water. This minimizes the potential for cross-contamination among heads of lettuce being minimally-processed very close to the timing of consumption.

The core messages we feel are supported by the outcomes of our studies include;

1. Having some validated antimicrobial added as a wash aide is both the prudent and imperative action for retail foodservice and supermarket outlets to take to protect their consumers and their brand.

2. There are multiple options available; in response to interest among retail parties in the U.S. and E.U. for data with chlorine alternatives, suitable for small-scale systems, ozone was identified as a preferential candidate.
3. In produce handling systems likely to have a low microbial burden and low "oxidizer demand", such as cored but not shredded head lettuce, ozone has been shown to have a good fit with end-user defined requirements.
4. This low burden, extended soak- contact time, and branding around "fresh in-store prep" makes ozone an excellent choice due to the absence of residues and undesirable disinfection-by-products on product.
5. The low oxidizable burden on produce such as whole lettuce, pre-washed tomatoes, pre-washed herbs and similar 'clean' wholesale product supports the potential effective performance of a compact and low ozone-output generation design for in-store uses
6. Continual ozone injection during a batch wash and soak appears to be a reliable way to achieve process control in this type of system
7. For head lettuce specifically, we observed no evidence for bacteria moving from outer leaves to inner leaves during batch soak time intervals, under study conditions.
8. As expected, greater log-reduction was observed in the 'cleanest' batch water with low COD, exceeding 5-log reduction of pathogen numbers in two minutes. Pathogen surrogates released from the surface of individual inoculated lettuce leaves accumulated in water without ozone treatment but not in water when continually injected with ozone during the soak-interval.
9. Within the experimental design, prevention of cross-contamination potential was demonstrated.
10. The accomplishments to date support an expectation that this ozone system approach is transferrable to other applications at points of retail produce handling and food preparation.

Summary of Findings and Recommendations

Benefit to industry

Though preliminary relative to a standard optimization and comparative research study, this project has developed data which better defines and determines the performance of an ozonated wash water system pilot design. The ozonated wash maintained its efficacy in suppression of microbial accumulation in the water by continual injection during washing of iceberg lettuce heads, thereby preventing cross contamination. These results serve as a foundation for future studies that enhance guidelines for food service companies to use as an additional step for improving the continuity of produce safety from farm to fork.

Background and Literature Citations

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APPENDICES

Publications and Presentations (required)

A limited verbal overview of the study outcomes was presented by T. Suslow at the 2014 Produce Safety Meeting in Yuma, AZ on 11 February, 2014. The meeting was attended by diverse growers, processers, and retailers.

Chelsea Kaminski will present this research project at the International Association of Food Protection (IAFP) in August. An abstract was submitted in January 2014.

A manuscript, based on this Final Report, is in development for submission to a peer-reviewed journal.

Budget Summary (required)

The allocated budget was fully utilized to execute this project. As is typical, it is very difficult to write a budget, within the parameters to obtain support from multiple Proof of Concept sponsors that anticipate over-expenditures required by the necessity to repeat or modify experiments based on in-study outcomes, to take advantage of 'real-time' observations and outcomes, and to respond to data development needs that emerge from communication and dialogue with project sponsor stakeholders. Therefore, for future CPS reference, it is clear that this project was somewhat under-budgeted in Time and Supplies to achieve the reported outcomes. Regardless, we are very appreciative of the timely and generous funding that was provided and the comparatively simple and streamlined process that is in place to respond to off-cycle opportunities.

The additional generosity of DEL Ozone in covering some of the supply resource replenishments to conduct extra experimental assessments within the project period is gratefully acknowledged.

Tables and Figures

Table 1: Seasonal variation in total heterotroph (TPC) and total coliform (TC) populations on unwashed iceberg lettuce. LOD: 0.65 log CFU/g. Values are means ± SD (*n = 10*).

