FINAL PROJECT REPORT **YEAR:** No Cost Extension

Project Title: Critical limits for antimicrobials in dump tank systems

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Cooperators: WA packinghouses

Total Project Request: **Year 1**: $86,183 **Year 2**: $93,414 **Year 3**: $8,660
Other funding sources
None

Budget 1
Organization Name: Washington State University
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Item	2019	2020	2021
Salaries	38,245	39,775	
Benefits	2,538	2,639	
Wages			
Benefits			
Equipment			
Supplies	42,000	50,000	8,660
Travel	3,400	1,000	
Miscellaneous			
Plot Fees			
Total	86,183	93,414	8,660

Footnotes:
Salaries: In year 1, $38,245, and year 2, $39,775, is requested for a Graduate Research Assistantship for a MS student to work on all objectives.

Benefits: $2,538 and $2,639 are requested for benefits tied to the Graduate Research Assistantship for a MS student to work on all objectives for years 1 and 2, respectively.

Supplies: Supply costs of $42,000 in year 1, $50,000 in year 2 and $8,660 in year 3 are requested to pay for disposable supplies such as glassware, microbiological media, pipettes, water attribute measurement instrumentation and calibration standards, and water makeup analysis.

Travel: $3,400 and $1,000 is requested in years 1 and 2, respectively, for mileage and associated travel costs at a rate of $0.535/mi and adhering to all university policies for per diem associated with overnight travel. Increased travel costs in year 1 are associated with cost of traveling to participating facilities to collect water samples associated with objective 1.

Objectives:

1. Establish the carbohydrate, protein, and mineral makeup of dump tank water during production in addition to the attributes of chemical oxygen demand (COD), temperature, pH, oxidation reduction potential (ORP), turbidity, and conductivity.
2. Determine the impact of free chlorine, peroxyacetic acid, chlorine dioxide or ozone concentration on the survival of Shiga toxigenic *E. coli*, *Salmonella*, or *Listeria monocytogenes* over time in water which has the similar composition as water evaluated in objective 1 and is representative of water chemistries observed throughout production in dump tank systems.

Significant Findings

- Mean COD value (preliminary data) was 592 mg/L, with considerable variation amongst sites and over time.
- Chlorine efficacy was highly dependent on organic load and exposure time.
- *L. monocytogenes* was more resistant to PAA than *Salmonella* or STEC.
- PAA efficacy increased with exposure time, while chlorine remained unchanged.
- Chlorine dioxide and ozone were not as effective as PAA or chlorine and showed little or no efficacy at low COD conditions.

Methods

Objective 1. Establish the carbohydrate, protein, and mineral makeup of dump tank water during production in addition to the attributes of chemical oxygen demand (COD), temperature, pH, oxidation reduction potential (ORP), turbidity, and conductivity.

Packinghouse selection and descriptions. Three commercial apple packinghouses were recruited into the study which encompass different industry management practices for managing flumes. One packinghouse has a single flume which is used up to 68 hr or until water changeover is needed (e.g. conventional to organic break). There is no filtration within the system. The second packinghouse has a single flume up to 68 hr or until water changeover is needed (e.g. conventional to organic break). There is a flocculation system installed. The third packinghouse utilizes two flumes, the first is used for the same duration as the first two packinghouses and has a filtration system installed. The secondary downstream flume is utilized for up to 10 days and also has a filtration system installed. The initial plan of work included data collection for only the 24hr of production. To encompass the full period water was used within the flumes, sampling periods were changed as shown in figure 1. Timing was set to occur throughout the packing season to encompass natural differences which occur as apples are held in storage. The first sampling event for all packinghouses occurred November-December of 2019. There was a slight delay due to COVID in the spring, but sampling resumed in the summer. Production variables such as additives to the flume system (e.g. acid, antimicrobials), flume capacity, varieties packed, storage conditions, % culls, line speed, was provided by the packinghouse and noted for each data collection period.

Water sample collection. Two 500 mL water samples were taken at 0, 4, 8, 12, 18, 24, 36, 48, 60, 72, 84 h at a consistent location from the flume. Once samples was shipped to a third party lab to deteremine carbohydrate, protein, and mineral content. The other sample was used for in real-time water quality parameters of chemical oxygen demand (COD), oxidation reduction potential (ORP), conductivity, pH, turbidity, temperature and amount of aniticmicrobial/acid present. All samples were held at 4°C (39.2°F) if not analyzed in real-time.

Establishing carbohydrate, protein, and mineral makeup of dump tank. Samples were shipped overnight for analysis with Merieux Nutrisciences. Target analytes were as follows: carbohydrates [simple sugars (fructose, glucose, maltose, sucrose), starch, and fiber (pectin, cellulose, and hemicellulose)], protein, and minerals (calcium, iron, magnesium, phosphorus, potassium, and sodium). Based upon outcomes from the first replication, certain analytes may be discontinued if they consistently are below the limit of detection for the analyses.

Quantifying water chemistry attributes of dump tanks. Chemical oxygen demand was calculated using a reactor digestion method with colorimetric quantification *(4)* using the Hach DRB200 Reactor and DR900 multiparameter colorimeter. The colorimeter was also used to measure sample turbidity. A multiparameter meter (Hach probe model 5048) determined pH, ORP, conductivity, and temperature during real time during collection.

Statistical analysis. A completely randomized design was used to evaluate significant differences of water attributes and nutritional compounds.

