CENTER for PRODUCE SAFETY

CPS 2014 RFP
FINAL PROJECT REPORT

Project Title
Validation of chlorine level in sanitization system to avoid cross-contamination

Project Period
January 1, 2015 – December 31, 2016

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Objectives
1. *Develop a novel contact time x chlorine dose-sensitive method and response curves to determine the conditions needed to prevent cross-contamination by planktonic and biofilm E. coli O157:H7 and Salmonella in the presence of organic matter loads during fresh-cut wash water operations.*

2. *Identify salient operational variables for measuring the performance of hypochlorous acid wash water sanitizer for fresh-cut operations.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

FINAL REPORT

Abstract
Determination of the minimum free chlorine (FC) concentration needed to prevent pathogen survival/cross-contamination during produce washing is essential for development of science-based food safety regulations and practices. Although the trend of chlorine concentration–contact time on pathogen inactivation is generally understood, specific information on chlorine and the kinetics of pathogen inactivation at less than 1.00 second is urgently needed by the produce processing industry. In addition, in the presence of organic load arising from the fresh produce, such an investigation becomes more challenging due to rapid FC depletion, which results in an ever-changing FC concentration in the produce washing systems. To address the first concern, a novel microfluidic mixer with adjustable mixing time was designed and employed to evaluate the sanitization efficacy of chlorine in sub-second periods. Test results indicated that a 5-$\log_{10}$ reduction of *E. coli* O157:H7 required exposing *E. coli* O157:H7 cells to a solution containing 1.0 mg/L FC for at least 1.00 s, or a solution containing 10.0 mg/L FC for 0.25 s. To overcome the second challenge, a sustained chlorine decay approach was employed to study the stabilized FC, chloramine, and chemical oxygen demand (COD) at desired levels. Our study showed a clear decrease in sanitization efficacy as the organic load increased. At 5 s residence time and pH 6.5, a minimum of 0.5 and 7.5 mg/L FC was needed to achieve a 5-log reduction at 0 and 900 mg/L COD, respectively. The decrease in sanitization efficacy was more pronounced at lower FC, higher COD, higher pH, and shorter residence time values. The organic load–associated interference with FC measurement and disruption of chlorine and bacteria interactions were identified as possible reasons for the inadequate sanitization. This project demonstrated feasible methods for studying the sanitization efficacy under industrially relevant conditions and provided valuable recommendations on the minimal FC levels required to mitigate pathogenic cross-contamination.

Background
The presence of sufficient sanitizer concentration in wash water for fresh and fresh-cut produce is critical for preventing pathogen survival in the wash water and its consequential transfer to clean produce washed in the same tank and/or flume of water. However, maintaining high levels of sanitizers in the wash water is a practical challenge to the produce industry due to rapid reactions of organic matter with sanitizers, especially for widely used hypochlorous acid (chlorine). Therefore, it is critical to establish a minimum chlorine concentration that is effective for preventing pathogen cross-contamination, and also is achievable by the industry. While a plethora of information is available regarding chlorine concentration on pathogen inactivation, information regarding the minimum chlorine concentration for preventing pathogen cross-contamination is scarce. One reason for such scarcity may be the technical difficulties associated with conducting such studies, i.e., controlling and manipulating liquids (e.g., sanitizers, bacterial inoculum, and stopping solutions) in short time frames (about or below 1 second, which is considered relevant to the occurrence of cross-contamination) without tedious work. This necessitates a convenient and accurate mechanism to control the reaction time and chlorine concentration needed for pathogen inactivation.

It has been well established that the sanitizing efficacy is constantly compromised by the organic load, which has been attributed to rapid chlorine depletion and unsuccessful maintenance of residual FC (Fukumoto et al., 2002; Luo et al., 2011; Yang et al., 2012). However, no study has been reported to date on the direct comparison between the sanitizing power of two chlorinated water samples, one with and the other without organic load, both at the same FC level. In other words, it remains unknown

whether 1 mg/L of FC is capable of achieving equally sufficient log reduction with or without the presence of organic load. With proper experimental design, e.g., a comparison at a same, stable FC content and varying COD level, one can separate the impact of individual parameters without the interference from other factors. This provides valuable insights into the possible mechanisms underlying the current observations, i.e., the influence of different parameters on the interaction among chlorine, organic load, and the bacteria, and the effect of those interactions on the sanitizing process. Ultimately, this would help identifying the key compound or interaction that compromises the sanitizing efficacy significantly, which benefits the technological advancement in preventing water-mediated cross-contamination. However, due to the lack of proper methodology to maintain stable washing conditions, especially at high organic load levels, there remains a considerable knowledge gap with this respect.