Bacterial species	Lettuce sample	Bacterial populations on unwashed heads of iceberg lettuce (log CFU/g) — Sampling date: 11/22/13	12/6/13	12/19/13	1/6/14	1/16/14	1/28/14
TPC	Inner leaves	2.10 ± 0.56 A [a]	1.73 ± 0.57 AB	1.85 ± 1.10 AB	2.26 ± 0.61 A	2.12 ± 0.88 A	1.07 ± 0.54 B
	Outer leaves	5.01 ± 0.63 ABC	5.29 ± 0.77 AB	5.86 ± 0.38 A	5.83 ± 0.43 A	4.52 ± 0.97 BC	3.99 ± 1.18 C
TC	Inner leaves	1.88 ± 0.36 AB	1.03 ± 0.49 B	1.27 ± 0.65 AB	1.45 ± 1.00 AB	0.76 ± 0.22 B	0.79 ± 0.43 B
	Outer leaves	3.70 ± 1.42 AB	4.31 ± 0.79 A	4.26 ± 1.53 A	4.40 ± 1.24 A	2.61 ± 1.10 B	2.38 ± 1.24 B

[a] Means with different capital letters represent populations that differed significantly ($P < 0.05$) between samples in a row.

Table 2: Water samples collected during in-store lettuce preparation at a retail outlet. Values are means ± SD (*n = 4*).

Retail Outlet Lettuce Wash Water Survey	
Sample	**Mean ± SD**
TPC (log CFU/100 ml)	6.33 ± 0.75
Coliforms (log MPN/100 ml)	4.42 ± 0.61
Temperature (˚C)	9.9 ± 7.7
pH	7.33 ± 0.16
ORP (mV)	77 ± 21
COD (mg O_2/L)	78 ± 41
EC (mS)	0.57 ± 0.28
Turbidity (NTU)	3 ± 3

[a] Abbreviations as follows: Total heterotrophic bacteria (TPC), total coliforms (TC), oxidation-reduction potential (ORP), chemical oxygen demand (COD), electro-conductivity (EC)

Table 3: Population reductions of *Salmonella* Typhimurium in water samples with recirculating ozone with or without oxidizer bio-burden.

Salmonella Typhimurium log reductions in wash water containing ozone (0.1 to 10 ml)			
	Wash water		
Time (min)	No lettuce	Shredded lettuce	Filtered Lettuce Juice
1	8.13	0.36	0.12
2	8.65	6.78	1.14
3	NR	7.16	1.45
4	NR	8.01	2.24
5	NR	7.71	0.41
10	NR	8.01	0.91
15	NR	8.36	1.56
20	NR	8.36	2.70
25	NR	8.31	4.11
30	NR	8.36	5.36

Ozone concentration with no added lettuce tissue was approximately 0.6 to 1.0 mg/L
NR – none recoverable

Table 4: Wash water parameters obtained during recirculating ozone generation in water with or without different varieties of organic materials.

Wash water parameters during processing with varying organic materials								
Parameter	Wash water	Sampling time (min)						
		0	5	10	15	20	25	30
Temperature (˚C)	No lettuce	6.4	6.2	6.8	7.4	7.9	8.6	9.1
	Shredded lettuce	4.1	4.8	5.6	6.4	7.1	7.8	8.6
	Lettuce liquid	6.6	4.8	5.6	6.1	6.9	7.5	8.1
pH	No lettuce	8.54	8.34	8.15	8.07	8.05	7.97	7.95
	Shredded lettuce	9.4	7.93	7.67	7.59	7.53	7.54	7.61
	Lettuce liquid	7.81	7.88	7.84	7.85	7.83	7.83	7.96
ORP (mV)	No lettuce	410	851	977	996	1003	1006	1007
	Shredded lettuce	432	684	678	693	697	674	628
	Lettuce liquid	218	260	297	303	292	260	270
COD (mg O_2/L)	No lettuce	16	6	4	9	4	16	5
	Shredded lettuce	17	25	25	25	38	29	33
	Lettuce liquid	308	277	313	268	262	289	256
EC (mS)	No lettuce	0.4	0.37	0.37	0.38	0.38	0.39	0.37
	Shredded lettuce	0.35	0.37	0.38	0.39	0.39	0.39	0.4
	Lettuce liquid	0.46	0.42	0.41	0.42	0.43	0.42	0.42
[Ozone] (mg O_3/L)	No lettuce	na [a]	0.6	1.07	0.87	0.63	1.06	1.52
	Shredded lettuce	na	0.41	0.66	0.47	0.53	0.4	0.58
	Lettuce liquid	na	0.15	0.06	0.03	0.06	0.11	0.55