Objective 2. Determine the impact of free chlorine (FC), peroxyacetic acid (PAA), chlorine dioxide or ozone concentration on the survival of Shiga toxigenic *E. coli* (STEC), *Salmonella*, or *Listeria monocytogenes* over time in water which ha similar composition as water evaluated in objective 1 (year 2).

Water composition. Water quality measurements used in this part of the study were developed to represent standard features of washwater used in packinghouses in Washington. Three variations of dump tank water quality were used to represent postharvest water quality features which are inclusive of real-life conditions as determined by objective 1.

Microbial cultures. A five-strain cocktail of STEC, *Salmonella*, and *L. monocytogenes* associated with an outbreak were used for this objective. Bacterial strains are as follows: STEC cocktail [O104 (2011 European outbreak), O111 (apple juice outbreak), O103 (venison outbreak), O157 F4546 (alfalfa sprout outbreak) and O157 321 (spinach outbreak)]; *Salmonella* cocktail [Agona (alfalfa sprout outbreak), Montevideo (tomato outbreak), Gaminara (orange juice outbreak), Michigan (cantaloupe outbreak), and Saint Paul (pepper outbreak)]; *L. monocytogenes* cocktail [390-1 (cantaloupe outbreak), 390-2 (cantaloupe outbreak), 1452 (caramel apple outbreak), 108 (hard salami outbreak), 310 (goat cheese outbreak)]. Each strain of Shiga-toxigenic *E. coli* and *Salmonella* were individually grown in Tryptic Soy Broth (TSB) at 37°C (98.6°F) for 24 h with three successive transfers prior to inoculation of Tryptic Soy Agar (TSA) plates with each individual strain. TSA was incubated at 37°C (98.6°F) for 24 h to achieve a lawn of each strain. Each plate was flooded with 10 ml of Buffered Peptone Water (BPW) to harvest cells. *E. coli* and *Salmonella* strains were combined in equal volumes to create the five-species cocktail for inoculation. The same process was used for *L. monocytogenes*, with the exception that each strain was individually grown in Tryptic Soy Broth with Yeast Extract (TSBYE) at 32°C (89.6°F).

Sanitizer concentration. Three concentrations plus a no sanitizer control was evaluated for chlorine and PAA, while one concentration plus no sanitizer control was evaluated for chlorine dioxide (3 ppm) and ozone (1 ppm). The upper limit was based upon EPA label (chlorine, PAA or chlorine dioxide) or 1 ppm for ozone (which does not have an EPA label as it is an EPA registered device). To determine the efficacy of chlorine, as per industry practice, the pH of the system was maintained at 6.5 with the addition of a 1 in 10 dilution of 50% (v/v) of phosphoric acid.

Determining impact of sanitizers on pathogen survival. Simulated washwater treatments were inoculated and bacteria enumerated to estimate survival after 15, 30 and 60 seconds of exposure. All samples were neutralized with sodium thiosulphate to arrest sanitizer activity, then are serially diluted and plated onto both TSA or TSYE and selective media and incubated at 37°C (98.6°F; STEC and *Salmonella*) and 32°C (89.6°F; *L. monocytogenes*) for 48 h to enumerate surviving bacteria.

Statistical analysis. Each experiment is being independently replicated three times with three technical replicates (n=9) for reach sanitizer concentration evaluated. A completely randomized design with analysis of variance (ANOVA) was conducted. Post-hoc analyses was also conducted to determine significant differences between survival rates between and within treatments.

Results and Discussion

Mean, minimum and maximum values obtained for real-time physicochemical measurements for all replicates of objective 1 are presented in Table 1. Given the natural variation within and between the data set, it is important not to over analyze any values given that they may vary considerably. Based upon

the significant amount of variation, no significant correlations were observed amongst any parameters over time ($p>0.05$). Replication amongst sites helped determine mean values for the parameter COD over production time. These values were used to determine the water quality parameters in objective 2.

Table 1. Observed physicochemical attributes for flume water chemistry (n=104).

	pH	ORP (mV)	Conductivity (µS/cm)	Temperature °C (°F)	Turbidity (FAU)	COD (mg/L)	PAA (ppm)	Free Chlorine (ppm)
Mean	5.21	562.99	386.30	20.33 (68.6)	72.57	592.37	62.42	11.46
Min	2.46	194.30	2.41	11.70 (53.1)	0.00	10.00	2.00	0.50
Max	7.46	969.00	1574.00	34.30 (93.7)	250.00	2510.00	150.00	65.00

The first replicate complex chemical analyses were returned below the limit of detection for the assay, with the exception of ICP-MS, which had several minerals above the limit of detection. Therefore, the research team determined it is most cost effective to continue with only the ICP-MS and forgo carbohydrates [simple sugars (fructose, glucose, maltose, sucrose), starch, and fiber (pectin, cellulose, and hemicellulose)], and protein analysis.From the data analysis we have found a lack of correlation to any analyte and production time, but have reported mean, minimum and maxium values in Table 2.

Table 2. ICP mineral analysis for flume water (n=72).

