

Evaluation of a novel commercial ground beef production system using a chlorinated nanobubble antimicrobial technology to control Shiga toxin-producing *Escherichia coli* and *Salmonella* spp. surrogates

by

Amanda Jean Wilder

B.S., University of Idaho, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

Major Professor
Dr. Randall Phebus

Copyright

© Amanda Jean Wilder 2016.

Abstract

A variety of antimicrobial processes are used to reduce pathogen risks on commercially processed raw beef. Little research has evaluated chlorinated water on beef tissues, especially in a processing water dip scenario. Interest in nanobubble technology has increased due to its proposed surfactant properties, but it is undetermined whether this improves antimicrobial effectiveness of chlorine-based solutions in food applications. Benchtop studies were conducted to evaluate chlorinated nanobubble waters (0 to 11.94 ppm) against Shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O121, O145, and O157:H7 (STEC-7), *Salmonella* spp., and USDA-approved non-pathogenic STEC surrogates 1) in pure culture with the goal of characterizing the lethality contributions of pH (5 or 7), temperature, free available chlorine level (FAC), inclusion of nanobubbles, or a combination thereof; 2) in select chlorinated nanobubble “red water” (water containing 0.1% beef purge) solutions; and 3) on the surface of lean and fat beef tissue. In pure culture solutions, surrogates demonstrated greater resistance ($P \leq 0.05$) to chlorinated solutions (3.4-5.5 log CFU/mL reductions) with increased reductions at the higher (11.94 ppm) FAC levels. STEC-7 and *Salmonella* population reductions were also notably reduced (3.3-7.1 log CFU/mL) by the higher FAC concentrations. No definitive impacts of temperature, nanobubble inclusion, or acidic pH were observed. At an average 5.23 ppm FAC in red water, all microbial populations were reduced by > 6 log CFU/mL after 60 minutes. Reductions of target organisms on inoculated lean and fat tissues were ≤ 1 log CFU/g in red water; likely due to the inability to maintain FAC levels above 0.7 ppm in the presence of organic loading. An in-plant antimicrobial validation study of a proprietary raw beef manufacturing process was conducted to determine the effectiveness of a recirculating acidic nanobubble water system, chlorinated to 5 ppm FAC using EO water generated concentrate, against the USDA-approved STEC surrogates. Preliminarily, inoculated beef trim was introduced into the system targeting 5 ppm FAC; chlorine concentrate reinfusion rates were determined to establish applicable operational parameters and sampling strategies for the system. An optimized in-plant study was conducted. Meat inoculated at ~ 7 log CFU/g was introduced into the recirculating chlorinated nanobubble system every other day over 6 days, achieving an average 1.6 log CFU/g surrogate reduction on inoculated meat throughout the manufacturing

process. Approximately 2.7 log CFU/g of residual surrogates were recovered on non-inoculated meat ~35 minutes after inoculated meat entered the system, indicating that harborage of microbial contamination on processing equipment can lead to subsequent contamination carry-over that must be controlled during processing. Surrogate organisms were recovered by enrichment only from non-inoculated meat 24 h after inoculated meat processing on alternate days, likely stemming from inadequately sanitized processing equipment after inoculated batch processing. Control of the residual surrogate population in the system following inoculation was accomplished through daily equipment sanitation and boosting recirculated processing water to 50 ppm during a 4-h sanitation period (no beef entering system). The optimized study will be used as an antimicrobial process validation against STEC and *Salmonella* spp. in beef manufacturing.

Table of Contents

List of Figures	ix
List of Tables	xiii
Acknowledgements.....	xiv
Chapter 1 - Introduction	1
Chapter 2 - Literature Review.....	4
2.1 Foodborne Illness.....	4
2.1.1 Shiga Toxin-producing <i>Escherichia coli</i>	4
2.1.1.1 Public health effects of Shiga toxin-producing <i>E. coli</i>	6
2.1.1.2 Shiga toxin-producing <i>E. coli</i> in the beef industry	8
2.1.2 <i>Salmonella</i> Species.....	10
2.1.2.1 <i>Salmonella</i> in the beef industry	12
2.2 Interventions to Control Foodborne Pathogens during Commercial Beef Processing	14
2.2.1 Chlorine-based Antimicrobials.....	15
2.2.1.1 Electrolyzed water	23
2.2.1.2 Chlorine disinfection by-products.....	24
2.2.2 Nanobubble Technology in the Food Industry	26
2.2.3 Use of Non-pathogenic Surrogates to Validate Antimicrobial Interventions for In-Plant Studies	28
Chapter 3 - Research Questions	30
Chapter 4 - Efficacy of Chlorinated Nanobubble Solutions to Control Shiga Toxin-producing <i>E. coli</i> , <i>Salmonella</i> spp., and Non-pathogenic Surrogate <i>E. coli</i> in a Model Beef Processing System—Laboratory Benchtop Studies.....	31
4.1 Determining Minimum Bactericidal Concentrations of Chlorinated Waters in Pure Cultures.....	31
4.1.1 Introduction	31
4.1.2 Materials and Methods.....	32
4.1.2.1 Bacterial cultures and inoculum preparation	32
4.1.2.2 Antimicrobial water solutions	33

4.1.2.3	Application of chlorinated water treatments to pure cultures	34
4.1.2.4	Microbial analysis.....	35
4.1.2.5	Statistical analysis	35
4.1.3	Results and Discussion	36
4.1.4	Conclusion	45
4.2	Determining Lethality of Pathogens and Surrogates in the Presence of Red Water	46
4.2.1	Introduction	46
4.2.2	Materials and Methods.....	46
4.2.2.1	Generation of red water solutions	46
4.2.2.2	Preliminary chlorine loss determination resulting from varying levels of purge addition	47
4.2.2.3	Bacterial cultures and inoculum preparation	48
4.2.2.4	Application of chlorinated water treatments to reduce target bacterial populations in the presence of 0.1% red water	48
4.2.2.5	Microbial analysis.....	49
4.2.3	Results and Discussion	49
4.2.3.1	Preliminary evaluation of chlorine loss	49
4.2.3.2	Pathogen survival.....	53
4.2.4	Conclusion	56
4.3	Efficacy of Chlorinated Nanobubble Solutions on Beef Lean and Fat in the Presence of Red Water	57
4.3.1	Introduction	57
4.3.2	Materials and Methods.....	57
4.3.2.1	Meat source	57
4.3.2.2	Bacterial cultures and inoculum preparation	58
4.3.2.3	Antimicrobial water solutions.....	58
4.3.2.4	Experimental procedure	58
4.3.2.5	Microbial analysis.....	59
4.3.3	Results and Discussion	60

4.3.3.1	Free available chlorine, pH, and ORP of red water solutions	60
4.3.3.2	Pathogen recovery in water samples	62
4.3.3.3	Pathogen recovery on lean and fat.....	64
4.3.4	Conclusion	66
Chapter 5 - Evaluation of a Chlorinated Nanobubble Water System to Control Shiga Toxin-		
producing <i>E. coli</i> Surrogates in a Novel Commercial Ground Beef Manufacturing Process. 68		
5.1	Introduction	68
5.2	Unit Components of the Novel Ground Beef Processing System.....	70
5.3	Materials and Methods.....	75
5.3.1	Preliminary Validation Study	75
5.3.1.1	Antimicrobial treatment water source	75
5.3.1.2	Meat source	75
5.3.1.3	Bacterial cultures and inoculum preparation	76
5.3.1.4	Preliminary water-only study inoculation study parameters.....	77
5.3.1.5	Parameters for the preliminary inoculated meat processing study.....	78
5.3.1.6	Pre-operation and environmental sponges.....	79
5.3.1.7	Chemical analysis of process water	80
5.3.1.8	Microbiological analysis.....	80
5.3.2	Optimized Validation Study to Evaluate the Effectiveness of the Cesco-Bauer Recycling Process Water System for Controlling STEC Surrogates during a 6-Day Processing Scenario.....	81
5.3.2.1	Antimicrobial process water source	81
5.3.2.2	Meat source	82
5.3.2.3	Bacterial cultures and inoculum preparation	82
5.3.2.4	System sanitation and disinfection.....	82
5.3.2.5	Water-only inoculation study to evaluate effectiveness of an optimized set of operational parameters and an elevated chlorine disinfection step	83
5.3.2.6	Inoculated meat study parameters	84

5.3.2.7	Pre-operation environmental sponges to identify presence of viable surrogate bacteria	85
5.3.2.8	Chemical analysis of Cesco-Bauer processing water samples	86
5.3.2.9	Microbiological analysis of meat and process water samples	87
5.3.2.10	Statistical analysis of meat sample microbiological data	88
5.4	Results and Discussion	88
5.4.1	Water Samples	88
5.4.1.1	Preliminary water studies	89
5.4.1.2	Chlorine demand and surrogate recovery during meat processing	90
5.4.1.1	Chlorine by-product results in recycled processing water	96
5.4.2	Pre-Operation and Environmental Samples	98
5.4.3	Meat Samples	102
5.5	Conclusion	107
	References	109
	Appendix A - Temperature Charts from In-Plant Validation Study	123
	Appendix B - Cesco-Bauer Chemical Readings during In-Plant Studies	131
	Appendix C - Extra Graphs from In-Plant Study	134
	Appendix D - SAS Code	137
	Appendix E - Aquaox 5000™ SDS	142

List of Figures

Figure 2.1 Schematic diagram of bubble size in aqueous solution; adapted from Agarwal, Ng, & Liu, 2011	26
Figure 4.1 Profile Plot of 5-Way Interaction between Organism, Temperature, pH (Acidic or Neutral), Presence of Nanobubbles (NB), and Media Type. Least Square Means (Avg. Log CFU/mL) of Log CFU/mL reductions are reported for each combination.	39
Figure 4.2 Average Log CFU/mL Reductions Based on Type of Media; A-B different letters indicate significant differences ($P \leq 0.05$).....	40
Figure 4.3 Profile Plot of 4-Way Interaction between Level of Chlorine (high, medium, low, zero FAC), pH (Acidic or Neutral), Presence of Nanobubbles (NB), and Media Type. Least Square Means (Avg. Log CFU/mL) of Log CFU/mL reductions are reported for each combination.	41
Figure 4.4 Profile Plot of 4-Way Interaction between Level of Chlorine (high, med., low, zero), Type of Organism, Presence of Nanobubbles (NB), and Media Type. Least Square Means (Avg. Log CFU/mL) of Log CFU/mL reductions are reported for each combination.....	42
Figure 4.5 Average Log CFU/mL Reductions Based on Type of Organism; A-B different letters indicate significant differences ($P \leq 0.05$).....	43
Figure 4.6 Average Log CFU/mL Reductions Based on Level of FAC; A-C different letters indicate significant differences ($P \leq 0.05$).....	43
Figure 4.7 Residual Free Available Chlorine after Addition of 5% Purge to Cesco-Bauer Water Solutions.....	50
Figure 4.8 Residual Free Available Chlorine after Purge Addition (0-0.25%) to 25 ppm FAC Cesco-Bauer Water Solutions	52
Figure 4.9 Residual Free Available Chlorine after Purge Addition (0.05-0.10%) to 23-30 ppm FAC Cesco-Bauer Water Solutions with No Re-infusion of Fresh Solution	53
Figure 4.10 Average Recovery of Surrogates After Exposure to Red Water for 60 min in the Presence of 0.1% Purge on Injury Recovery Media	54
Figure 4.11 Average Recovery of STEC-7 After Exposure to Red Water for 60 min in the Presence of 0.1% Purge on Injury Recovery Media.....	55