Research Methods and Results

Dose-dependent inactivation of pathogen by chlorine in sub-second level periods without organic load
To address the need of investigating sub-second period bacterial inactivation, we developed a novel microfluidic device with adjustable contact time. The microfluidic mixer with "Y" inlet junction and chaotic mixer designs, as shown in **Figure 1**, was fabricated in three steps as follows: design printing, mold fabrication (Friend & Yeo, 2010), and device assembly (Comina et al., 2014). The as-prepared microfluidic chip was then validated for its mixing capacities. The mixing efficiency and pattern were evaluated by modeling scalar mixing using the Computational Fluid Dynamics software (Autodesk Simulation CFD Inc., v. 2014). The chlorine diffusion coefficient in water used in modeling was 1.38×10^{-5} cm^2/s. The incoming fluids were assumed as laminar flow (evidenced by low Reynolds number), incompressible, and non-reactive. The modeled result indicated that chlorine homogenously diffuses inside the chaotic mixer and mixes with the bacterial solution to achieve the contact time. Additionally, an inversely proportional relation between the mean contact time and flow rate was observed, suggesting the feasibility of precise control over contact time by flow rate adjustment. It was noteworthy that the contact time of different fluid elements in the mixer follows a nearly normal distribution, so that not all the fluid elements were mixed for equal amount of time. Finally, both chlorine concentration (tested at 5.0 mg/L) and bacterial suspension (tested at 10^7 MPN/mL) were stable for the entire 20 min test period during continuous pumping at a flow rate of 0.16 mL/min, or the equivalent of a 0.75 s contact time. The deviation of chlorine concentration was no more than 3%. *E. coli* O157:H7 concentrations varied by less than 2.5% from the respective expected values.

Using this well-characterized microfluidic device, the research team tested the survival of pathogens at different contact times and FC contents. Three strains of *E. coli* labeled genetically with two biochemical labels, i.e., green fluorescence protein (GFP) and nalidixic acid (NA), were mixed to make a cocktail containing 10^7 CFU/ml. The bacterial survival tests were performed by pumping chlorine, bacteria, and neutralizing solution (sodium thiosulfate) through the respective inlets and collecting the solution from the outlet. The surviving pathogen was enumerated by modified most probable number (MPN) procedures.

Two major conclusions were drawn from the microfluidic assay. First, as shown in **Table 1**, pathogen inactivation was significantly affected by FC concentration ($P < 0.0001$), sub-second reaction time ($P < 0.0001$), and their interactions ($P < 0.0001$). Second, the current industry practice of using 1.0 mg/L FC will require more than 1.00 s total contact to achieve a 5-$\log_{10}$ reduction of an *E. coli* O157:H7 population, whereas a 10.0 mg/L FC solution will achieve a 5-$\log_{10}$ reduction within as little as 0.25 s.

At FC concentrations higher than 10 mg/L and contact times longer than 0.25 s, a ≥7-$\log_{10}$ reduction of was observed for *E. coli* under experimental conditions. Moreover, the pathogen inactivation kinetics were fitted to three mathematical models. Both the Watson (P = 0.860) and Hom (P = 0.841) models reflected the pathogen inactivation scenarios with short contact times, and the computed parameters are globally fitted to contact times between 0.10 and 1.00 s. These findings provided critical information regarding the determination of minimum FC concentration required to prevent pathogen survival and cross-contamination during fresh produce wash operations.

Dose-dependent inactivation of pathogen by chlorine as a function of pH and organic load
Romaine lettuce extracts were prepared and diluted to designated COD levels (225, 450, and 900 mg/L). The diluted lettuce extract was then mixed with different chlorine solutions whose FC content ranged from 50 to 160 mg/L. The pH of the mixture was monitored and maintained at the desired values in the first 10 min of the experiment by using 1 M NaOH, and it remained stable throughout the whole incubation process afterwards (preliminary data). Samples of 2 mL were drawn periodically from the mixture and analyzed for the free and total chlorine content. As shown in **Figure 2**, a similar trend of FC level was observed under all tested conditions. To begin with, over 60% and 80% of the FC was depleted within the first 5 and 20 min of incubation, respectively. This was followed by a slow, continuous decrease in the FC level; more specifically, the FC level reached 10 mg/L after approximately 90 min of incubation and further decreased to about 1 mg/L at 4 h. Accompanying the depletion of FC was the accumulation of chloramine, the major disinfection byproduct identified by far, which accumulated rapidly in the first 20 min of experiment (**Fig. 2**, Chloramine). After reaching a maximum, the chloramine level remained virtually unchanged over a relatively long period, ranging from 30 min to 2 h, depending on the experimental condition, and it decreased by up to 26% afterwards. In the meantime, the total chlorine (TC) level underwent a decrease of over 50% in the first 5 min of incubation. Since TC is defined by the sum of FC and chloramines, the rapid decrease in TC level suggested the extensive conversion of chlorine to byproducts other than chloramines. Lastly, a separate set of experiments was carried out to compare the chlorine depletion in the presence of filtered and non-filtered lettuce extract. Non-filtered lettuce extract exhibited a COD of 43,000 mg/L according to our preliminary results, which was approximately one third higher than that of the filtered one. However, when the unfiltered lettuce extract was diluted to 900 mg/L of COD, it depleted FC at a slower rate compared to a filtered lettuce extract diluted to the same COD level (**Fig. 2**, Free chlorine). This result suggested that the water-insoluble fraction existing in the unfiltered lettuce extract did not contribute to a significant amount of chlorine demand.