	Mean (std. dev.)	Min	Max
Alumium	0.78 (2.82)	0.01	21.1
Barium	0.06 (0.05)	0.01	0.28
Calcium	52.94 (49.53)	15.3	306.0
Chromium	0.01 (0.05)	0.00	0.20
Copper	0.05 (0.12)	0.00	0.49
Iron	0.67 (1.72)	0.00	7.40
Magnesium	7.25 (3.98)	1.37	22.9
Manganese	0.09 (1.09)	0.00	8.79
Phosphorous	13.94 (132.42)	0.08	757.0
Potassium	10.53 (47.81)	0.82	398.0
Sodium	24.88 (19.38)	6.75	87.0
Stronium	0.29 (0.43)	0.04	2.8
Zinc	0.28 (4.36)	0.00	22.30

COD parameters for objective 2 were determined based upon observations in objective 1 and were set at 30, 500, and 2500 ppm for low, medium and high COD categories. Distinct differences in inactivation curves between free chlorine (FC) and peroxyacetic acid (PAA) were seen for all organisms (Figures 1, 2, and 3). When bacteria were exposed to FC, there was a sharp significant ($p<0.05$) initial reduction in bacterial populations within the first 15 s, after which the resulting populations remained rather stable for the remaining exposure time. In contrast, when exposed to PAA, bacteria first exhibited a slower initial inactivation with a more rapid decline after 15 s. PAA is a peroxide of acetic acid generated

from the reaction of acetic acid and hydrogen peroxide (*11*). Having a large oxidation potential, even greater than chlorine, PAA's mode of antimicrobial activity is similar to other peroxides and oxidizers (*38*). It has been theorized that PAA oxidizes sulfhydryl and disulfide bonds located in the cell wall and in other cellular components (*9*, *16*). Through rupture of these bonds, the fluidity of the cellular membrane is altered, proteins are denatured, and enzymes and metabolite functions are disrupted, causing detrimental effects to the cell (*9*, *16*).

A two-slope inactivation was seen when bacteria were exposed to PAA (Figure 1 B, D, F, Figure 2 B, D, F, and Figure 3 B, D, F). PAA has been shown to oxidize organic compounds at a slower rate than FC, which could help explain the differences seen in inactivation curves (*20*). Additionally, it is thought that the cellular membrane of the bacterial cells may exhibit initial resistance to PAA diffusing into the cell when exposed for shorter periods of time, also resulting in a slower inactivation rate (*14*). Gereffi et al. (*10*) found that *Salmonella* populations in round green tomato flume water were undetectable (<1 log CFU/mL) after 30 s regardless of organic load when exposed to 25 ppm PAA. In the same study *Salmonella* populations were only recoverable 2 s after of exposure to 25 ppm FC demonstrating the quick inactivation mechanism of chlorine (*10*). A rapid decline in microbial populations when exposed to varying FC concentrations was also seen in this study for all organisms (Figure 1 A, C, E, Figure 2 A, C, E, and Figure 3 A, C, E), with the greatest decline in microbial populations achieved within the first 15 s of exposure.

Figure 4 demonstractes the efficacy of chlorine dioxide (3 ppm) when exposed to low COD conditions for target foodborne pathogens. This concentration is the highest which can be used for direct product contact based upon FDA regulations. It is known that chlorine dioxide can have excellent efficacy, but based upon current regulatory limits it is generally considered rather ineffective when COD increases in water systems. That trend was apparent in these results, with <1 log inactivation of *L. monocytogenes* (Figure 4B) and no significant reduction of STEC or *Salmonella* (Figure 4A and 4C).

Ozone was generated on site to achieve 1 ppm in solution. Similar to chlorine dioxide, limited efficacy was found under low COD conditions, but this concentration achieved a 1 to 3 log reduction of target organisms under these conditions, which was significantly different from water-only controls (Figure 5 A-C; $p<0.05$). Hilighting once more the impact organic load plays in efficacy and limited use for this compound in recirculated systems where organic load easily climbs.

When introduced into an aqueous solution, hypochlorite dissociates into sodium or calcium ions and hypochlorite (OCl^-) (*6*). OCl^- gains hydrogen atoms to become at equilibrium with hypochlorous acid (HOCl). In the pH range of 4-7, chlorine is predominantly in the form of HOCl (*17*). Due to its neutral net charge, HOCl penetrates through the bacterial cell's lipid bilayer membrane by passive diffusion, easily gaining access to the intercellular components and begins to quickly attack and oxidize multiple nucleophilic intercellular components (*4*, *6*, *8*). Due to its similar molecular size to water, HOCl can also attack the outer part of the cell, likely contributing to its quick inactivation rate (*8*). This rapid inactivation was also observed in a study conducted by Van Haute et al. (*6*), where inactivation of *E. coli* O157:H7 populations using a chlorine based sanitizer at 20, 35, and 50 ppm FC occurred within the first minute in standardized process water with a COD level of 500 ppm (*18*).

Results from this study also show the influence COD plays on sanitizer efficacy. In general, as sanitizer concentration and exposure time were kept constant an increase in organic loading decreased the efficacy of both sanitizers. Low organic loading conditions demonstrated that 30 ppm of FC was sufficient to cause a 5.08, 6.42, and 3.47 log-reduction in STEC, *L. monocytogenes* and *Salmonella* sp. populations, respectively, after just 15 seconds of exposure (Figure 1A, 2A, and 3A). However, increasing the amount of organic matter to reach a

COD level of 500 mg/L (mid), the same concentration of 30 ppm FC resulted in a 3.85, 4.65 and 2.81 log-reduction, for the same organisms (Figure 1C, 2C, and 3C). The efficacy of the FC was further decreased when the organic loading in the system was increased by approximately five times as much.

At the highest organic loading level, 30 ppm FC only decreased the initial microbial load by 2.46, 3.41, and 1.11 log CFU/mL in STEC, *L. monocytogenes*, and *Salmonella* populations, respectively (Figure 1E, Figure 2E and Figure 3E). The high COD level required 50 ppm of FC to achieve at least a 3-log inactivation within 60s for STEC, *Salmonella* and *L. monocytogenes*. In the presence of 50 ppm PAA, over a 3-log reduction was achieved within 60s for STEC and *Salmonella*, but not *L. monocytogenes* (1.81 log CFU/mL) (Fig. 1F, Figure 2F, and Figure 3F).