Figure 4.12 Average Recovery of <i>Salmonella</i> After Exposure to Red Water for 60 min in the Presence of 0.1% Purge on Injury Recovery Media	55
Figure 4.13 Average Free Available Chlorine in Red Water over 42 hours	61
Figure 4.14 Average pH of Red Water over 42 hours	62
Figure 4.15 Average ORP of Red Water over 42 Hours	62
Figure 4.16 Average Recovery of Organisms in Red Water over 42 Hours on Injury Recovery Media	64
Figure 4.17 Average Recovery of Organisms on Inoculated Meat Before and After ~60 second Exposure to the Antimicrobial Red Water on Injury Recovery Media	65
Figure 4.18 Average Recovery on Non-Inoculated Pick-Up Meat Introduced into the System 15 and 45 minutes after Inoculated Meat on Injury Recovery Media	65
Figure 4.19 Average Recovery of Target Organisms on Non-Inoculated Meat Introduced to Red Water 24 hours after Inoculated Meat on Injury Recovery Media	66
Figure 5.1 Novel Ground Beef Manufacturing System Utilizing Chlorinated Nanobubble Process Water	74
Figure 5.2 Recovery of Surrogates (ECC) Before and After the Cryofreeze Tunnel; A-B different letters indicate significant differences ($P \leq 0.01$).	103
Figure 5.3 Recovery of Surrogates (ECC) Averaged Across All Sampling Points in Contact with Chlorinated Nanobubble Process Water	104
Figure 5.4 Average Recovery of Surrogates (ECC) on Inoculated Meat at Different Sampling Points Throughout the System, A-B different letters indicate significant differences ($P \leq 0.05$)	104
Figure 5.5 Average Recovery of Surrogates (ECC) on Same-Day Pick-Up Meat at Different Sampling Points Throughout the System, A-B different letters indicate significant differences ($P \leq 0.05$)	105
Figure 5.6 Average Recovery of Surrogates (ECC) on 24-hour Pick-Up Meat at Different Sampling Points Throughout the System at the Beginning, Middle, and End of the production Day.....	106
Figure 5.7 Temperature of Processing Room during Preliminary In-Plant Study.....	123

Figure 5.8 Temperature of water in recirculating solution during preliminary water experiment	123
Figure 5.9 Temperature of water in recirculating water, Day 2 Preliminary Study	124
Figure 5.10 Temperature of water in recirculating water, Day 1 Preliminary Study	124
Figure 5.11 Temperature of recirculating water, Day 4 Preliminary Study.....	125
Figure 5.12 Temperature of recirculating water, Day 3 Preliminary Study.....	125
Figure 5.13 Temperature of recirculating water, Day 6 Preliminary Study.....	126
Figure 5.14 Temperature of recirculating water, Day 5 Preliminary Study.....	126
Figure 5.15 Temperature of recirculating water, Preliminary water and Day 1 of Optimized Study	127
Figure 5.16 Temperature of Processing Room during Optimized Validation Study.....	127
Figure 5.17 Temperature of recirculating water, Day 3 Optimized Study.....	128
Figure 5.18 Temperature of recirculating water, Day 2 Optimized Study.....	128
Figure 5.19 Temperature of recirculating water, Day 5 Optimized Study.....	129
Figure 5.20 Temperature of recirculating water, Day 4 Optimized Study.....	129
Figure 5.21 Temperature of recirculating water, Day 6 Optimized Study.....	130
Figure 5.22 Free Available Chlorine at point of meat contact (Vortex) in Recirculating Water over 6-day Preliminary Study.....	131
Figure 5.23 pH of Recirculating Water over 6-day Preliminary Study, recorded at flotation tank	131
Figure 5.24 ORP of Recirculating Water over 6-day Preliminary Study, recorded at flotation tank	132
Figure 5.25 Free Available Chlorine in Recirculating Water over 6-day Optimized Validation Study	132
Figure 5.26 pH of Recirculating Water over 6-day Optimized Validation Study, recorded at flotation tank.....	133
Figure 5.27 ORP of Recirculating Water over 6-day Optimized Validation Study, recorded at flotation tank.....	133
Figure 5.28 Average Recovery of Organisms on Inoculated Meat during Preliminary Study	134

Figure 5.29 Average Recovery of Total Aerobic Plate Counts on Inoculated Meat during
Optimized Study..... 134

Figure 5.30 Average Recovery of Organisms on Pick-Up Meat during Preliminary Study 135

Figure 5.31 Average Recovery of Total Aerobic Plate Counts on Pick-Up Meat during Optimized
Study 135

Figure 5.32 Average Recovery of Total Aerobic Plate Counts on 24-hour Pick-Up Meat during
Optimized Study..... 136

List of Tables

Table 2.1 Major Foodborne Illness Outbreaks Attributed to STEC.....	7
Table 2.2 Number of Beef Recalls Due to STEC from 2010 to 2015	9
Table 2.3 Major Foodborne Illness Outbreaks Attributed to <i>Salmonella</i> in the United States ...	11
Table 2.4 Number of Beef Recalls Due to <i>Salmonella</i> from 2010 to 2015	12
Table 2.5 Use of Chlorinated Antimicrobials Approved by the United States Department of Agriculture Food Safety and Inspection Service	17
Table 4.1 Parameters Evaluated in Cesco-Bauer Water During Determination of Minimum Bactericidal Concentrations of Chlorinated Waters in Pure Cultures Experiment.....	35
Table 4.2 Type 3 Fixed Effects of Split-Split-Split Plot Analysis	36
Table 4.3 Level of Free Available Chlorine (ppm) in Each Solution During 0.1% Purge Experiment at Each Sampling Point.....	56
Table 5.1 Water-only Study Results, Preliminary Validation Study.....	89
Table 5.2 Water-only Study Results, Optimized Validation Study.....	90
Table 5.3 Free Available Chlorine (FAC), Total Chlorine, pH, ORP of Recirculating Water during Preliminary Study	93
Table 5.4 Free Available Chlorine (FAC), Total Chlorine, pH, ORP of Recirculating Water during Optimized Study.....	95
Table 5.5 Trichloromethane Analysis of Recirculating Water during Optimized Validation Study	97
Table 5.6 Preliminary Study Pre-Operation Equipment Samples.....	98
Table 5.7 Preliminary Study Indicators of Equipment Cleanliness	99
Table 5.8 Preliminary Study Environmental Samples.....	99
Table 5.9 Optimized Study Pre-Operation Equipment Samples.....	100
Table 5.10 Optimized Study Indicators of Equipment Cleanliness.....	101
Table 5.11 Optimized Study Environmental Samples.....	102

Acknowledgements

This project is supported by Agriculture and Food Research Initiative Grant No. 2012-68003-30155 from the USDA National Institute of Food and Agriculture, Prevention, Detection and Control of Shiga Toxin-Producing *Escherichia coli* (STEC) from Pre-Harvest Through Consumption of Beef Products Program – A4101.

The success of these combined projects would not have been possible without the support of the Kansas State Food Safety and Defense Laboratory (Nick Severt, Matt Krug, Carla Schwan, Minto Michael, Donka Milke, Jennifer Acuff, Keyla Lopez, Saurabh Kumar, Sara Jones, Austin McDaniel, and Robert Scholl), and numerous Kansas State University faculty and students including Dr. Christopher Vahl and Dr. J. Scott Smith (and his food analysis lab team, particularly Dr. Faris Hussain and Mostafa Taghvaei). I would like to thank my committee members Dr. Randall Phebus, Dr. Sara Gragg, and Dr. Harsha Thippareddi for their support and guidance. A special thanks goes to Drs. John Luchansky and Anna Porto-Fett from the USDA ARS Eastern Regional Research Center, Karl and Paul with Cesco Solutions/Cesco-Bauer Technologies, and to the commercial beef processor who owns this proprietary ground beef processing patent rights for presenting me a challenging opportunity for research and scientific growth.

I would like to acknowledge and thank the Kappa Delta Sorority Foundation, Meat Industry Supplier's Alliance, Food Marketing Institute Foundation, the United Dairymen of Idaho, and the Kansas State University Graduate School for providing scholarships that financially supported my education while at Kansas State University.

Chapter 1 - Introduction

Beef products, especially ground beef, have been associated with human illness, disease outbreaks, and product recalls due to contamination from Shiga toxin-producing *E. coli* (STEC) and *Salmonella enterica*, both cattle harbored foodborne pathogens. Between 2002 and 2011, ground beef tainted with *Salmonella* accounted for 35% of the beef associated outbreaks (Laufer et al., 2015). Seven strains of enterohemorrhagic *E. coli* have been declared adulterants in raw, non-intact beef—O26, O45, O103, O111, O121, O145, and O157: H7—due to the severity of illness caused after human consumption (CDC, 2012). STEC infection traditionally induces hemorrhagic colitis and, in severe cases, hemolytic uremic syndrome (HUS) in high-risk populations such as children. Similar to pathogenic *E. coli*, *Salmonella* infections are characterized by gastrointestinal illness. However, *Salmonella* illness usually is self-limiting within 72 hours, but in severe cases death can occur from complications related to the illness, usually dehydration (FDA, 2012).

Numerous antimicrobials, including lactic and peroxyacetic acids, are widely used in raw beef processing to reduce the presence of foodborne pathogens such as STEC and *Salmonella*; however, there is interest in exploring combinations of various technologies to enhance antimicrobial effects on pathogens and to support proprietary developments in beef processing. Of particular interest are antimicrobial interventions that can be effectively applied at the beef trim and/or ground beef stages of manufacturing, as these would address pathogen risks closer to the consumer. Chemical residues from traditional antimicrobials such as organic acids can lead to deterioration of quality attributes, including appearance, texture, and taste, and create potential human health hazards (Tsuge, 2014). Therefore, interventions requiring very low levels of active chemicals are of interest. Chlorinated water is the most commonly used antimicrobial in the U.S. produce and poultry industries due to its low cost and its efficacy against pathogens (Sohaib et al., 2016; Yang et al., 2012). Chlorine can be applied as an antimicrobial in a variety of forms as directed by the USDA FSIS Safe and Suitable Ingredients list (USDA FSIS, 2016) for meat and poultry. One method of generating chlorine in water solutions using low levels of chemicals is with electrolyzed-oxidized (EO) water. The application

of EO water at 5-7 pH minimizes detrimental health effects from Cl₂ gassing off (Guentzel, Liang Lam, Callan, Emmons, & Dunham, 2008), while maintaining the antimicrobial effects of hypochlorous acid (HOCl) present in solution. HOCl is the most effective form of chlorine as a disinfectant; at pH 5, HOCl constitutes 97% of total chlorine in solution (Park, Hung, & Chung, 2004).