[a] na: value not applicable

Table 5: Total heterotroph (TPC) and total coliform (TC) populations from water samples obtained during pilot-scale processing of uninoculated lettuce with and without recirculating ozone. LOD: 0.48 log CFU/100 ml, -0.05 log MPN/100 ml. Values are means ± SD (*n = 3*).

Bacterial populations in wash water											
Bacterial species	Wash water	Sampling time (min)									
		0		10		20		30		40	
TPC (log CFU/100 ml)	Water	2.88 ± 0.88	A [a]	2.62 ± 0.58	A	3.52 ± 1.17	A	4.53 ± 0.78	A	5.78 ± 0.97	A
	Ozone	2.72 ± 0.72	A	2.22 ± 0.42	A	2.57 ± 0.60	A	2.20 ± 0.43	B	1.97 ± 0.03	B
TC (log MPN/100 ml)	Water	1.31 ± 2.31	A	1.02 ± 1.56	A	2.23 ± 0.79	A	2.88 ± 0.85	A	2.92 ± 0.72	A
	Ozone	-0.05 ± 0.00	A	0.07 ± 0.20	A	0.61 ± 1.13	A	0.58 ± 0.55	B	0.15 ± 0.30	B

[a] Means with different letters represent populations that differed in significance (*P* < 0.05) within a column in terms of wash water.

Table 6: Total heterotroph (TPC) and total coliform (TC) populations from lettuce samples obtained during pilot-scale processing of uninoculated iceberg lettuce with and without recirculating ozone. LOD: 0.65 log CFU/g. Values are means ± SD (*n = 3*).

Bacterial populations on lettuce leaves (log CFU/g)												
Bacterial species	Lettuce sample	Wash water	Washing time (min)									
			0		20		26		32		38	
TPC	Outer leaves	Water	5.05 ± 0.43	A [a]	4.99 ± 0.47	A	3.94 ± 1.05	A	4.56 ± 1.41	A	4.08 ± 1.03	A
		Ozone	5.58 ± 0.11	A	3.98 ± 0.94	A	4.59 ± 1.03	A	4.31 ± 0.60	A	4.64 ± 0.19	A
	Inner leaves	Water	3.16 ± 1.02	A	2.95 ± 1.12	A	3.30 ± 0.51	A	3.11 ± 0.20	A	3.87 ± 1.02	A
		Ozone	3.64 ± 0.66	A	2.12 ± 0.50	A	2.74 ± 0.33	A	2.56 ± 0.30	A	2.72 ± 0.61	A
TC	Outer leaves	Water	3.43 ± 0.90	A	2.77 ± 0.95	A	2.61 ± 1.82	A	2.99 ± 1.43	A	3.19 ± 0.65	A
		Ozone	4.95 ± 0.51	A	1.93 ± 1.78	A	3.37 ± 2.37	A	3.76 ± 0.49	A	2.98 ± 2.06	A
	Inner leaves	Water	1.53 ± 0.13	B	1.25 ± 0.54	A	2.03 ± 0.66	A	1.67 ± 0.20	A	1.30 ± 0.56	A
		Ozone	3.16 ± 0.95	A	1.33 ± 0.68	A	1.79 ± 0.98	A	1.41 ± 0.66	A	2.04 ± 1.20	A

[a] Means with different letters represent populations that differed in significance (*P* < 0.05) within a column in terms of wash water.

Table 7: *E.* coli O157:H7, *Salmonella* Typhimurium, and *L. innocua* populations from the outermost lettuce leaves obtained during pilot-scale processing with and without ozone. LOD: 0.65 log CFU/g. Values are means ± SD (*n = 3*).