The impact of organic load on sanitizer efficacy in water systems has been previously reported (*7*, *10*, *11*, *13*, *15*). As the organic matter increases in the solution, a greater dose of sodium hypochlorite is needed to achieve appropriate FC concentrations, increasing the total amount of chlorine in the system. However, total chlorine is not indicative of an increase in efficacy of chlorine-based solutions (*17*). Total chlorine is the addition of both FC and combined chlorine present in the solution (*17*). Although combined chlorine compounds are a more stable compound than FC, their reaction kinetics to inactivate microorganisms is much slower (*17*). Furthermore, upon introduction of chorine into a system with high organic load, FC quickly reacts with the organic material, depleting sanitizer efficacy and generating toxic byproducts (*13*). Keeping FC concentration consistent at 30 ppm, a significant decrease ($p<0.05$) of microbial inactivation was observed in all three microorganisms as the organic load increased.

Conversely, in previous studies, PAA has been shown to be more resistant to organic matter compared to chlorine-based sanitizers due to its slower reaction with organic matter and greater resistance to self-decomposition in the presence of organic matter (*5*, *11*, 20). Resiliency to organic load was also seen in this study. After 60 s of exposure, 80 ppm of PAA was sufficient to reduce the microbial populations of STEC and *Salmonella* below the limit of detection regardless of organic load, demonstrating PAA efficacy even under high loading conditions (Figure 1 B, D, F and Figure 2 B, D, F).

A positive correlation was observed between bacterial inactivation and sanitizer concentration. Apart from organic load, increasing FC concentrations resulted in significant differences ($p<0.05$) in microbial populations. Significant reductions in STEC, *Salmonella* and *L. monocytogenes* populations were also seen after 30 s of exposure when PAA concentrations increased ($p<0.05$). Independent of COD, 25 ppm of PAA had little inactivation (<1 log) for any of the three microorganisms studied.

Currently there is little information available on the concentration of commercial antimicrobials that are needed to effectively mitigate cross-contamination risk in recirculation systems where water quality is constantly changing. Furthermore, the critical limits established for one commodity, may not have the same effectiveness in another commodity, leaving process operators guessing about adequate dosing strategies that should be used in their processing environment. Results from this study highlight the important roles water quality, sanitizer concentration and exposure time all play in inactivation of pathogenic microorganisms. These findings provide inactivation rates of target foodborne pathogens when exposed to PAA, FC, chlorine dioxide, and ozone using similar water quality parameters for apple packers. This information provides evidence to base their programs on in order to enhance the scientific basis of their food safety plan based upon their own water quality parameters.

Citations

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Figure 1. Bacterial survival (log CFU/mL) of a five-strain Shiga–toxigenic *Escherichia coli* (STEC) cocktail in simulated processing water with different sanitizer concentrations (ppm) of either free chlorine (A, C, E) or peroxyacetic acid (B, D, F) and varying levels of COD 30 mg/L (A, B), 500 mg/L (C, D), or 2500 mg/L (E, F). Data points represent the mean of log transformed STEC populations with three biological replications (n=18 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL.