A relatively new concept to the food industry, nanobubble technology, is showing promise to aid in the development of improved food safety interventions. There is increased interest in nanobubbles due to their proposed surfactant abilities or cleaning effect. Small particles in water can be effectively removed by introducing micro- or nanobubbles of opposing charge and zeta potential, which is controlled by the pH of the solution (Tsuge, 2014). Nanobubbles also provide increased surface area-to-volume ratio per mass as compared to standard water or other aqueous solutions ("Bauer Nanobubbles," 2016), which theoretically enhances the efficiency of any dissolved or suspended antimicrobial components in solution. Nanobubble treatment, as a sanitation method, has been evaluated against norovirus surrogates in oyster bodies and was found to inactivate more than 99% of active virus after 12 hours (Tsuge, 2014).

In the current studies, a combination antimicrobial intervention treatment as a key component in a novel ground beef manufacturing process (U.S. Patent No. 9167843) was evaluated at a non-inspected commercial-scale pilot plant facility, with all manufactured beef products during the period of the inoculated studies being sent to inedible rendering operations. Briefly, this patented system utilizes higher-fat commercial beef trimmings to separate predominantly lean tissues from predominantly fatty tissues while the trimmings are being transported through a recirculating chilled antimicrobial fluid conduit system. Through proprietary operating conditions, lean tissues are recovered for further processing into raw ground beef demonstrating similar quality characteristics of traditionally manufactured product. An added benefit of this trim processing approach is the submersion of all beef tissue surfaces (fat and lean) in the recirculating fluid, which if adequately antimicrobial, provides a unique pathogen control opportunity in the raw ground beef manufacturing process. The portion of the trim treatment process utilizing the recirculating fluid is envisioned as operating

over a multi-day period with continuous re-infusion of fresh make-up antimicrobial solution at defined points and intervals. Thus, such a system must be capable of inherent disinfection to prevent microbial build-up over the extended processing period.

Two inoculated in-plant studies were conducted using USDA-approved non-pathogenic *E. coli* surrogate cultures to evaluate the effectiveness of the recirculating antimicrobial fluid—an acidic (pH 5) Cesco-Bauer water [i.e. municipal water that is continuously chlorinated by infusion of concentrated chorine produced through an electrolyzed-oxidized (EO) water process, acidified by introduction of CO₂ gas, and then passed through a patented nanobubble generator (Patent No. 8454837)]. The first study was conducted to determine preliminary inefficiencies in operational variability/stability of the novel commercial scale ground beef system over a determined 6-day continuous production run and to optimize inoculation, sampling and testing protocols. The second study incorporated necessary processing system modifications to enable characterization of lethality of the antimicrobial process water on inoculated beef trimmings and equipment components included in the recirculating conduit system that support multi-day continuous processing. This optimized study will be used by the ground beef manufacturing company as scientific validation of this novel system for controlling enteric pathogens such as STEC and *Salmonella* spp. in their future commercial ground beef operations. Before the commercial studies could be conducted, a series of benchtop experiments were required to 1) determine the lethality contributions of pH, presence of nanobubbles, level of free available chlorine, temperature, or a combination thereof against STEC, *Salmonella* spp., and USDA-approved STEC surrogates in pure solution, 2) determine the effectiveness of select chlorinated nanobubble solutions against STEC, *Salmonella* spp., and surrogates in red water; and 3) evaluate the effectiveness chlorinated nanobubble solutions against STEC, *Salmonella* spp., and surrogates on the surface of lean and fat tissue submerged in red water.

Chapter 2 - Literature Review

2.1 Foodborne Illness

Based on the data collected from five pathogen surveillance sources [namely FoodNet, the National Notifiable Diseases Surveillance System (NNDSS), the Cholera and Other *Vibrio* Illness Surveillance (COVIS) system, the National Tuberculosis Surveillance System (NTSS), and the Foodborne Disease Outbreak Surveillance System (FDOSS)], Scallan et al. (2011) estimated that 31 pathogens cause 37.2 million illness annually in the United States; of these, 36.4 million were acquired domestically, 9.4 million were attributed to food sources, and 3.6 million, or 39%, of these illnesses were caused by bacteria. Norovirus is the leading cause of foodborne illness in the U.S., followed by *Salmonella*, *Clostridium perfringens*, and *Campylobacter* spp. (Scallan et al., 2011). The U.S. Centers for Disease Control and Prevention (CDC) estimates the annual burden of foodborne disease to be 48 million sick, 128,000 hospitalized, and 3,000 dead, higher than previously stated due to the high number of unreported and undiagnosed illnesses (CDC, 2014b). Many food commodities have been associated with foodborne pathogen risks, recalls, and illness outbreaks. Produce—including fruits and nuts, and leafy, root, sprout, and vine-stalk vegetables—accounts for 46% of attributed illnesses; whereas, meat and poultry account for only 22% of total illnesses but cause the highest incidence (29%) of deaths, (CDC, 2014b). Therefore, it is important to identify and control foodborne bacterial pathogens in the food system, particularly in meat products.

2.1.1 Shiga Toxin-producing *Escherichia coli*

Of the thousands of strains of bacteria, less than 200 species have been isolated as a cause of human disease (Donnenberg, 2013). *Escherichia coli* is the most widely studied organism of all time with relatively few strains having been associated with human illness. *E. coli*, members of the Enterobacteriaceae family, are short Gram-negative rods that are usually motile, non-sporulating, and exhibit a non-fastidious facultative metabolism (Astridge et al., 2013; Hussein & Bollinger, 2005). *E. coli* can grow in a broad range of conditions. They have been documented to grow between 7 and 46°C, 4.4 and 10 pH, and above 0.95 water activity (a_w) with optimum ranges between 35-40°C, 6-7 pH, and at 0.995 a_w (Astridge et al., 2013).

Moreover, *E. coli* has been known to be more acid-tolerant and heat resistant in its stationary growth phase compared to its log phase (Astridge et al., 2013).

Pathogenic varieties of *E. coli* have been classified into 6 groups: Enteropathogenic *Escherichia coli* (EPEC), Enterotoxigenic *Escherichia coli* (ETEC), Enteroaggregative *Escherichia coli* (EAEC), Enteroinvasive *Escherichia coli* (EIEC), Diffusely Adherent *Escherichia coli* (DAEC), and Enterohemorrhagic *Escherichia coli* (EHEC)(Bettelheim, 2007;CDC, 2012). EHEC are often referred to as STEC or VTEC, due to their ability to produce toxins called either Shiga toxin (Stx) or verotoxin (Vtx), named for their similarity to *Shigella* toxins and their effect on Vero (i.e. African Green monkey kidney) cells (Astridge et al., 2013;Hussein & Bollinger, 2005). These pathotypes are grouped strains of a single species, *E. coli*, that cause a similar disease using a common set of virulence factors.

The pathogenicity of EHEC is due to each strain's specific genetic composition including presence of shiga toxins (Stx1 and Stx2) and attaching and effacing lesions. In EHEC, diverse prophages encode primary virulence characteristics. Stx1 and Stx2 are prophage genes, which indicates that they are silent during growth and reproduction and usually only activated under stress conditions such as exposure to antibiotics and sublethal levels of antimicrobials (CDC, 2012; Donnenberg, 2013). The process of gene amplification, gene duplication in particular, leads to increased virulence which is common of Stx2 in *E. coli* O157:H7 outbreak strains (Donnenberg, 2013). Stx toxin depurinates specific residues of host cell ribosomes, inhibiting protein synthesis, and binds to endothelial cells in the intestine, kidney, and brain causing damage to capillary beds (Hussein & Bollinger, 2005). Initially discovered in EPEC, attaching and effacing (A/E) lesions, internalized within the locus enterocyte effacement (LEE) pathogenicity island, are important for attachment and colonization of STEC within a host (Donnenberg, 2013; Sperandio & Hovde, 2015). LEE is described as a locus divided into 5 major operons housing the genes for adhesion and A/E lesion formation, regulatory proteins Ler, GrIA, and GrIR, and the translocated receptor protein Tir and is not present in non-pathogenic *E. coli* cells (Sperandio & Hovde, 2015). However, the presence of LEE is not necessarily essential for illness as several STEC outbreaks causing hemolytic uremic syndrome (HUS) and hemorrhagic colitis have been LEE-negative (Bettelheim, 2007). Intimin (*eae*) and enterohemolysin (*ehxA*) are the most

important A/E proteins enhancing virulence characteristics responsible for cellular attachment to host intestinal cells and for enterocyte damage, respectively (Hussein & Bollinger, 2005). Type III secretion system (T3SS) effector proteins, specifically Cif, EspF_U, EspK, and NleA, are prophages that are commonly expressed by adding phenotypic traits necessary for colonization and competition (Donnenberg, 2013). T3SS mediates the translocation of A/E lesion proteins directly into host cells thus enhancing virulence (Bhunia, 2008; Sperandio & Hovde, 2015).

Classification as an STEC requires that an *E. coli* isolate produces one or both Stx genes and demonstrates the presence of intimin (CDC, 2012); FDA, 2012). STEC serogroups are designated by their 'O' and/or 'H' type antigen groups where 'O' antigens are somatic lipopolysaccharides and 'H' antigens correspond to bacterial flagella found in motile strains (Hussein & Bollinger, 2005). Serotypes O1, O2, O4, O5, O6, O18, O26, O45, O50, O68, O91, O103, O104, O111, O113, O114, O121, O125, O126, O128, O145, O157, and O165 have been classified as STEC. Of these, those in O groups O26, O103, O111, O145, and O157 are most commonly isolated from humans and clearly recognized as prominent pathogens (Sperandio & Hovde, 2015).

2.1.1.1 *Public health effects of Shiga toxin-producing E. coli*

First recognized as a public health threat in the early 1980's, STEC currently cause more than an estimated 265,000 illnesses, 3,600 hospitalizations, and 30 deaths each year in the United States alone (CDC, 2012). The estimated costs of treating and identifying non-O157 and O157 attributed cases is \$27 million and \$271 million annually, respectively ("USDA ERS - Cost Estimates of Foodborne Illnesses," n.d.).