Bacterial populations on outer lettuce leaves (log CFU/g)											
Bacterial species	**Wash water**	**Washing time (min)**									
		0		**20**		**26**		**32**		**38**	
***E. coli* O157:H7**	**Water**	**4.42 ± 0.19**	**A** [a]	**3.09 ± 0.29**	**A**	**3.15 ± 0.57**	**A**	**3.52 ± 0.21**	**A**	**3.49 ± 0.34**	**A**
	Ozone	**4.41 ± 0.25**	**A**	**2.77 ± 0.24**	**A**	**2.62 ± 1.14**	**A**	**3.44 ± 0.31**	**A**	**2.80 ± 0.58**	**A**
***Salmonella* Typhimurium**	**Water**	**4.55 ± 0.11**	**A**	**3.08 ± 0.31**	**A**	**3.15 ± 0.62**	**A**	**3.33 ± 0.22**	**A**	**3.38 ± 0.25**	**A**
	Ozone	**4.31 ± 0.24**	**A**	**3.01 ± 0.68**	**A**	**2.34 ± 1.17**	**A**	**3.35 ± 0.09**	**A**	**2.72 ± 0.46**	**A**
L. innocua	**Water**	**4.65 ± 0.02**	**A**	**3.07 ± 0.55**	**A**	**3.33 ± 0.67**	**A**	**3.63 ± 0.19**	**A**	**3.64 ± 0.27**	**A**
	Ozone	**4.57 ± 0.10**	**A**	**2.87 ± 0.30**	**A**	**2.56 ± 1.20**	**A**	**3.68 ± 0.16**	**A**	**2.97 ± 0.60**	**A**

[a] Means with different letters represent populations that differed in significance ($P < 0.05$) within a column in terms of wash water.

Table 8: Bacterial populations from water samples obtained during pilot-scale processing of lettuce inoculated with *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. innocua* with and without recirculating ozone. LOD: -1.52 log MPN/ml. Values are means ± SD (*n = 3*).

Bacterial populations in wash water (log MPN/ml) *(% Positive Enrichments)*											
Bacterial species	**Wash water**	**Sampling time (min)**									
		0		**10**		**20**		**30**		**40**	
E. coli O157:H7	Water	-0.55 ± 0.86	*(66.7)* A [a]	-1.49 ± 0.03	*(33.3)* A	-1.51 ± 0.03	*(66.7)* A	-0.76 ± 0.65	*(66.7)* A	-0.79 ± 0.86	*(33.3)* A
	Ozone	-1.52 ± 0.00	*(0)* A	-1.52 ± 0.00	*(0)* A	-1.08 ± 0.76	*(33.3)* A	-1.52 ± 0.00	*(0)* A	-1.52 ± 0.00	*(33.3)* A
Salmonella Typhimurium	Water	-0.80 ± 0.96	*(66.7)* A	-1.35 ± 0.30	*(33.3)* A	-1.21 ± 0.55	*(33.3)* A	-0.82 ± 0.68	*(66.7)* A	-1.31 ± 0.37	*(0)* A
	Ozone	-1.51 ± 0.03	*(33.3)* A	-1.52 ± 0.00	*(0)* A	-1.27 ± 0.43	*(33.3)* A	-1.52 ± 0.00	*(33.3)* A	-1.52 ± 0.00	*(0)* A
L. innocua	Water	-0.36 ± 0.92	*(33.3)* A	-1.35 ± 0.30	*(33.3)* A	-1.52 ± 0.00	*(0)* A	-0.47 ± 0.64	*(33.3)* A	-0.61 ± 0.91	*(33.3)* A
	Ozone	-1.51 ± 0.03	*(0)* A	-1.52 ± 0.00	*(0)* A	-1.03 ± 0.85	*(33.3)* A	-1.52 ± 0.00	*(0)* B	-1.52 ± 0.00	*(0)* A

[a]. Means with different letters represent populations that differed in significance ($P < 0.05$) within a column in terms of wash water

Table 9: Wash water parameters obtained during pilot-scale processing of uninoculated iceberg lettuce with or without recirculating ozone. Values are means ± SD (*n = 3*).