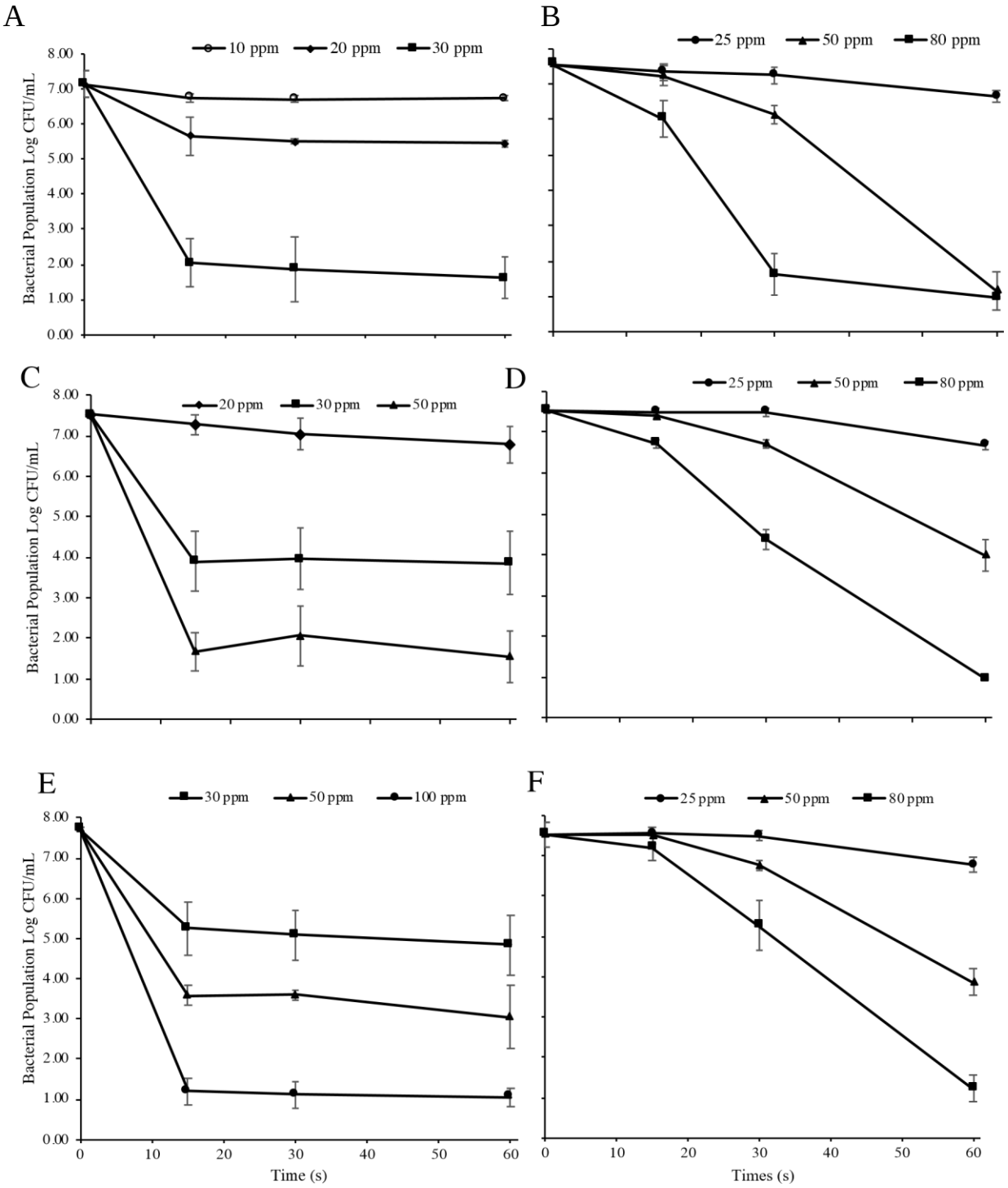


Figure 2. Bacterial survival (log CFU/mL) of a five-strain *Listeria monocytogenes* cocktail in simulated processing water with different sanitizer concentrations (ppm) of either free chlorine (A, C, E) or peroxyacetic acid (B, D, F) and varying levels of COD 30 mg/L (A, B), 500 mg/L (C, D), or 2500 mg/L (E, F). Data points represent the mean of log transformed STEC populations with three biological replications (n=18 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL.