Human symptoms of STEC infection include hemorrhagic colitis characterized by abdominal cramps and bloody diarrhea and, in severe cases, HUS or thrombotic thrombocytopenia purpura (TTP) (CDC, 2012). Approximately 3-7% of symptomatic cases develop HUS, most prominently children, or TTP, most prominently adults; 3-5% of these cases are fatal (FDA, 2012; Hussein & Bollinger, 2005). However, many asymptomatic illnesses go undiagnosed and unreported accounting for an estimated 96,500 illnesses attributed to *E. coli* O157 and 168,700 illnesses attributed to non-O157 STEC (CDC, 2012). Sickness is commonly caused by consumption of contaminated foods but can also be transmitted through direct

contact with infected persons or animals or the environment (CDC, 2012). Approximately 85% of STEC infections are transmitted by food (Astridge et al., 2013). Consumption of as few as 10 cells has been found to cause illness (Astridge et al., 2013; FDA, 2012). After ingestion, symptoms generally begin following a 3-4 day incubation period and will persist for an average of 8 days (FDA, 2012). Severity of symptoms varies based on host susceptibility with increased morbidity seen in the immunocompromised, pregnant women, children, and the elderly. STEC cells cause illness by attaching to epithelial cells in the intestine via LEE-encoded factors and produce Shiga toxin that is internalized (Astridge et al., 2013); Sperandio & Hovde, 2015). Stx2 and *eae* are associated with higher severity of illness (Donnenberg, 2013). High levels of Stx toxin within a human host results in significant damage to blood vessels in the colon which leads to subsequent hemorrhagic colitis and, in severe cases, HUS characterized by impaired kidney and neurological function (Astridge et al., 2013). STEC-related food recalls and illnesses have been attributed to a wide range of agricultural commodities including wheat flour, sprouts, lettuce, and beef.

Table 2.1 Major Foodborne Illness Outbreaks Attributed to STEC

Year	Serotype	Total No. Cases (deaths)	Food Matrix	Country
2016	O121, O26	63	Flour	USA
2015	O157:H7	19	Costco Rotisserie Chicken Salad	USA
2015	O26	55	Chipotle Mexican Grill Restaurants	USA
2014	O121	19	Raw clover sprouts	USA
2014	O157:H7	12	Ground beef	USA
2013	O121	35	Frozen food products	USA
2012	O26	39	Raw clover sprouts	USA
2012	O145	18 (1)	None identified	USA
2011	O157:H7	60	Romaine lettuce	USA

2011	O104:H4	3,842 (53)	Fenugreek sprouts/seeds	Germany, 13 other European countries, USA, Canada
2010	O145	26	Romaine lettuce	USA
2009	O157:H7	80	Cookie dough	USA
2006	O157:H7	205 (3)	Spinach	USA
2006	O157:H7	71	Taco Bell restaurants	USA
1996-1997	O157:H7	490 (20)	Cooked meat products	Scotland
1996	O157:H7	12, 680 (3)	White radish sprouts	Japan
1995	O111	161 (1)	Fermented mettwurst	Australia
1992-1993	O157:H7	731 (4)	Jack-in-the-Box hamburgers	USA

* Table modified from (Astridge et al., 2013; CDC, 2016; CDC, 2016; CDC, 2015; Schwan, 2015; Sperandio & Hovde, 2015)

2.1.1.2 *Shiga toxin-producing E. coli in the beef industry*

Approximately 200 serotypes of STEC have been recognized; however only 7 strains have been declared adulterants in raw, non-intact beef—*E. coli* O26, O45, O103, O111, O121, O145, and O157: H7), known as the ‘Big 7’ (CDC, 2012; USDA FSIS, 2011). According to the United States Food and Drug Administration (2012), a food is considered adulterated if it:

”1) contains a poisonous or otherwise harmful substance that is not an inherent natural constituent of the food itself [i.e. STEC], in an amount that poses a reasonable possibility of injury to health, or 2) a substance that is an inherent natural constituent of the food itself; is not the result of environmental, agricultural, industrial, or other contamination; and is present in an amount that ordinarily renders the food injurious to health”.

Since the 1990s, following a multi-state outbreak of *E. coli* O157:H7 in undercooked beef hamburgers at Jack-in-the-Box quick-serve restaurants, controlling Shiga toxin-producing *E. coli* has been a high priority for regulatory agencies and beef processors. Cattle are recognized as a primary reservoir for EHEC (Bhunja, 2008; Sperandio & Hovde, 2015). STEC are often transferred to humans, or other animals and the environment, via fecal shedding. *E. coli* O157 is found on nearly all cattle farms, although the prevalence of any the ‘Big 7’ STEC in cattle herds is highly variable (Sperandio & Hovde, 2015). In the United States, herd prevalence of

O157 can vary between types of cattle and by season with a range of 0.2-27.8% and 0.2-48.8% for O157 and 2.1-70.1% and 0.4-74% for non-O157 STECs in beef cattle and dairy cattle, respectively (Hussein & Bollinger, 2005; Hussein & Sakuma, 2005; Stephens et al., 2007). In the warmest summer months, colonized cattle can shed STEC cells for up to 10 weeks at levels as high as 1.1×10^5 CFU/g feces (Fegan, et al., 2004). Animals excreting greater than 10^4 CFU/g are termed 'super-shedders' and typically only make up a small fraction of the cattle population; however, these animals are arguably responsible for a majority of cattle-to-cattle and environmental contamination (Sperandio & Hovde, 2015). In most prevalence studies, the highest rates of STEC in beef are reported during the warmest spring and summer months which is consistent with the timing of most human infections (Hussein & Bollinger, 2005). Prevalence of STEC in feces often leads to contamination of beef hides and subsequent transfer to the carcass during slaughter. Higher levels of STEC on carcass surfaces may persist throughout the beef processing chain into ground beef and other consumer products.

Table 2.2 Number of Beef Recalls Due to STEC from 2010 to 2015

Year	Number of STEC-caused beef recalls	Total Number of Beef Recalls	Pounds of beef related due to STEC
2010	12	28	2,313,423
2011	13	35	1,002,971
2012	5	19	63,467
2013	7	20	89,919
2014	5	22	1,840,533
2015	8	41	214,613*
Total	50	165	5,524,926

* Recall with an undefined number of pounds included

** Table adapted from FSIS Recall Summaries (FSIS, 2015; FSIS, 2015; FSIS, 2015; FSIS, 2015; FSIS, 2015; FSIS, 2016; Schwan, 2015).

In slaughter plants, the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) tests for *E. coli* O157:H7 in beef trimmings, raw ground components (i.e. cheek meat, head meat, heart meat) generated onsite, and any raw meat that is ground and/or formed into patties. Between 1 (very small processors <1001 lbs/day) and 4

(large processors >600,000 lbs/day) samples are taken per month. Bench trim produced offsite and used to produce secondary trimmings or raw ground product is also subject to testing (USDA FSIS, 2015). Tests are also conducted for the 'Big 6' non-O157 STEC (O26, O45, O103, O111, O121, and O145) in raw beef trimmings (USDA FSIS, 2015). Raw, non-intact beef is of special concern to the industry due to the nature of the product where the potentially contaminated exterior muscle surface is either comminuted, in the case of grinding, or translocated, in the case of blade-tenderization, to the sterile interior muscle.

2.1.2 *Salmonella* Species

Similar to *E. coli*, *Salmonella* species is a Gram-negative rod and member of the Enterobacteriaceae family (FDA, 2012). These bacteria are non-fastidious, facultative aerobes that are mostly mobile due to peritrichous flagella (Bhunia, 2008; Cliver & Riemann, 2002). *Salmonella* can grow in a broad range of conditions. They have been documented to grow between 5 and 45°C at 4.4 and 9.4 pH, with an optimum temperature range of 35-37°C (Bhunia, 2008). Historically, *Salmonella* species have been named based on their places of origin (ex. Heidelberg, Tennessee, Dublin, Montevideo); this system has been discontinued and new strains are now identified by their susceptibility to different phages and resistance to antibiotics. Similar to *E. coli*, *Salmonella* spp. are also grouped based on their O and H antigens (Bhunia, 2008).

Also similar to STEC, *Salmonella* virulence factors are encoded on pathogenicity islands (SPI). There are 12 SPIs, however, only 10 of them encode virulence genes for *S. enterica*, Enteritidis, and Typhimurium: SPI-1—T3SS, invasion, iron uptake; SPI-2—T3SS, invasion, systemic infection; SPI-3—Mg²⁺ uptake, macrophage survival; SPI-4—macrophage survival; SPI-5—enteropathogenicity; SPI-6—fimbriae; SPI-9—Type 1 secretion system, RTX-like toxin; SPI-10—Sef fimbriae; SGI-1—antibiotic resistance genes; and HPI—affinity for iron uptake, septicemia (Bhunia, 2008). Invasion genes, encoded in SPI-1, are maximally expressed during the late phase of growth at high osmolarity, neutral pH, and 37°C (Bhunia, 2008). *Salmonella* and STEC both use a Type III secretion system to enter host cells.

Salmonella causes 3 forms of disease: typhoid fever, non-typhoidal gastroenteritis, and bacteremia. Gastroenteritis is the most common form of infection from foodborne sources.

Unlike *E. coli*, *Salmonella* species cause gastrointestinal illness and damage primarily by invading cells and colonizing within a host. *Salmonella* invades host cells by using a ruffling mechanism, also called the ‘trigger mechanism’, to bind to a host cell receptor inducing pinocytosis. This occurs in the enterocytes and M-cells of the large and small intestines subsequently activating an LPS-induced inflammatory response (Bhunias, 2008; Cliver & Riemann, 2002). This response evokes symptomatic illness in the host. Invasive *Salmonella* will also enter macrophages to evade death from the host’s natural responses. *Salmonella* survival in macrophages facilitates the organism’s ability to evade and colonize other areas of the host including lymph nodes, the liver, kidney, spleen, and blood vessels (Cliver & Riemann, 2002). Independent of M-cell-mediated entry, *Salmonella* will invade apical epithelium in the ileum, cecum, and proximal colon, which is characterized by neutrophil infiltration, necrosis, edema, and fluid secretion (Bhunias, 2008). Invasion is mediated by 3 mechanisms: 1) phagocytosis by M-cells, as described above, 2) phagocytosis by dendritic cells, and 3) induced phagocytosis by epithelial cells. When enclosed in a dendritic cell, *Salmonella* secretes effector proteins and, similar to macrophage engulfment, is transported to different extra-intestinal sites for dissemination of bacteria (Bhunias, 2008). Induced phagocytosis by epithelial cells is facilitated by T3SS causing cell lysis upon *Salmonella* uptake (Bhunias, 2008).