Parameter	Wash water	Sampling time (min) 0		10		20		30		40	
Temperature (˚C)	Water	13.4 ± 7.6	A [a]	13.1 ± 5.2	A	11.8 ± 1.7	A	10.4 ± 1.8	A	9.9 ± 2.3	A
	Ozone	15.7 ± 1.7	A	11.6 ± 1.3	A	10.6 ± 0.6	A	10.4 ± 0.8	A	10.9 ± 0.6	A
pH	Water	8.50 ± 0.53	A	8.19 ± 0.36	A	8.24 ± 0.26	A	8.33 ± 0.11	A	8.18 ± 0.03	A
	Ozone	7.91 ± 0.11	A	8.27 ± 0.31	A	8.37 ± 0.36	A	8.42 ± 0.21	A	8.46 ± 0.27	A
ORP (mV)	Water	428 ± 118	A	393 ± 182	B	407 ± 176	B	381 ± 118	B	219 ± 186	B
	Ozone	276 ± 115	A	706 ± 62	A	784 ± 98	A	697 ± 130	A	571 ± 28	A
COD (mg O_2/L)	Water	15 ± 8	A	14 ± 9	A	40 ± 21	A	20 ± 8	A	26 ± 6	A
	Ozone	17 ± 18	A	18 ± 6	A	28 ± 5	A	18 ± 4	A	25 ± 6	A
EC (mS)	Water	0.48 ± 0.06	A	0.48 ± 0.03	A	0.45 ± 0.02	A	0.46 ± 0.02	A	0.46 ± 0.02	A
	Ozone	0.50 ± 0.03	A	0.47 ± 0.03	A	0.46 ± 0.01	A	0.45 ± 0.01	A	0.46 ± 0.01	A
[Ozone] (mg O_3/L)	Water	na [b]		na		na		na		na	
	Ozone	na		0.36 ± 0.28		0.43 ± 0.16		0.14 ± 0.10		0.07 ± 0.08	

[a] Means with different letters represent values that differed in significance ($P < 0.05$) within a column in terms of wash water.
[b] na: value not applicable

Table 10: Wash water parameters obtained during pilot-scale processing of inoculated iceberg lettuce with or without recirculating ozone. Values are means ± SD (*n = 3*).

Wash water parameters during pilot-scale processing											
Parameter	**Wash water**	**Sampling time (min)**									
		0		**10**		**20**		**30**		**40**	
Temperature (˚C)	Water	16.9 ± 1.2	A [a]	13.4 ± 0.9	A	9.8 ± 1.8	A	9.0 ± 0.2	B	8.6 ± 0.1	B
	Ozone	14.2 ± 4.2	A	10.0 ± 0.4	A	10.0 ± 0.4	A	9.7 ± 0.1	A	10.2 ± 0.2	A
pH	Water	8.07 ± 0.63	A	7.99 ± 0.18	A	8.01 ± 0.05	A	7.96 ± 0.23	B	7.80 ± 0.32	B
	Ozone	8.13 ± 0.77	A	8.51 ± 0.49	A	8.46 ± 0.40	A	8.47 ± 0.17	A	8.60 ± 0.13	A
ORP (mV)	Water	424 ± 106	A	591 ± 21	B	573 ± 72	B	548 ± 25	B	493 ± 50	B
	Ozone	559 ± 47	A	742 ± 28	A	951 ± 44	A	952 ± 26	A	947 ± 29	A
COD (mg O_2/L)	Water	22 ± 13	A	28 ± 4	A	24 ± 6	A	16 ± 2	A	13 ± 7	A
	Ozone	18 ± 11	A	26 ± 18	A	28 ± 17	A	17 ± 7	A	17 ± 12	A
EC (mS)	Water	0.54 ± 0.03	A	0.49 ± 0.03	A	0.47 ± 0.01	A	0.47 ± 0.01	A	0.46 ± 0.00	A
	Ozone	0.52 ± 0.04	A	0.46 ± 0.00	A	0.46 ± 0.01	A	0.45 ± 0.01	A	0.46 ± 0.01	A
[Ozone] (mg O_3/L)	Water	na [b]		na		na		na		na	
	Ozone	na		0.38 ± 0.20		0.64 ± 0.22		0.47 ± 0.15		0.45 ± 0.34	