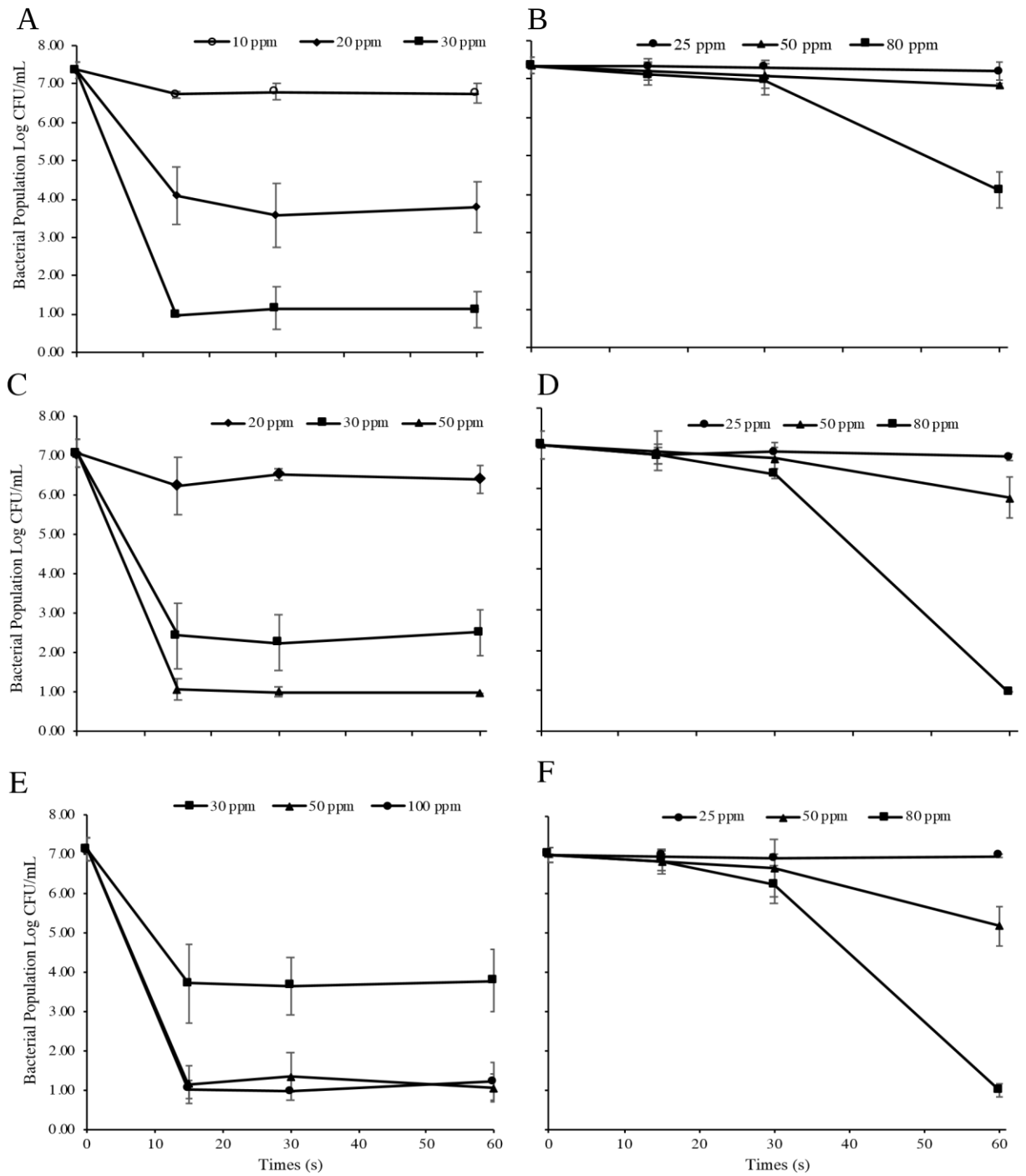


Figure 3. Bacterial survival (log CFU/mL) of a five-strain *Salmonella* spp. cocktail in simulated processing water with different sanitizer concentrations (ppm) of either free chlorine (A, C, E) or peroxyacetic acid (B, D, F) and varying levels of COD 30 mg/L (A, B), 500 mg/L (C, D), or 2500 mg/L (E, F). Data points represent the mean of log transformed STEC populations with three biological replications (n=18 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL.

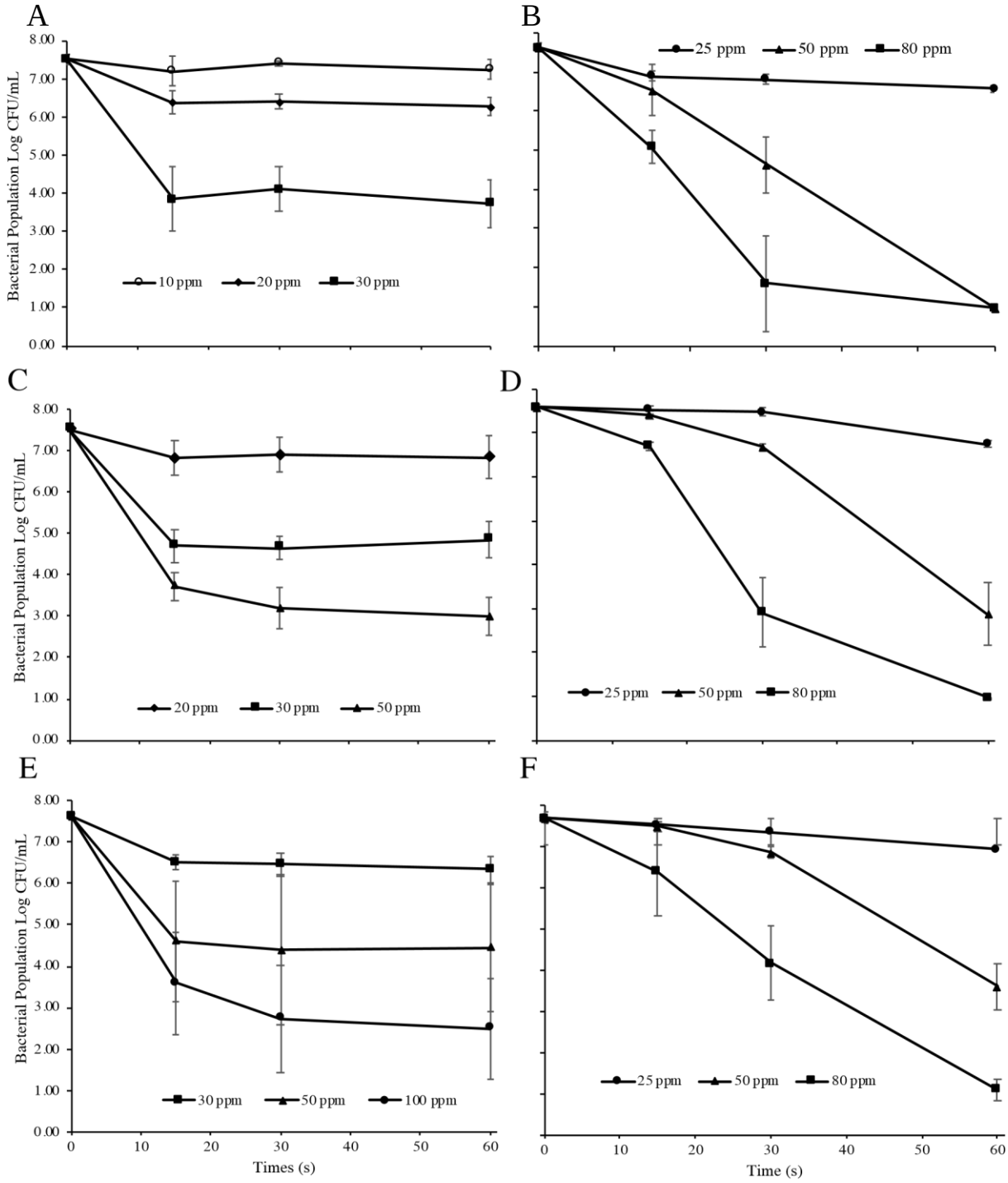


Figure 4. Bacterial survival (log CFU/mL) of a five-strain Shiga toxigenic *Escherichia coli* (STEC) (***A),*** *Listeria monocytogenes* (***B***) and *Salmonella* spp. cocktail (***C***) in deionized water (**−*)*** or simulated processing water with a COD level of 30 mg/L (----) when exposed to natural water conditions (●) or 3 ppm of residual chlorine dioxide (▲). Data points represent the mean of log transformed bacterial populations with two biological replications (n=12 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL .

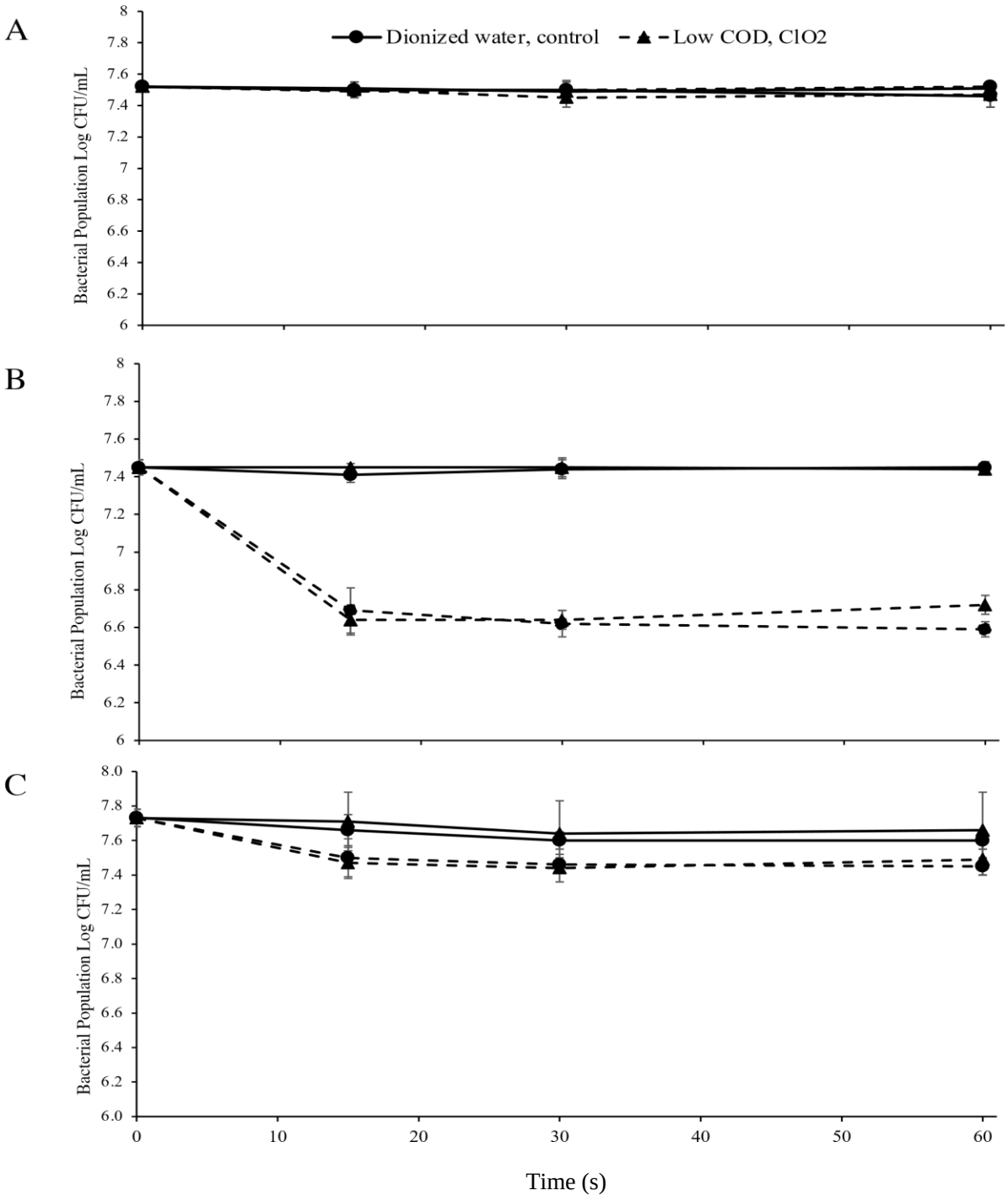
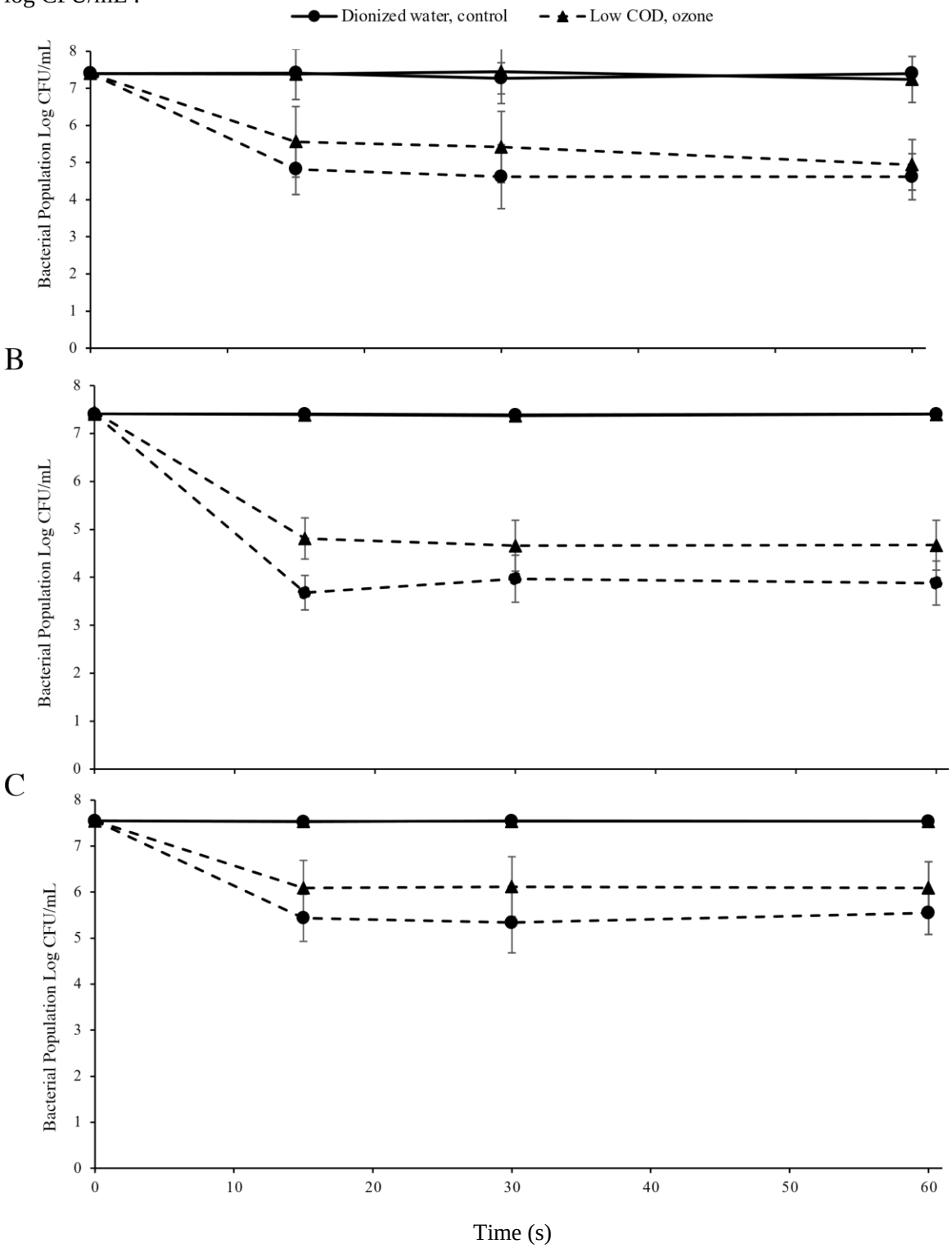