We further explored the impact of pH on the sanitizing process. **Figure 3** (Free chlorine) presents the chlorine depletion profile at three pH levels (6.5, 5.0, and 3.0), one FC_0 level (150 mg/L), and two COD levels (600 and 900 mg/L). These values are representative of the typical conditions that we experienced in our field study. Irrespective of the experimental condition, all samples exhibited a biphasic FC depletion profile. However, with the same level of COD, a significantly higher rate of FC depletion was observed at lower pH. For example, the FC content decreased from 150 to 4.42 and 12.3 mg/L at pH 3.0 and 5.0, respectively, after 1 h of incubation. This result was in sharp contrast to the observation at pH 6.5, where the FC level remained at 22.3 mg/L after 1 h. From a practical point of view, our results suggested that a considerably greater amount of hypochlorous acid needs to be added into the wash system at lower pH to compensate its consumption, which may produce larger quantity of unwanted byproducts. Similar to the FC content, the chloramine level increased at significantly different rates at different pH levels (**Fig. 3**, Chloramine). At 900 mg/L COD, maximum chloramine levels of 47.5, 38.7,

and 32.7 mg/L were observed at pH 3.0, 5.0, and 6.5, respectively. Even when the COD was lowered to 600 mg/L, the maximal chloramine content was as high as 44.6 mg/L at pH 3.0. Moreover, the time needed for reaching the chloramine level maximum was 5, 10, and 20 min at pH 3.0, 5.0, and 6.5, respectively.

The sanitizing experiment was initiated by introducing 0.1 mL of *E. coli* inoculum (10^8 CFU/mL in 100 mM phosphate buffer without chloride, pH 6.5) to the above mentioned mixtures (1.9 mL). After 5 or 20 s of incubation under constant stirring, sodium thiosulfate ($Na_2S_2O_3$, 1 mL, 0.75 mg/mL) was added to quench the chlorine, and the resulting dispersion was subjected for enumeration by both standard plate counting and most probable number (MPN) methods. For comparison purpose, another group of experiments was conducted following the same procedure as described above, except that the lettuce extract–containing mixtures were replaced with pure NaClO solutions with various FC levels (0.5, 2.5, 5, 7.5, and 10 mg/L). A control was performed by substituting the lettuce extract–containing mixtures with pure water. The sanitizing power expressed in log reduction was calculated by comparing the MPN readings for the treatments with the control. As shown in **Figure 4**, the sanitizing efficacy was significantly reduced in the presence of organic load. Such an adverse effect of organic load was more pronounced at high COD levels, low FC contents, or shorter residence time. These results were a continuum of the preliminary data presented in the last reporting period. Meanwhile, as shown in **Figure 5**, pH also exerted a considerable effect on the sanitizing efficacy. For example, at pH 3.0, 5 s of residence time, and 900 mg/L COD (44.3 ± 1.1 mg/L chloramine), the log reduction achieved by 10, 7.5, 5, 2.5, and 1.25 mg/L FC increased by 0.15, 1.27, 2.29, 2.02, and 1.92 units over that obtained at pH 6.5 and the same COD level. Nevertheless, those numbers remained considerably lower than those achieved without any organic load. A similar trend in the sanitization efficacy was also observed at pH 5.0 and 900 mg/L of COD (29.5 ± 0.6 mg/L chloramine), except that no significant improvement was evidenced at 1.25 mg/L FC. Regardless of the pH and/or FC level, the sanitization efficacy was improved as the residence time was extended to 20 s.

Table 2 summarizes the minimal FC content required to achieve at least a 5-log reduction in *E. coli* O157:H7 under different experimental conditions. With no organic load present, free chlorine at a level as low as 0.5 mg/L was able to achieve sufficient sanitization within 5 s. As the level of organic load increased, the FC level required increased gradually, reaching a maximum of 7.5 (5 s) and 5 mg/L (20 s) at pH 6.5 and 900 mg/L COD. Lowering the pH to 5.0 or 3.0 resulted in a significant decrease in the FC level required, suggesting the potential benefit of maintaining lower pH during the washing process. However, the potential quality deterioration of fresh produce at low pH should be considered when this strategy is applied in practice. Our study underscored the potential risk of insufficient bacterial inactivation in the presence of organic load at FC levels previously demonstrated effective for drinking water disinfection procedures (e.g., 0.5 mg/L), and it necessitates the proper validation of sanitization procedures based on produce type, processing facilities, and operational parameters.