Table 2.3 Major Foodborne Illness Outbreaks Attributed to *Salmonella* in the United States

Year	Strain	Total No. Cases (deaths)	Food Matrix
2016	Reading, Abony	30	Alfalfa sprouts
2016	Virchow	33	RAW Meal Organic Shake and Meal Products
2015	Poona	907 (6)	Cucumbers
2015	MDR I 4, 12:i:-; Infantis	192	Pork
2014	Enteritidis	115	Bean Sprouts
2013	MDR Heidelberg	634	Foster Farms Chicken
2013	Montevideo, Mbandaka	16 (1)	Tahini Sesame Paste
2013	Typhimurium	22	Ground Beef
2012	Enteritidis	46	Ground Beef

2012	Bareilly, Nchanga	425	Raw Scraped Ground Tuna
2011	Typhimurium	20	Ground Beef
2011	Agona	106	Fresh Papaya
2011	Heidelberg	136 (1)	Ground Turkey
2008-2009	Typhimurium	714 (9)	Peanut Butter
2008	Saintpaul	1,442 (2)	Jalapeno and Serrano Peppers, Tomatoes
2006	Tennessee	628	Peter Pan/Great Value Peanut Butter

(CDC, 2016; CDC, 2015; CDC, 2015; CDC, 2014a); CDC, 2013b; CDC, 2013a; CDC, 2007; CDC, 2009; CDC, 2011a; CDC, 2011b)

2.1.2.1 *Salmonella* in the beef industry

Cattle are well known reservoirs of *Salmonella* species. This has been confirmed in studies where *Salmonella* has been isolated in 76-100% of cattle tested (Gragg et al., 2013; McEvoy et al., 2003; Stephens et al., 2007). Similar to STEC transmission, *Salmonella* present in cattle feces leads to the contamination of hides and subsequent contamination of carcasses and beef products for human consumption. Although not declared an adulterant in beef products, *Salmonella* contamination is a major food safety hazard that is considered reasonably likely to occur in beef. Laufer et al. (2015) reported that out of the 1,965 *Salmonella* outbreaks reported to the CDC from 1953-2011, 96 outbreaks were attributed to beef. Before 1987, delicatessen-style roast beef was the predominant type of beef associated with illness; however, ground beef emerged as the major contributor of beef associated salmonellosis, accounting for 35% of the *Salmonella* outbreaks between 2002 and 2011 (Laufer et al., 2015).

Table 2.4 Number of Beef Recalls Due to *Salmonella* from 2010 to 2015

Year	Number of <i>Salmonella</i>-caused beef recalls	Total Number of Beef Recalls	Pounds of beef related due to <i>Salmonella</i>
2010	2	28	1,815,700
2011	2	35	126*
2012	1	19	29,339
2013	2	20	1,050
2014	0	22	0
2015	0	41	0

Total	7	165	1,846,215
--------------	----------	------------	------------------

* Recall with an undefined number of pounds included. Initiated by salmonellosis outbreak with 11 of 14 ill persons reporting having consumed ground beef and 10 of 14 reporting having purchased ground beef at Hannaford grocery stores between Oct. 12 and Nov. 20 (FSIS, 2011)

** Table adapted from FSIS Recall Summaries (FSIS, 2015; FSIS, 2015; FSIS, 2015; FSIS, 2015; FSIS, 2015; FSIS, 2016)

Although *Salmonella* has not been implicated in any beef recalls recently, USDA FSIS is under pressure to determine why people are still getting sick from contaminated beef. Less than 1% of beef carcasses test positive for *Salmonella* at the end of the slaughter process (Gabbett, 2016), whereas, 4.2 to 10% of ground beef tests positive (Bosilevac et al., 2009; Mani-López et al., 2012). The low percentage of carcass surface contamination indicates that the problem is independent of the success of carcass-level antimicrobial interventions (Barkocy-Gallagher et al., 2003). A growing number of studies indicate that *Salmonella* can be harbored in bovine deep tissue lymph nodes (DTLNs) embedded in adipose tissues that are frequently included in ground beef, thus serving as the primary vehicle for transferring pathogens into the final product (Gragg et al., 2013; Li et al., 2015). Contaminated lymph nodes also add a considerably higher level of contamination to ground beef ($\sim 10^3$ CFU/DTLN) than post-intervention carcass surfaces ($\sim 10^{1.5}$ CFU/8000 cm²) (Li et al., 2015). A diverse population of *Salmonella* strains have been recovered from beef; within-animal diversity of *Salmonella* strains recovered from lymph nodes and beef carcasses is common (Gragg et al., 2013; McEvoy et al., 2003).

In 2014, FSIS announced that the agency would begin analyzing all raw beef samples collected for STEC analysis for *Salmonella* to gather necessary data to determine an updated prevalence in ground beef and beef trim to propose new performance standards (FSIS, 2014). The current *Salmonella* Performance Standard for ground beef is 7.5% or a maximum of 5 positives per establishment (FSIS, 2013). Following FSIS Directive 10,010.1, beef trimmings, raw ground and any other raw meat that is ground into patties in slaughter facilities, and bench trim generated offsite and processed into secondary trimmings or raw ground product is also

subject to testing for *Salmonella*; however, beef patties and fabricated steaks are not subject for testing (USDA FSIS, 2015).

2.2 Interventions to Control Foodborne Pathogens during Commercial Beef Processing

Beef processors have a moral and ethical responsibility to provide safe meat to consumers across the globe. Thermal- and chemical-based intervention technologies to inactivate biological hazards are commonly utilized throughout the beef processing industry. Specific processing steps, such as live animal or hide washing/antimicrobial sprays, knife trimming, steam vacuuming, hot water washes, and organic acid washes of carcasses and subprimals have been added to the beef slaughter process to mitigate the transfer of pathogenic organisms (Sofos, 2008; Sohaib et al., 2016). Multiple hurdle strategies are recognized to be most effective in reducing microorganisms throughout the beef slaughter operation and during subsequent fabrication processes (Sofos, 2008).

Organic acids are commonly used antimicrobials in the beef industry due to their ease of application, low cost, and generally recognized as safe (GRAS) status (Mani-López et al., 2012). Microbial activity is generally affected by two primary mechanisms: 1) cytoplasm acidification with subsequent uncoupling of energy production and regulation, and 2) accumulation of dissociated acid at toxic levels within the cell (Mani-López et al., 2012). Organic acids, specifically lactic, citric, acetic, and peracetic or a combination thereof, sprayed at ~4.0% concentrations onto carcasses are effective in reducing *Salmonella* and *E. coli* bacterial loads by ~1-2 log cycles; however, spraying higher levels can result in undesirable bleaching of the muscle tissue (Mani-López et al., 2012; Sohaib et al., 2016). Lactic acid is the most commonly used organic acid in the beef industry because of its effective kill against pathogenic microorganisms, with a comparatively lesser effect on the color and flavor of meat compared to other organic acids (Sohaib et al., 2016; Schwan, 2015).

A variety of novel technologically based interventions are being evaluated and implemented in contrast to chemical use. Some of these include high hydrostatic pressure

(HHP) processing, pulsed electric fields, ultrasonic waves, oscillating magnetic fields, use of bacteriophages, and antimicrobial packaging ingredients (Sofos, 2008).

2.2.1 Chlorine-based Antimicrobials

Chlorine is applied as antimicrobial in a variety of forms as directed by the USDA FSIS Safe and Suitable Ingredients list (USDA FSIS, 2016) for meat and poultry (Table 2.5 Use of Chlorinated Antimicrobials Approved by the United States Department of Agriculture Food Safety and Inspection Service). Free chlorine, unbound available chlorine, is much more effective as an antimicrobial and therefore is the most important measurement of a sanitizer's antimicrobial activity. Factors contributing to stability of chlorine in solution include pH, low temperature, absence of catalysts, high alkalinity, and absence of organic material. In aqueous solution, free available chlorine (FAC) can be present as either hypochlorous acid (HOCl), at pH levels below 6.8, or hypochlorite (ClO⁻): $\text{Cl}_2 + \text{H}_2\text{O} \rightarrow \text{HOCl} + \text{H}^+ + \text{Cl}^-$ (Block, 1991). Although the exact mechanism by which FAC destroys microorganisms has not fully been elucidated, chlorine has been found to be effective against both Gram-positive and Gram-negative bacteria due to its strong oxidative potential which disrupts bacterial cell walls resulting in cleavage of DNA (CDC, 2009; Sohaib et al., 2016). Being uncharged, it is believed that hypochlorous acid effectively inactivates *E. coli* cells by acidifying the cytoplasm forcing the organism to dissociate from the inside and by disrupting metabolism by specifically inhibiting the transfer of needed fermentative and respiratory substrates, glucose and succinate, thus irreversibly abolishing ATP production even in the presence of nutrient sources (Block, 1991; Barrette et al., 1989; Najjar & Meng, 2009). On the other hand, the bactericidal effect of the less germicidal hypochlorite is characterized by the penetration of germicidal ingredients into the cell and the subsequent formation of toxic complexes (N-chloro compounds) in the cell protoplasm (Block, 1991).

pH has the greatest impact on the effectiveness of chlorine solutions (Block, 1991). Hypochlorous acid is the primary disinfection agent, and at pH 5, HOCl constitutes 97% of total chlorine in solution (Park et al., 2004). The dissociation of hypochlorous acid ($\text{HOCl} \leftrightarrow \text{H}^+ + \text{ClO}^-$) is highly dependent on pH; as pH increases, the disinfection capacity of chlorine decreases (Block, 1991). The effectiveness of chlorine as a disinfectant is dependent on acidic pH (i.e. presence of hypochlorous acid), concentration of chlorine maintained in solution, and contact

time (Najjar & Meng, 2009; Zhou et al., 2015). Lethality of chlorine based solutions increases as temperature increases, although, temperature does not affect the coefficient of pH. It has been observed that at 25 ppm concentration hypochlorite solution at pH 5, 7, and 10, the necessary exposure time to kill bacteria was increased by up to 2.3 times with each 10°C drop in temperature (Block, 1991).

In poultry and produce processing, chlorinated water, often sprayed or added to chill tank water, is the most common and widely used antimicrobial in the United States due to its low cost and efficacy against pathogens (Sohaib et al., 2016; Yang et al., 2012). However, bacterial reductions on poultry show variable results. The incorporation of 18-25 ppm chlorine into chill water has been found to significantly reduce *Salmonella* (Sohaib et al., 2016). In poultry chiller tanks, chlorination of water has been found to be most effective if an initial level of 50 ppm FAC is used and maintained at 5 ppm residual chlorine (Najjar & Meng, 2009). The produce industry uses high levels of chlorine, 25-250 ppm FAC, to inactivate pathogens due to a short contact time (Najjar & Meng, 2009; Stopforth et al., 2008). Most commercially available chlorine based sanitizers reduce pathogens on the surface of produce by 1-2 log cycles (Stopforth et al., 2008; Yang et al., 2012). While chlorine has been found to be an effective pathogen control, its capacity to inactivate pathogens on the surface of products, especially produce, is limited. Chlorine, however, is extremely effective at controlling pathogen levels in wash water (Zhou et al., 2015). Chlorinated water can also limit the growth of biofilms on food processing equipment (Najjar & Meng, 2009). Chlorine based sanitizers are utilized to clean in-plant utensils, large equipment, and food contact surfaces with hypochlorite based 50-200 ppm FAC for a minimum of 10 seconds or longer (Block, 1991). Block (1991) reported the capacity of hypochlorite solutions to inhibit immediate biofilm growth when exposed to 0.5 and 5 ppm solutions or create extended antimicrobial effects after exposure to 50 ppm FAC solutions. As the FAC level in solution increases, logically, the antimicrobial capacity of the solution also increases as long as all other factors including pH, temperature, and organic content remain constant (Block, 1991).