Figure 5. Bacterial survival (log CFU/mL) of a five-strain Shiga toxigenic *Escherichia coli* (STEC) (***A),*** *Listeria monocytogenes* (***B***) and Salmonella spp. cocktail (***C***) in deionized water (**−**) or simulated processing water with a COD level of 30 mg/L (----) when exposed to medical grade oxygen (●) or ozone (▲) when generated at 4 liters per minute for five minutes. Data points represent the mean of log transformed bacterial populations with two biological replications (n=12 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL .



EXECUTIVE SUMMARY

Project Title: Critical limits for antimicrobials in dump tank systems

Key words: *Postharvest washing, organic load, chlorine, PAA, chlorine dioxide, ozone*

Abstract:

Studies have shown the risk of cross-contamination in fruit and vegetable recirculating washing systems (e.g., flumes and dump tanks) when improperly managed with commercial antimicrobials (e.g. sanitizers). However, there is little evidence regarding minimum concentrations needed to effectively inactivate target organisms and mitigate cross contamination risk. The majority of Environmental Protection Agency (EPA) labels for antimicrobials used in these systems do not include information on control for microorganisms that are a public health concern. The goal of this study was to determine the efficacy of commonly used antimicrobials [free chlorine, peroxyacetic acid (PAA), chlorine dioxide, and ozone] against Shiga-toxigenic *Escherichia coli* (STEC), *Listeria monocytogenes*, and *Salmonella enterica* in apple wash water with similar characteristics seen in industry. Three commercial apple packinghouses were visited during a packing season to obtain water quality data (n=104) from their recirculated washing systems. Water samples were collected from dump tanks with clean water (0 h) and throughout production (up to 84 h of recirculated water). Samples were analyzed for chemical oxygen demand (COD), turbidity, oxidation-reduction potential (ORP), conductivity, and pH. Based on the information collected from the packinghouses, simulated wash water with COD levels of 30, 500, and 2500 mg/L ppm were created in the laboratory. Sanitizers were added to the water to achieve 10, 20, 30, 50, or 100 ppm free chlorine; 25, 50, or 80 ppm PAA; 3 ppm chlorine dioxide; 1 ppm ozone. A five-strain cocktail of *Salmonella, L. monocytogenes*, or STEC was inoculated into the water, and aliquots were taken over 1 min to determine microbial inactivation. The efficacy of sanitizers was highly dependent on COD level, sanitizer concentration, and exposure time. Maintaining consistent the sanitizer concentration and time, increasing organic load resulted in a significant ($p<0.05$) reduction in efficacy of PAA and chlorine for all organisms evaluated. Exposure to 100 ppm free chlorine or 80 ppm PAA for 60 s resulted in at least a 3-log reduction for all microbial populations regardless of organic load. Limited efficacy was seen in chlorine dioxide or ozone under low COD conditions.

This study can be utilized as supporting documentation to base current postharvest sanitizer concentrations in recirculzted systems. Concentrations of PAA and chlorine have been determined which result in rapid inactivation of pathogens in water with similar properties to that observed during production. Chlorine dioxide and ozone, while fit for use in single-pass systems, are influenced substantially by organic load which will accumulate in dump tanks and flumes. This is especially important with the focus of HACCP-based approaches for managing food safety risks which require critical limits (minimum concentrations of sanitizers) to be specified for dump tank systems to mitigate the risk of cross-contamination.