A follow-up study was performed to identify the chief factors that may lead to lower sanitizing efficacy in the presence of organic substances. To begin with, since inorganic chloramines are known for their interference during FC measurement by the conventional DPD method (i.e., using N,N diethyl-1,4 phenylenediamine sulfate), we hypothesized that organic chloramine formed by organic nitrogen-containing compounds (e.g., proteins and nucleic acids) may also result in biased readings in the FC level (Kouame & Haas, 1991). To test this hypothesis, we compared the FC levels measured by the DPD method and a novel indophenol method. The latter method demonstrates extraordinary capability in

distinguishing FC and chloramines (Hach Company, 2014). As shown in **Table 3**, the overestimation of FC by the DPD method compared with the indophenol method escalated when the measured FC level decreased, and the difference reached up to 60% when the reading by the DPD method was 0.54 mg/L. It should be noted that the DPD method is applied widely as a convenient calibrating method for chlorine measurement by the fresh produce processing industry. Therefore, the interference from chloramine on the determination of FC levels should not be overlooked, especially at low FC levels. It is advised that both FC and chloramine levels are recorded by the industry during produce washing, in order to better estimate the killing power and avoid insufficient sanitization.

We further studied the chlorine demand of different types of compounds in the lettuce extract (as per Weng et al., 2016). Model compounds, including glucose, sodium caseinate and gallic acid, were incubated with a NaClO solution (pH 6.5, 200 mg/L FC) at room temperature. Additional aliquots (200 mg/L) of NaClO solution were introduced when the FC level decreased below 10 mg/L. The time-dependent chlorine demand of these mixtures was recorded. As shown in **Figure 6**, glucose exhibited minimal chlorine demand within up to 3 h of incubation, whereas gallic acid depleted the highest amount of chlorine within 5 min. Sodium caseinate was able to react with chlorine at a moderate but steady rate, showing an increasing chlorine demand during 3 h of incubation. In a follow-up study, glucose (added at 1 mg/mL to a pure chlorine solution) did not reduce the sanitization efficacy of hypochlorous acid (1 mg/L FC). This result was consistent with the low chlorine demand of glucose, and it also indicated that the existence of small molecules, such as glucose, did not hinder the reaction between chlorine and bacteria.

Comparison between FC measurement methods

We compared the measurement of FC by three commercially available assays: indophenol method using iodometric titration for FC according to AWWA standard methods (iodometric method 4500-Cl B; Eaton et al., 2005); standard DPD method with a lab-prepared formula according to Eaton (4500-Cl G; Eaton et al., 2005); and an alternative assay using DPD powder (with a commercial PPD-2 DPD powder pop dispenser for chlorine testing; HF Scientific). As shown in **Figure 7**, a positive bias of the measured FC with both DPD methods was observed even at very low residual FC. This result was further explored by measuring the FC until no further changes in FC measurements occurred, which was after 250 min; the experiment was repeated three times. After 250 min, the indophenol method showed a residual of 0.07 ± 0.03 mg/L (so basically there was no residual left). However, for both the DPD methods, a significant residual FC was still observed.

Outcomes and Accomplishments

This project provided scientific data to determine effective minimum chlorine concentrations needed to avoid microbial contamination from cross-contamination in produce wash water. With the aid of the novel microfluidic mixing device, we clearly determined the conditions needed (with precision at sub-second levels) to prevent cross-contamination by planktonic *E. coli* O157:H7 without the presence of organic load during fresh-cut wash water operations. The microfluidic flow device were tested primarily with chlorine, but the device and methodologies developed are applicable to all other sanitizers, such as ozone and peroxyacetic acid, used for produce wash water. The sustained chlorine decay study employed in achievement of Objective 2 provided a feasible, convenient, and robust method to test the sanitization efficacy of chlorine under well-maintained conditions, which are relevant to the fresh produce washing industry. Our study underscored the risk of insufficient inactivation of bacteria in the presence of high organic load, even at FC levels that had been considered adequate in drinking water

with low organic load. It also emphasized on the importance of accurate measurement of FC levels, in that conventional DPD measurement is subject to positive interference by organic chloramines, which are much less active than FC. The results from this project have been documented in two scientific manuscripts, one published in *Food Microbiology* (Zhang et al., 2015) and the other submitted to the same journal. The results have also been presented at several conferences, including the annual CPS Research Symposiums. In addition to the scientific impacts, this research again demonstrated the benefits of collaborations between CPS, produce industry, academia, and government research agencies.

Summary of Findings and Recommendations

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

Chlorine is commonly used for preventing water-mediated cross-contamination during fresh produce washing. The sanitizing efficacy is constantly compromised by the organic load, which has been attributed to rapid chlorine depletion and unsuccessful maintenance of residual FC. However, little is known about whether chlorine performs equally effectively with or without organic load at the same, well-maintained residual level. In this project, the efficacy of *E. coli* O157:H7 inactivation by chlorine was evaluated at various FC levels, COD levels, and pH, under an experimental setting relevant to fresh produce washing procedures. A chlorine depletion technique was employed for the first time to maintain the above mentioned parameters over a sufficiently long period, allowing the accurate evaluation of sanitization efficacy under stable conditions. The chlorine depletion and chlorination byproduct formation proceeded at higher rates at either COD levels or lower pH. A significant decrease in the sanitization efficacy was observed in the presence of diluted lettuce extract exhibiting 450 and 900 mg/L COD, compared to that obtained without organic load. Such deterioration of performance was more noticeable at shorter residence time, higher COD levels, or elevated pH. In an extreme case, the sanitization efficacy of 1.25 mg/L FC (pH 6.5, 5 s residence time) was a 5.76- and 0.20-log reduction at 0 and 900 mg/L COD, respectively. Whereas 0.5 mg/L FC was sufficient to reduce the bacterial population by 5 log units, a minimum of 7.5 and 5 mg/L of FC was required to achieve a 5-log reduction at pH 6.5 within 5 and 20 s of residence time. The overestimation of the FC level in the presence of chloramines may partly have accounted for the decreased sanitization efficacy, but other factors associated with the formation and interaction between chlorination byproducts should be explored extensively. This study underscored the potential risk of insufficient bacterial inactivation in the