[a] Means with different letters represent values that differed in significance ($P < 0.05$) within a column in terms of wash water.
[b] na: value not applicable

Table 11. Comparison of untreated and ozone-treated water in survival of bacteria inoculated onto lettuce leaves and released to the soak basin adjusted to a COD of ~60 mg O2/ml

Water Samples		
***E. coli* (PTVS 155) (log MPN/ml)**		
	Water	**Water + Ozone**
0	**1.83**	**1.83**
10	**2.01**	**1.50**
20	**2.03**	**0.92**
30	**2.06**	**0.23**
40	**1.97**	**-0.70**
***Salmonella* (x3985) (log MPN/ml)**		
	Water	**Water + Ozone**
0	**1.81**	**2.03**
10	**1.90**	**1.84**
20	**2.01**	**1.75**
30	**1.99**	**0.88**
40	**1.95**	**0.07**
***Listeria innocua* (TVS 451) (log MPN/ml)**		
	Water	**Water + Ozone**
0	**1.82**	**1.91**
10	**1.75**	**1.59**
20	**1.86**	**1.24**
30	**1.83**	**0.44**
40	**1.87**	**-0.40**

Table 12. Wash water parameters associated with experimental conditions of ‘high organic-burden’ water used in cross-contamination tests

Parameters					
Water					
	t = 0	t = 10	t = 20	t = 30	t = 40
Temp	13.60	13.30	11.6	10.1	9.6
pH	7.77	8.30	8.36	8.45	8.37
ORP	305.00	99.00	139	240	259
COD	60.00	59.00	90	130	70
EC	0.51	0.50	0.49	0.47	0.46
Turbity	0.00	3.00	5	3	4
Ozone	---	---	---	---	---

Water + Ozone

	t = 0	t = 10	t = 20	t = 30	t = 40
Temp	8.60	11.10	11.6	11.7	12.6
pH	7.04	8.07	8.3	8.54	8.87
ORP	80.00	281.00	288	248	297
COD	49.00	118.00	56	69	101
EC	0.43	0.47	0.46	0.48	0.47
Turbidity	3.00	1.00	0	0	0
Ozone	---	0.44	0.17	0.07	0.47

FIGURES

Figure 1 : A) Total heterotroph (TPC) and B) Total coliform (TC) populations from water samples obtained during pilot-scale processing of uninoculated lettuce with (■) and without (◆) recirculating ozone. Values are means ± SD (*n = 3*).

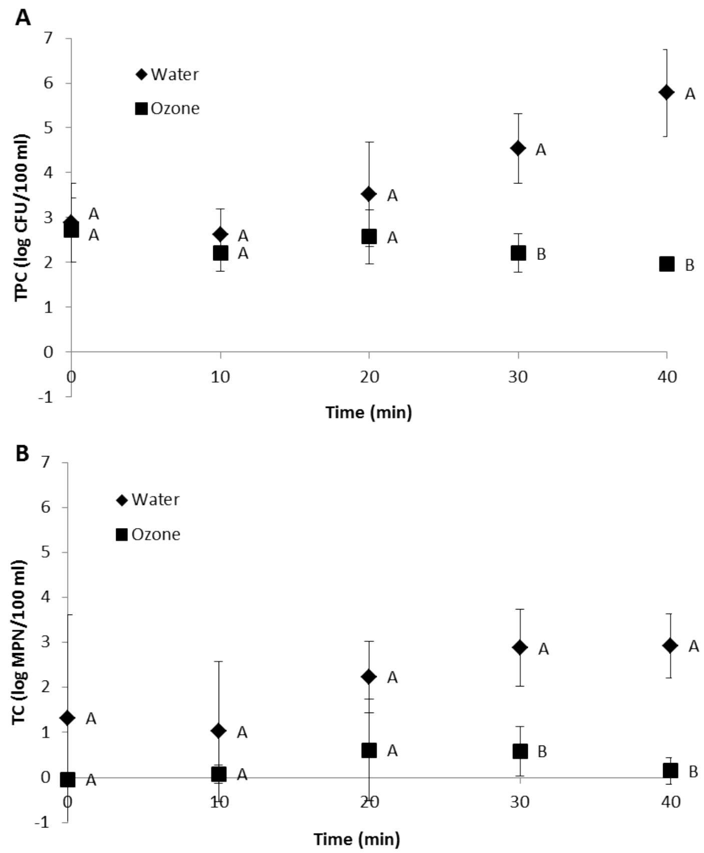


Figure 2: Visual shelf-life of iceberg lettuce at 5°C washed with or without ozone.