A major limitation of chlorine in the meat and poultry industry is that it is easily bound and deactivated by organic matter (Najjar & Meng, 2009; Block, 1991; Sohaib et al., 2016). The

difference between the chlorine that is bound by organic matter and the residual chlorine that remains is referred to as chlorine demand (Block, 1991). ‘Breakpoint’ chlorination is often used to account for chlorine demand by adding levels of chlorine in solution that satisfy initial demand and provide residual chlorine at levels necessary for antimicrobial action (Block, 1991). In drinking water, where very low levels of organic materials are present, low levels of residual chlorine are effective. Higher levels of chlorine are required in the meat industry where high organic loads are encountered (Najjar & Meng, 2009). However, in the presence of proteins (specifically amine, amide, imine, or imide N-groups) HOCl will form chloramines and retain some level of antimicrobial effect even when free available chlorine is reduced. Block (1991) reported 100% reductions of *Salmonella pullorum* in a 130 ppm hypochlorite solution with 5% organic matter, although there was no measurable level of FAC, thus showing the sanitizing capacity of chloramines. Aside from the presence and level of organic material in a solution, the initial chlorine levels, presence and level of catalysts (copper, nickel, cobalt), pH, temperature, and ultraviolet radiation are also factors that can decrease stability and effectiveness of chlorine in aqueous solution (Block, 1991).

Table 2.5 Use of Chlorinated Antimicrobials Approved by the United States Department of Agriculture Food Safety and Inspection Service

Substance	Product	Amount Allowed	Labeling Requirements
Acidified sodium chlorite*	Poultry carcasses and parts; meat carcasses, parts, and organs; processed comminuted, or formed meat food products (including RTE)	500 to 1200 ppm in combination with any GRAS acid at a level sufficient to achieve a pH of 2.3 to 2.9 in accordance with 21 CFR 173.325	None under accepted conditions of use
Acidified sodium chlorite*	Processed, comminuted or formed poultry products (including RTE)		None under accepted conditions of use
Acidified sodium chlorite*	Poultry carcasses, parts, trim, and organs	Mixing an aqueous solution of sodium chlorite with any	None under accepted conditions of use

		<p>GRAS acid to achieve pH of 2.2 to 3 then further diluting this solution with a pH elevating agent to a final pH of 3.5 to 7.5. When used in a spray or dip the final sodium chlorite concentration does not exceed 1200 mg/kg and chlorine dioxide concentration does not exceed 30 mg/kg. When used in a pre-chiller solution on poultry carcasses and parts the additive is used at a level that results in sodium chlorite concentrations between 50 and 150 ppm. Contact times may be up to several minutes at temperatures between 0 and 15°C</p>	
<p>Acidified sodium chlorite*</p>	<p>Red meat, red meat parts and organs, and on processed, comminuted, formed meat products (including RTE)</p>	<p>Applied as a spray or dip, the additive is produced by mixing an aqueous solution of sodium chlorite with any GRAS acid to achieve a pH range of 2.2 to 3, then further diluting this solution with a pH elevating agent such that the sodium chlorite concentration does not exceed 1200</p>	<p>None under accepted conditions of use</p>

		ppm, and the chlorine dioxide concentration does not exceed 30 ppm. The pH of the use solution is between 3.5 and 7.5.	
Calcium hypochlorite	Red meat carcasses down to a quarter of a carcass	Applied as a spray at a level not to exceed 50 ppm FAC measured prior to application	None under accepted conditions of use
Calcium hypochlorite	On whole or eviscerated poultry carcasses	Applied as a spray at a level not to exceed 50 ppm FAC as measured prior to application	None under accepted conditions of use
Calcium hypochlorite	In water used in meat processing	Not to exceed 5 ppm calculated as FAC	None under accepted conditions of use
Calcium hypochlorite	In water used in poultry processing (except product formulation)	Not to exceed 50 ppm calculated as FAC	None under accepted conditions of use
Calcium hypochlorite	Poultry chiller water	Not to exceed 50 ppm calculated as FAC (measured in the incoming potable water)	None under accepted conditions of use
Calcium hypochlorite	Poultry chiller red water (i.e. recirculated water)	Not to exceed 5 ppm FAC (measured at influent to chiller)	None under accepted conditions of use
Calcium hypochlorite	Reprocessing contaminated poultry carcasses	20 ppm FAC (Agency guidance has allowed use of up to 50 ppm FAC)	None under accepted conditions of use
Calcium hypochlorite	On giblets	Not to exceed 50 ppm FAC in the influent to a container for chilling	None under accepted conditions of use
	Beef primals	20 ppm FAC	None under accepted conditions of use
Chlorine Dioxide	Applied to red meat, processed, comminuted, or	Applied as a spray or dip at a level not to exceed 3 ppm	None under accepted conditions of use

	formed meat products	residual chlorine dioxide. The application of chlorine dioxide shall be followed by a potable water rinse or by blanching, cooking, or canning.	
Chlorine Dioxide	In water used in poultry processing	At levels not to exceed 3 ppm residual chlorine dioxide and in accordance with 21 CFR 173.300	None under accepted conditions of use
Chlorine Dioxide	In water used in poultry processing	At levels not to exceed 3 ppm residual chlorine dioxide as determined by method 45000-ClO ₂ -E or 4500-ClO ₂ -D modified with UV absorbance at 360 nm in the "Standard for Methods for Examination of Water and Wastewater" 20 th ed., or an equivalent method	None under accepted conditions of use
Chlorine Dioxide	Red meat, red meat parts and organs; processed, comminuted, or formed meat food products	Applied as a spray or dip at a level not to exceed 3 ppm residual chlorine	None under accepted conditions of use
Chlorine gas	Red meat carcasses down to a quarter of a carcass	Applied as a spray at a level not to exceed 50 ppm FAC measured prior to application	None under accepted conditions of use
Chlorine gas	On whole or eviscerated poultry carcasses		None under accepted conditions of use

Chlorine gas	In water used in meat processing	Not to exceed 5 ppm FAC	None under accepted conditions of use
Chlorine gas	In water used in poultry processing (except product formulation)	Not to exceed 50 ppm FAC	None under accepted conditions of use
Chlorine gas	Poultry chiller water	Not to exceed 50 ppm FAC (measured in incoming potable water)	None under accepted conditions of use
Chlorine gas	Poultry chiller red water (i.e. recirculating water)	Not to exceed 5 ppm FAC (measured at influent to chiller)	None under accepted conditions of use
Chlorine gas	Reprocessing contaminating poultry	20 ppm FAC (Agency guidance has allowed use of up to 50 ppm FAC)	None under accepted conditions of use
Chlorine gas	On giblets	Not to exceed 50 ppm FAC influent to a container for chilling	None under accepted conditions of use
Chlorine gas	Beef primals	20 ppm FAC	None under accepted conditions of use
Electrolytically generated hypochlorous acid	Red meat carcasses down to a quarter of a carcass	Applied as a spray at a level not to exceed 50 ppm FAC	None under accepted conditions of use
Electrolytically generated hypochlorous acid	On whole or eviscerated poultry carcasses	Applied as a spray at a level not to exceed 50 ppm FAC measured prior to application	None under accepted conditions of use
Electrolytically generated hypochlorous acid	In water used in meat processing	Not to exceed 5 ppm FAC measured prior to application	None under accepted conditions of use
Electrolytically generated hypochlorous acid	In water used in poultry processing (except for product formulation)	Not to exceed 50 ppm FAC	None under accepted conditions of use
Electrolytically generated hypochlorous acid	Poultry chiller water	Not to exceed 50 ppm FAC (measured in the incoming potable water)	None under accepted conditions of use
Electrolytically generated hypochlorous acid	Poultry chiller red water (i.e. recirculating water)	Not to exceed 5 ppm FAC (measured influent to chiller)	None under accepted conditions of use

Electrolytically generated hypochlorous acid	Reprocessing contaminating poultry	20 ppm FAC (Agency guidance has allowed use of up to 50 ppm FAC)	None under accepted conditions of use
Electrolytically generated hypochlorous acid	On giblets	Not to exceed 50 ppm FAC influent to a container for chilling	None under accepted conditions of use
Electrolytically generated hypochlorous acid	Beef primals	20 ppm FAC	None under accepted conditions of use
Sodium hypochlorite	Red meat carcasses down to a quarter of a carcass	Applied as a spray at a level not to exceed 50 ppm FAC	None under accepted conditions of use
Sodium hypochlorite	On whole or eviscerated poultry carcasses	Applied as a spray at a level not to exceed 50 ppm FAC measured prior to application	None under accepted conditions of use
Sodium hypochlorite	In water used in meat processing	Not to exceed 5 ppm FAC measured prior to application	None under accepted conditions of use
Sodium hypochlorite	In water used in poultry processing (except for product formulation)	Not to exceed 50 ppm FAC	None under accepted conditions of use
Sodium hypochlorite	Poultry chiller water	Not to exceed 50 ppm FAC (measured in the incoming potable water)	None under accepted conditions of use
Sodium hypochlorite	Poultry chiller red water (i.e. recirculating water)	Not to exceed 5 ppm FAC (measured influent to chiller)	None under accepted conditions of use
Sodium hypochlorite	Reprocessing contaminating poultry	20 ppm FAC (Agency guidance has allowed use of up to 50 ppm FAC)	None under accepted conditions of use
Sodium hypochlorite	On giblets	Not to exceed 50 ppm FAC influent to a container for chilling	
Sodium hypochlorite	Beef primals	20 ppm FAC	

Table adapted from FSIS Directive 7120.1 Safe and Suitable Ingredients list (USDA FSIS, 2016).

* In the presence of acid, sodium chlorite will form into chlorine byproducts chlorite (ClO^-), chlorine dioxide (ClO_2), and chloride (Cl^-) (Rao, 2007).