presence of organic load at FC levels previously demonstrated effective for drinking water disinfection procedures (e.g., 0.5 mg/L), and it necessitates the proper validation of sanitization procedures based on specific produce type, processing facilities, and operational parameters.

Accurate measurement of residual FC is critical for mitigating pathogen cross-contamination. Our study shows the significant interference by organic chlorination byproducts to the conventional DPD method, which could be minimized by using the indophenol method. In practice, the indophenol method cannot be used for rapid measurement of the residual FC during fresh-cut produce washing because the method reaction time takes 5 min. Rather, the indophenol method could be applied as a measurement to determine the bias of DPD colorimetric measurements from the true residual FC. However, for measuring very low residual FC, it should be noted that the DPD colorimetric method gives a considerable overestimation of residual FC in the wash water. When the DPD colorimetric method is applied, it is also important to measure the absorbance of the water sample at 515 nm (as a blank) before adding the DPD powder/solution in case significant color or turbidity interferes with the measurement.

APPENDICES

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Teng, Z.; Luo, Y.; Alborzi, S.; Zhou, B.; Chen, L.; Zhang, J.; Zhang, B.; Millner, P.; **Wang, Q**. Chlorine-based inactivation of *E. coli O157*:H7: impact of residual FC content, organic load, residence time, and pH. Submitted to *Food Microbiology.*

Wang, Q., Z. Teng, Y. Luo, P. Millner, B. Zhang, and B. Zhou. Validation of chlorine level in sanitization system to avoid cross-contamination. *Center for Produce Safety, Produce Research Symposium*, Seattle, WA, June 2016.

Wang, Q., Y. Luo, P. Millner, B. Zhang, and B. Zhou. Validation of chlorine level in sanitization system to avoid cross-contamination. *Western Food Safety Summit (WFSS)*, Hartnell College, Salinas, CA, May 2016.

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Budget Summary

Most of the project funds have been utilized for supporting students, purchasing materials and supplies, as outlined in our original budget. The remaining amount will be used for cover travel expenses to the 2017 CPS Research Symposium in Denver.

Tables and Figures

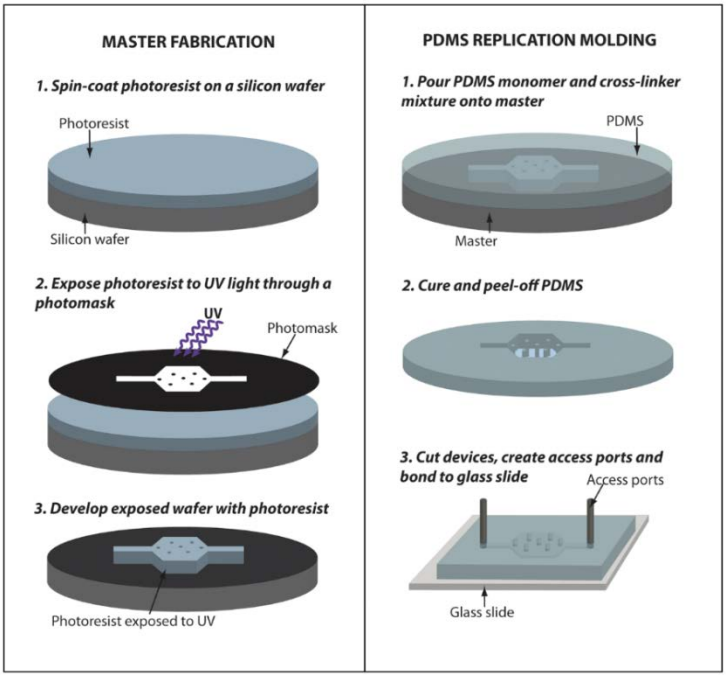

Figure 1. Fabrication of microfluidic mixer: mold fabrication (left) and device assembly (right)