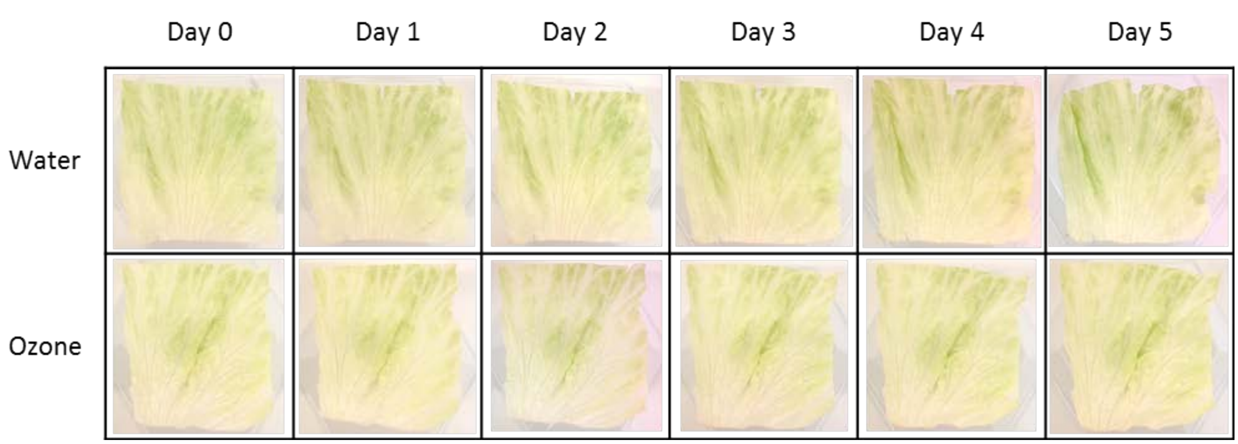
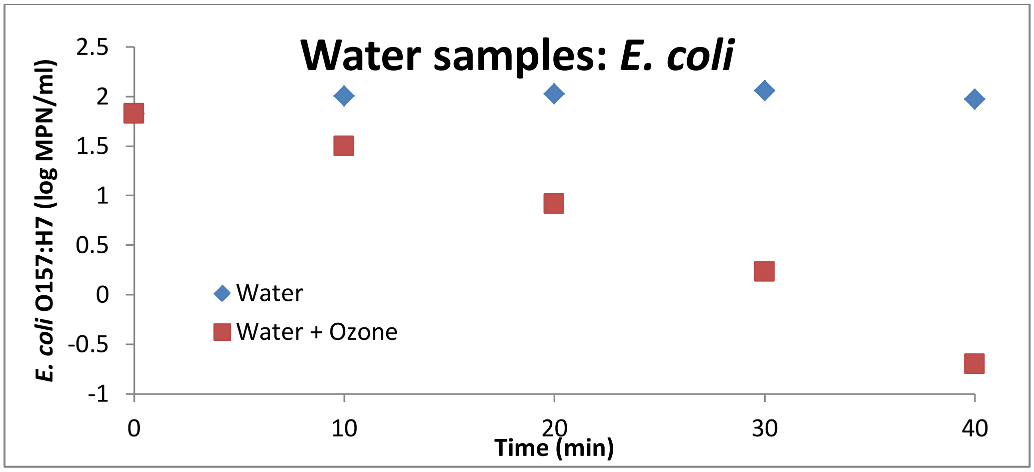
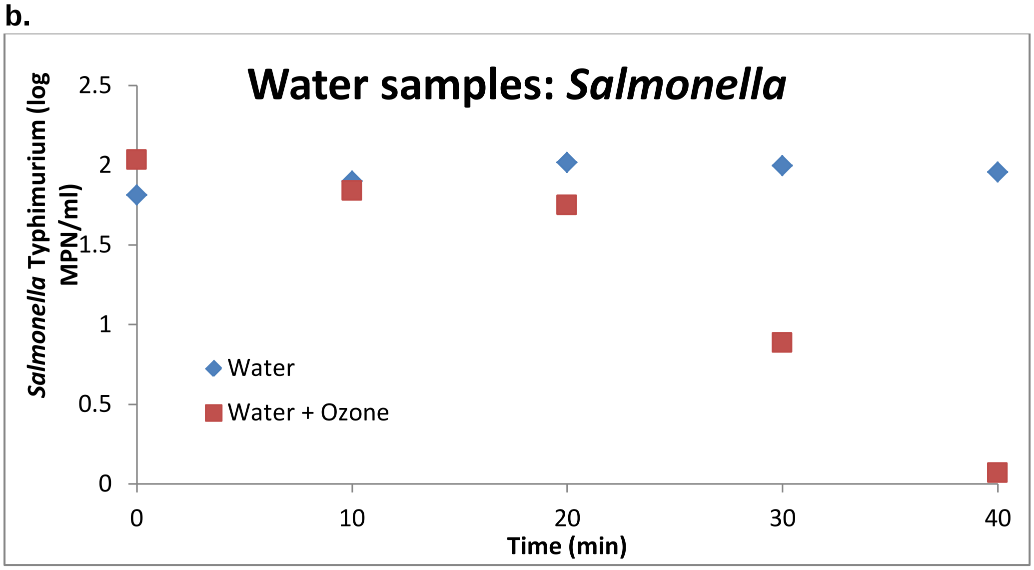