2.2.1.1 Electrolyzed water

Due to health and environmental concerns, interventions requiring very low levels of active chemicals are of interest. One method of generating chlorine in water solutions using low levels of chemicals is with electrolyzed-oxidized (EO) water, also referred to as electrolyzed water. EO water is generated by the passage of a dilute salt solution (~1% NaCl) through an electrochemical cell converting chloride ions and water molecules into chlorine oxidants (Guentzel et al., 2008) until a desired level of FAC is achieved. During electrolysis, NaCl and H₂O dissociate into positively charged (Na⁺, H⁺) and negatively charged (Cl⁻, OH⁻) ions which give up electrons to become oxygen, chlorine, or hydrogen gas and hypochlorite, hypochlorous acid, and hydrochloric acid (Hricova, Stephan, & Zweifel, 2008). When generated, two streams are formed; 1) a basic stream at pH ~11, -800 mV Oxidation Reduction Potential (ORP) and 2) an acidic stream at pH ~3, 10-90 ppm FAC, 1100 mV ORP (Hricova, Stephan, & Zweifel, 2008; Jadeja & Hung, 2014; Liao, Chen, & Xiao, 2007). EO water demonstrates an antimicrobial effectiveness ten times greater than traditional chlorine solutions of sodium hypochlorite (Soli et al., 2010). Acidic solutions of EO water contain primarily hypochlorous acid and a high ORP above 800 mV (Liao et al., 2007). A high ORP facilitates HOCl transfer into the cell by disrupting the outer membrane and metabolic processes of cells (Hricova et al., 2008). ORP levels over 848 mV have been proven to completely inactivate *E. coli* O157:H7 regardless of FAC present in the solution (Hricova et al., 2008; Liao et al., 2007). A major advantage of EO water over hydrochloric acid is that EO water is non-corrosive and environmentally friendly to produce (Y.-R. Huang et al., 2008). The application of EO water at 5-6.5 pH minimizes detrimental health effects from Cl₂ gassing off (Guentzel et al., 2008), while maintaining the antimicrobial effects of hypochlorous acid present in solution. Acidic EO water combined with cool storage temperatures and agitation resulted in stabilized residual FAC and ORP levels in solution and increased microbial reductions (Hricova et al., 2008).

EO water was initially used by the food industry as a disinfectant to control biofilm formation on food contact surfaces and equipment such as cutting boards and stainless steel (Hricova et al., 2008; Y.-R. Huang et al., 2008). Relatively little research has been conducted evaluating the efficacy of EO waters on beef products. EO water was found to be effective in

reducing *S. Typhimurium* and *E. coli* O157:H7 (0.65-1.09 log CFU/cm² reductions) on cattle hides as a potential pre-harvest intervention spray but not as effective as 5% lactic acid (2.75 log CFU/cm²) (Jadeja & Hung, 2014). Other researchers reported 3.5-4.3 log CFU/100 cm² reductions on cattle hides, similar to other traditional organic acid antimicrobials as measured by total plate and *Enterobacteriaceae* counts, after application of pressurized pH 11.2 EO water and pH 2.4 EO water (Hricova et al., 2008). EO water can be extremely bactericidal to *Listeria monocytogenes* and *E. coli* O157:H7 at low levels of FAC (1-2 ppm) at a wide pH range in a wash solution, indicating that low residual levels can still be effective antimicrobials (Park et al., 2004). Issa-Zacharia et al. (2011) reported statistically similar reductions in *E. coli* (2.7, 2.8 and 2.8 log CFU/g) and *Salmonella* spp. (2.7, 2.5, and 2.45 log CFU/g) populations on Chinese celery, lettuce, and daikon sprouts, respectively, with slightly acidic (5.0-6.5 pH) EO water (22.1 ± 0.7 ppm FAC) compared to 100 ppm FAC sodium hypochlorite solution. Whereas, Stopforth et al. (2008) reported higher reductions of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* (3.0-3.38 log CFU/g) on leafy greens treated with EO water compared to chlorine (2.1-2.8 log CFU/g). Antimicrobial effects of EO waters have also been studied on tuna fillets, tilapia, salmon, pork belly, frankfurters, broiler carcasses, chicken wings, strawberries, apples, sprouts, lettuce, alfalfa sprouts, carrots, spinach, potatoes, radish, and bell pepper (Y.-R. Huang et al., 2008). Overall, EO water has been found to reduce levels of contamination present in food processing environments, thus proving to be an appropriate alternative antimicrobial in a wash solution.

2.2.1.2 Chlorine disinfection by-products

While chlorine has proven to be an effective antimicrobial, it is important to manage and balance the risks between reducing microbial pathogens and the formation of chemical disinfection by-products at levels potentially harmful to human health (EPA, 2001). Use of chlorine as a disinfectant in water systems may lead to the formation of disinfection by-products (DBPs), specifically the trihalomethanes (THM) CHCl₃, CHCl₂Br, CHClBr₂, and CHBr₃ (A.-T. Huang & Batterman, 2009). The most recognized and predominant THM is trichloromethane, better known as chloroform (Vizzier-Thaxton et al., 2010). THM formation is often the result of chlorine residuals, from cleaning detergents or food contact disinfectants for example, and precursors present in foods such as protein (A.-T. Huang & Batterman, 2009). The most

important precursors in THM formation include total organic carbons (TOC), specifically natural organic material (NOM) and effluent organic matter.

THMs have been linked to detrimental health effects including cancers and birth defects in laboratory animals (EPA, 2001). However, chlorine is still widely used as an effective antimicrobial and disinfectant in the meat, poultry, and produce processing industries. In recent years there has been concern as to whether THMs, potential carcinogens, are formed on the surface of meat tissues exposed to chlorine in process water and washing systems (Vizzier-Thaxton et al., 2010). The risk of residual levels of disinfection by-products has been assessed on the surface of chicken tissues; chicken skin and fat exposed to a traditional chlorinated water chill tank (50 ppm) contained a median of 3.6 and 4.5 ppm chlorine, respectively (Najjar & Meng, 2009). Studies have shown significant reductions (from 255 ppb to 46 ppb) of THMs during cooking of meat exposed to chilled chlorinated water due to the boiling temperature of chloroform (65°C) (Najjar & Meng, 2009). These authors concluded that the chlorinated by-products generated by chilling poultry carcasses in 50 ppm chlorinated water posed minimal risk to human health, as they determined that 99% of humans' daily exposure to these chemical compounds is attributed to water, and poultry chilled in chlorinated water and subsequently cooked contributes only 0.3-1% of daily exposure.

Chlorine dioxide gas (ClO₂), although most commonly used in the treatment of drinking water and wastewater, is soluble in water and does not form THMs, partially due to its chemical nature of not forming hypochlorite or hypochlorous acid ($\text{Cl}_2 + 2\text{NaClO}_2 \rightarrow 2\text{ClO}_2 + 2\text{NaCl}$; Block, 1991). Thus, ClO₂ is an option for chlorination with lower possibility of harmful by-product formation. However, use of ClO₂ can be dangerous in workplace environments.

THM levels are regulated in drinking water. The maximum residual level for combined THMs is 0.08 mg/L, and a limit of 0.06 mg/L is set for dibromochloromethane (CHClBr₂) specifically (EPA, 2001). There are no regulations for THM concentrations in food industry process water or in foods. Maximum use levels have been set for food industry use and for drinking water in the United States, Australia, and EU to mitigate worker exposure to toxic levels of these disinfection products. Poultry chiller water maintained at 50 ppm chlorine accounts for 0.3-1% of human THM exposure, thus not posing a significant risk for cancer or

other health conditions from consuming poultry products (Najjar & Meng, 2009). It is important to recognize that the public health gains from reduced waterborne and foodborne illnesses by using chlorine in food processing outweigh the human health risks.

2.2.2 Nanobubble Technology in the Food Industry

Nanobubble technology is a relatively new concept in the food industry, showing promise to aid in the development of improved food safety interventions. Currently, there are two types of nanobubbles available: 1) oxygen-nanobubbles, produced from air, and 2) ozonated nanobubbles. Nanobubbles are generally formed from the collapsing of microbubbles through a process known as cavitation, and are difficult to quantify and measure

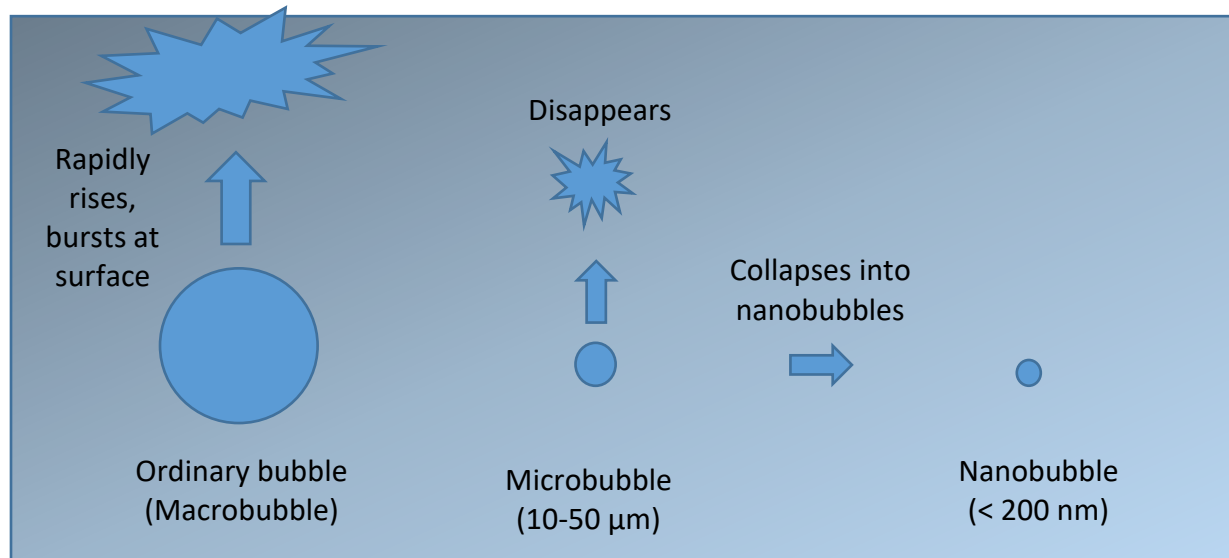


Figure 2.1 Schematic diagram of bubble size in aqueous solution; adapted from Agarwal, Ng, & Liu, 2011

due to their size.

Micro- and nanobubbles are generally formed by cavitation; cavitation can be caused by acoustic, hydrodynamic, optic and/or particle based methods (Agarwal, Ng, & Liu, 2011). Acoustic nanobubbles are formed by passage of ultrasonic waves through a liquid solution, while hydrodynamic cavitation is directed by varying pressure and flow. Within these modes of formation, gas-water circulation and pressurized decompression methods are implemented for gas dissolution (Agarwal et al., 2011). In the studies described in this thesis, nanobubbles are generated by a patented (U.S. Patent No. 8454837) generator that utilizes a hydrodynamic

method involving cavitation chambers and shear planes to initiate an endothermic reaction. This process produces a high concentration of paramagnetic oxygen nanobubbles with a mean particle size between 50 and 100 nm ("Bauer Nanobubbles," 2016). The presence of unpaired electrons and the subsequent realignment of electron paths caused by a magnetic field causes paramagnetic properties. It was previously believed that nanobubbles disappeared in solution, but it is now understood that the bubbles are stable up to months after the dispersion of microbubbles due to their electrical charge (Agarwal, Ng, & Liu, 2011; Tsuge, 2014). Less than 1 μm in size, as often determined by dynamic light scattering, nanobubbles are most useful when measured by zeta potential. Zeta potential is surface charge of a molecule when suspended in a fluid system or the degree of repulsion between similarly charged particles in colloidal dispersions (Particle Sciences, 2012; Tsuge, 2014). A high zeta potential will confer stability within a solution.