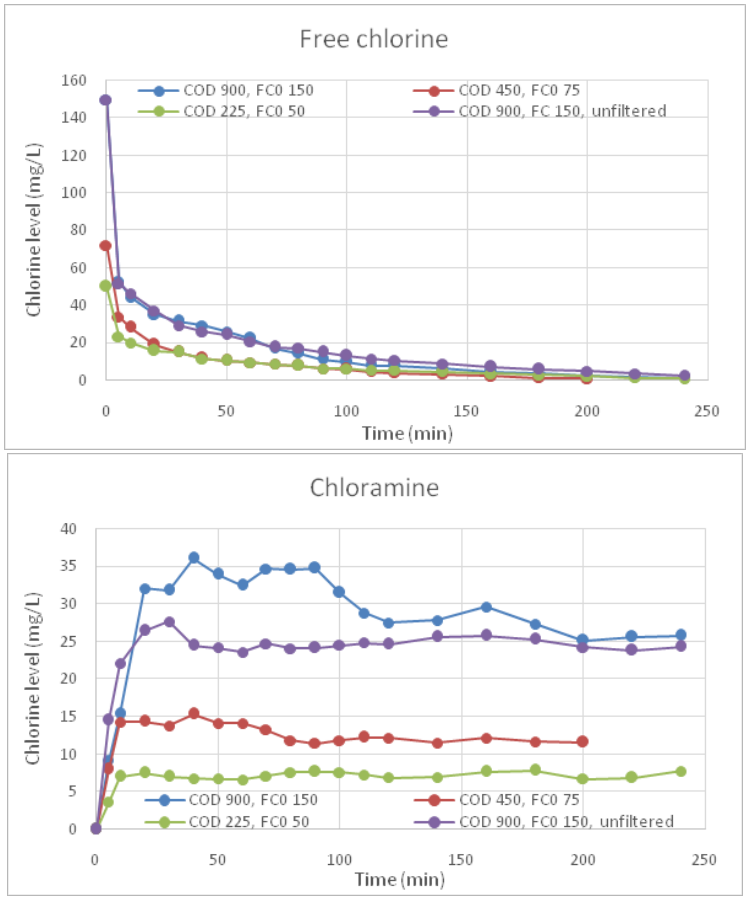

Figure 2. Effect of organic load on the depletion profile of free chlorine (FC) and accumulation of chloramine

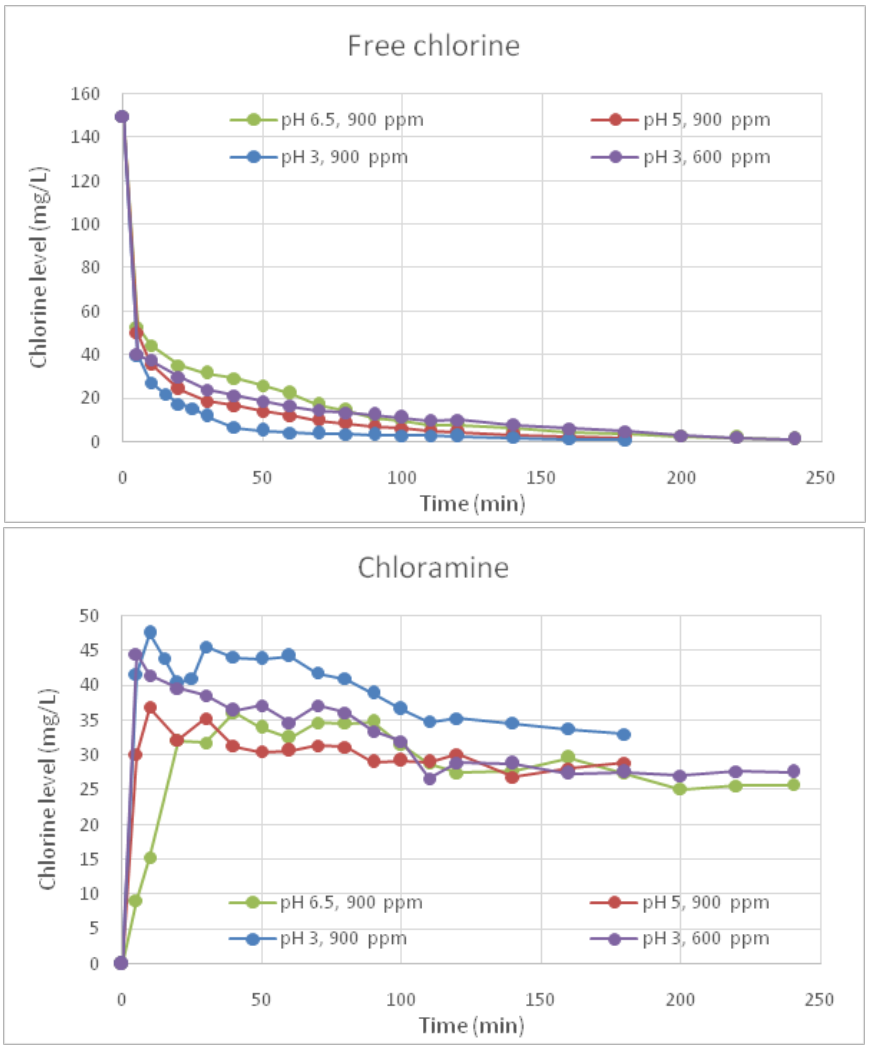

Figure 3. Effect of pH on the depletion profile of free chlorine (FC) and accumulation of chloramine