Fig. 3 – Graphic representation of data presented in Table 11 - Comparison of untreated and ozone-treated water in survival of bacteria inoculated onto lettuce leaves and released to the soak basin adjusted to a COD of ~60 mg O2/ml. Ozone injection was not initiated until after all inoculated lettuce was introduced to the batch system.

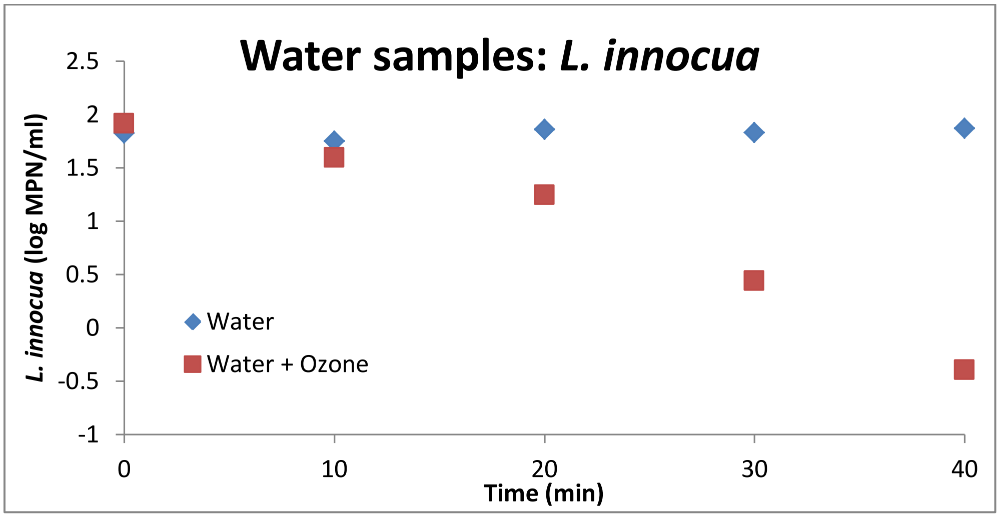
a.



b.



c.


Water samples: L. innocua
L. innocua (log MPN/ml)
2.5
2
1.5
1
0.5
0
-0.5
-1
Water
Water + Ozone
0
10
20
30
40
Time (min)