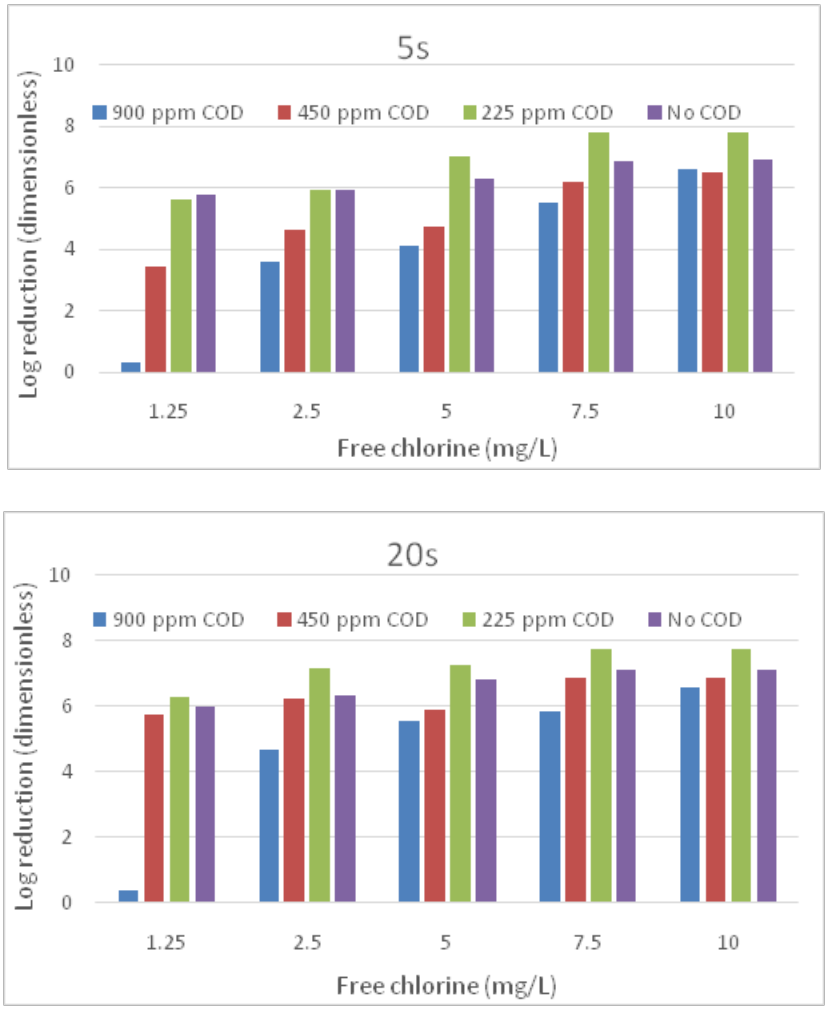

Figure 4. Effect of organic load on the sanitizing efficacy of free chlorine (FC) at pH 6.5

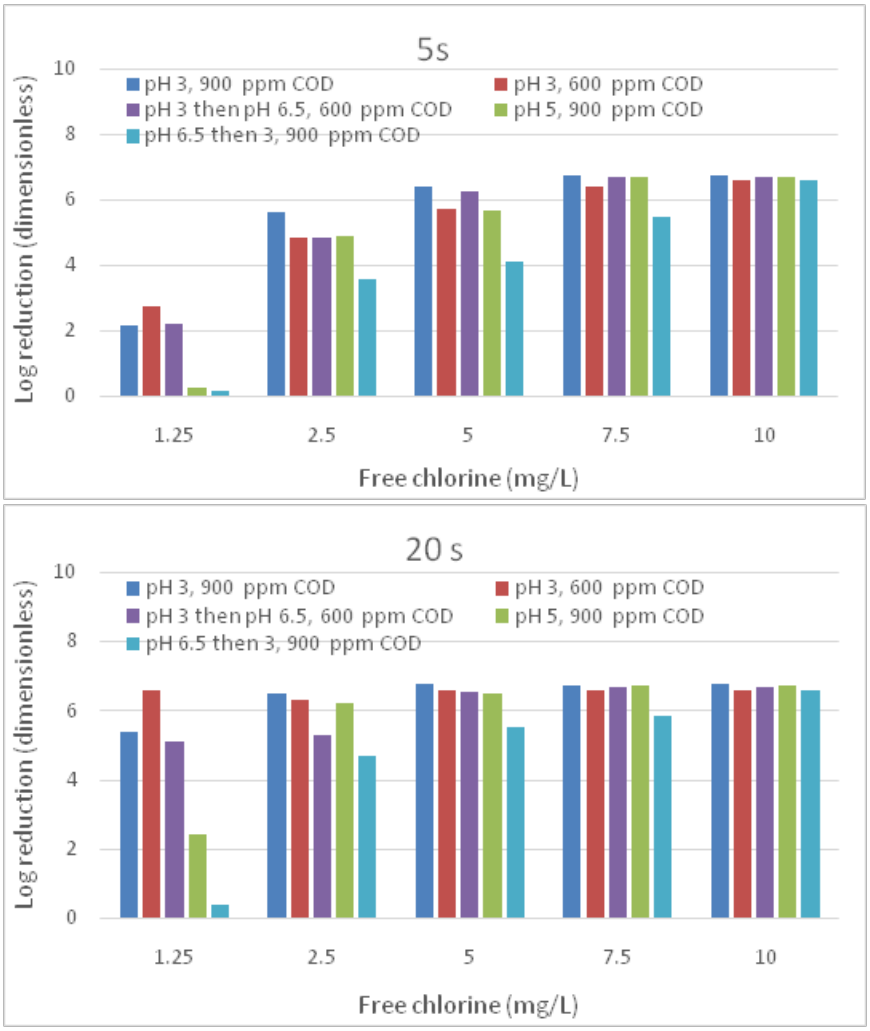

Figure 5. Effect of pH on the sanitizing efficacy of free chlorine (FC)

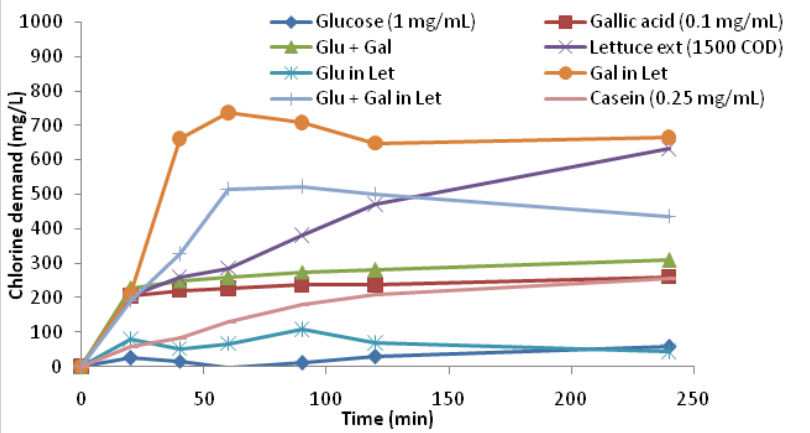

Figure 6. Chlorine demand of different model compounds: glucose (Glu), gallic acid (Gal), and casein (Cas), in water (pH 6.5) and lettuce extract (Let)

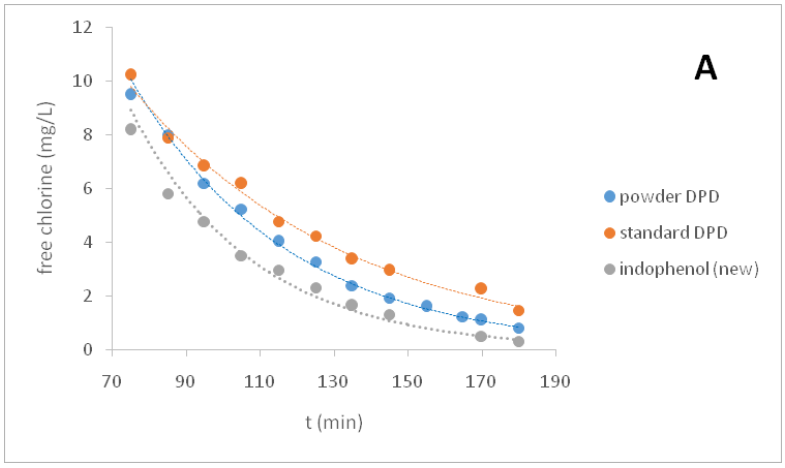

Figure 7. Residual free chlorine (FC) in lettuce wash water (225 mg/L COD, 22°C, pH 6.5) when dosing 75 mg/L FC

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

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

Table 2. Recommended free chlorine (FC) level for achieving 5-log reduction in *E. coli* O157:H7

pH	COD (mg/L)	Chloramine (mg/L)	FC (mg/L)	
			5s	20s
6.5	0	0	0.5*	0.5*
6.5	225	7.3±0.6	1.25	1.25
6.5	450	13.2±1.3	7.5	2.5
6.5	900	28.5±0.7	7.5	5
5.0	900	29.5±0.6	2.5	2.5
3.0	900	34.5±0.6	2.5	1.25
3.0	600	44.3±1.1	2.5	1.25

Table 3. Potential overestimation of free chlorine (FC) level by DPD method compared to indophenol method

FC – by DPD (mg/L)	**Chloramine – by DPD (mg/L)**	**FC – by indophenol (mg/L)**	**Difference (%)**
2.79±0.05	29.55±0.5	2.47±0.04	11±0.7
2.20±0.02	29.85±0.9	1.65±0.03	25±0.3
2.05±0.08	30.90±0.8	2.10±0.06	3±0.4
1.40±0.07	30.70±0.7	0.93±0.07	33±1.5
1.05±0.04	30.60±0.4	0.70±0.08	33±2.7
0.95±0.09	31.70±1.2	0.66±0.02	31±2.1
0.54±0.03	30.05±0.5	0.22±0.05	60±5.5