The interest in nanobubble technology has increased due to their proposed surfactant abilities or cleaning effect. Small particles in water can be effectively removed by introducing micro- or nanobubbles of opposing charge and zeta potential, which is controlled by the pH of the solution (Tsuge, 2014). Agarwal et al. (2011) reported inhibition and removal of protein build-up on solid surfaces and stainless steel, thus preventing fouling, after application of nanobubbles. Nanobubbles also provide increased surface area-to-volume ratio per mass as compared to standard water or other aqueous solutions ("Bauer Nanobubbles," 2016). This theoretically enhances the efficiency of any dissolved or suspended antimicrobial components in solution. Currently, nanobubble technology is used most commonly to aid in wastewater disinfection. Micro- and nanobubbles generate free radicals, thus catalyzing chemical reactions and enhancing detoxification efficiency (Agarwal et al., 2011). Agarwal et al. (2011) reported that implementation of nanobubble pretreatment to wastewater sources reduced overall biological, chemical, and physical loads and reduced the overall running costs of treating wastewater.

Currently, there are few reported applications of nanobubbles in the food industry due to limited knowledge and available data. However, inclusion of nanobubble technology has been found to beneficially impact Japanese sake fermentation and shorten the number of

growing days in hydroponic vegetable growing systems due to increased aeration in the soil (Tsuge, 2014). Nanobubble treatment, as a sanitation method, has been evaluated against norovirus surrogates in oyster bodies and was found to inactivate more than 99% of active virus after 12 hours (Tsuge, 2014). Ozonated nanobubbles have been observed to reduce *E. coli* by an additional 2 log cycles as compared to conventional ozone disinfection (Agarwal et al., 2011). Soli et al. (2010) determined that 30 ppm FAC with a sucrose fatty acid ester (SFAE) solution compared to 30 ppm FAC with SFAE solution with the pretreatment application of a microbubble exposure aided in decreasing natural flora ~1 log CFU on lettuce as opposed to pretreatments without microbubbles, thus showing a surfactant capability. Moreover, nanobubbles produced through hydrodynamic cavitation have been observed to have a high inactivation capacity against *E. coli* (Agarwal et al., 2011).

Current knowledge about the use of nanobubbles is relatively underdeveloped and should be studied further to determine practical uses in food safety applications. Laboratory-scale studies suggest that the cost of nanobubble production is higher than conventional chlorination techniques for use as a disinfectant (Agarwal et al., 2011). No data has been reported from evaluation of nanobubble water in combination, and possibly in synergy, with other antimicrobial constituents.

2.2.3 Use of Non-pathogenic Surrogates to Validate Antimicrobial Interventions for In-Plant Studies

Meat, poultry, seafood and juice establishments are required to implement Hazard Analysis and Critical Control Point (HACCP) plans which specifically mandate identification of biological, chemical, and/or physical hazards and critical operating parameters to control identified hazards (FSIS, 1996). Critical operating parameters must be based on scientific evidence. If sufficient data is not available in the scientific literature, an in-plant validation study should be conducted (USDA FSIS, 2015). Validation of in-plant antimicrobial processes against selected pathogens is critical; however, actual pathogens cannot be reasonably brought into food processing environments. Laboratory research, while valuable as a reference, is not a substitute for actual in-plant validation (Niebuhr et al., 2008). Therefore, the use of non-pathogenic indicator organisms, otherwise known as surrogates, can be valuable when

evaluating effectiveness of individual plant processes against pathogens such as STEC and *Salmonella*.

The USDA Food Safety and Inspection Service recommends that surrogate indicator organisms should be used with caution, following strict parameters. These precautions include using surrogates that are proven to be effective for their intended purpose, that the introduction of surrogates into a food processing environment does not create unsanitary conditions, and that an expert microbiologist trained in food science and experimental design is consulted (USDA FSIS, 2015). There are 5 strains of non-pathogenic *E. coli* approved by the USDA FSIS for use as surrogate organisms in in-plant validation studies. These are deposited with the American Type Culture Collection (ATCC) and are designated BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431. These surrogates can be utilized to mimic both STEC and *Salmonella* spp. in a beef processing environment (Cabrera-Diaz et al., 2009; Niebuhr et al., 2008).

Cabrera-Diaz et al. (2009) compared the growth parameters, thermal resistance, and acid resistance of these surrogate organisms to *E. coli* O157 and *Salmonella* Agona, Anatum, Montevideo and Typhimurium. The growth curves of the surrogate organisms were found to be statistically similar to the O157 and *Salmonella* cultures. Thermal resistance of the surrogates was the same or greater than the target pathogens, and acid resistance was found to be the same as *E. coli* O157 isolates and higher than the *Salmonella* isolates. When compared in a validation setting with 2% lactic acid sprays on beef carcasses, the surrogates were found to be statistically similar to the target pathogens, thus confirming them to be appropriate non-pathogenic inocula for use in organic acid-based in-plant studies for meat products.

Although the approved surrogate organisms exhibited similar properties as STEC and *Salmonella* in a variety of in-plant antimicrobial intervention scenarios (Cabrera-Diaz et al., 2009; Niebuhr et al., 2008), it is important to conduct preliminary research to ensure surrogates will mimic the target pathogens in a new situation, such as on beef in a chlorinated nanobubble dip solution.

Chapter 3 - Research Questions

- 1) What is the effect of chlorinated nanobubble solutions against Shiga toxin-producing *E. coli*, *Salmonella* spp., and non-pathogenic surrogates?
 - a. Does temperature, pH, presence of nanobubbles, level of free chlorine, or a combination thereof impact lethality?
 - b. Are the non-pathogenic surrogates comparable to STEC and/or *Salmonella* spp. in this scenario?
- 2) What is the effect of chlorinated nanobubble solutions against Shiga toxin-producing *E. coli*, *Salmonella* spp., and non-pathogenic surrogates in red water?
 - a. Are the non-pathogenic surrogates comparable to STEC and/or *Salmonella* spp. in this scenario?
- 3) What is the effect of chlorinated nanobubble solutions against Shiga toxin-producing *E. coli*, *Salmonella* spp., and non-pathogenic surrogates on lean and fat beef tissues in red water?
 - a. Are the non-pathogenic surrogates comparable to STEC and/or *Salmonella* spp. in this scenario?
- 4) What is the effect of a recirculating acidic (pH 5) Cesco-Bauer Water (i.e municipal water combined with electrolyzed (EO) water nanobubble technology) at a 5 ppm FAC against non-pathogenic surrogates on the surface of beef tissue in a continuous 6-day beef processing environment?

Chapter 4 - Efficacy of Chlorinated Nanobubble Solutions to Control Shiga Toxin-producing *E. coli*, *Salmonella* spp., and Non-pathogenic Surrogate *E. coli* in a Model Beef Processing System—Laboratory Benchtop Studies

4.1 Determining Minimum Bactericidal Concentrations of Chlorinated Waters in Pure Cultures

4.1.1 Introduction

Numerous antimicrobials, including lactic and peroxyacetic acids, are widely used in raw beef processing to reduce the presence of foodborne pathogens such as Shiga toxin-producing *E. coli* (STEC) and *Salmonella*; however, there is interest in exploring combinations of various technologies to enhance antimicrobial effects on pathogens and to support proprietary developments in beef processing. Chemical residues from traditional antimicrobials can lead to deterioration of quality attributes, including appearance, texture, and taste, and potential human health hazards (Tsuge, 2014). Therefore, interventions requiring very low levels of active chemicals are of interest.

Chlorinated water is the most commonly used antimicrobial in the U.S. produce and poultry industries due to its low cost and efficacy against pathogens (Sohaib et al., 2016; Yang et al., 2012). Chlorine can be applied as an antimicrobial in a variety of forms as directed by the USDA FSIS Safe and Suitable Ingredients list (USDA FSIS, 2016) for meat and poultry. One method of generating chlorine in water solutions using low levels of chemicals is with electrolyzed (EO) water. The application of EO water at 5-7 pH minimizes detrimental health effects from Cl₂ gassing off (Guentzel et al., 2008) while maintaining the antimicrobial effects of hypochlorous acid (HOCl) present in solution. HOCl is the most effective form of chlorine for disinfection; at pH 5 HOCl constitutes 97% of total chlorine in solution (Park et al., 2004).

A relatively new concept in the food industry, nanobubble technology shows promise to aid in the development of improved food safety interventions. There is interest in nanobubbles

due their proposed surfactant abilities and cleaning effect. Small particles in water can be effectively removed by introducing nanobubbles of opposing charge and zeta potential, which is controlled by the pH of the solution (Tsuge, 2014). Nanobubbles also provide increased surface area-to-volume ratio per mass as compared to standard water or other aqueous solutions (“Bauer Nanobubbles,” 2016), which theoretically enhances the efficiency of any dissolved or suspended antimicrobial components in solution. Nanobubble treatment, as a sanitation method, has been evaluated against norovirus surrogates in oyster bodies and was found to inactivate more than 99% of active virus after 12 hours (Tsuge, 2014).

EO water has been evaluated for use as a disinfectant and antimicrobial in food processing environments; however, research has not yet been conducted to determine if nanobubble technology aids in the antimicrobial effectiveness of chlorine based solutions. The primary goal of this experiment was to characterize lethality contributions of combinations of acidity (pH 5 or 7), level of free chlorine (zero, low, medium, or high), presence of nanobubble technology, and variation in processing temperature (1.6 or 5.5°C) in Cesco-Bauer water [i.e. municipal water that is chlorinated by infusion of concentrated chlorine produced through an electrolyzed (EO) water process, acidified by introduction of CO₂ gas, and then passed through a patented nanobubble generator] against STEC, *Salmonella* spp., and non-pathogenic surrogate organisms in pure solution.

4.1.2 Materials and Methods

4.1.2.1 Bacterial cultures and inoculum preparation

Five strains of rifampicin-resistant non-pathogenic surrogate *Escherichia coli* (ATCC BAA-1427 P1, BAA-1428 P3, BAA-1429 P8, BAA-1430 P14, and BAA-1431 P68) obtained from Dr. Gary Acuff (Texas A&M University, College Station, TX), five strains of *Salmonella* [four beef lymph node isolates (serotypes Lubbock, Mbandaka, and Montevideo) and one fecal (Mbandaka) isolate obtained from Dr. Guy Loneragan (Texas Tech University, Lubbock, TX) and *S. Typhimurium* ATCC 14028], and seven STEC strains [STEC-7; O26 (H30), O45 (CDC 96-3285), O103 (90-3128), O111 (JBI-95), O121 (CDC 97-3068), O145 (83-75) and O157:H7 (ATCC 35150), referred to as STEC-7], trained to be resistant to rifampicin (Laster et al., 2012) at 0.1 g/L,

obtained from Dr. John Luchansky (USDA Eastern Regional Research Center, Wyndmoor, PA) were used in this study. All cultures were received from their sources, transferred into fresh tryptic soy broth (TSB or TSB + rifampicin; Bacto, Becton Dickinson, Sparks, NJ, USA), incubated for 24 h at 37°C, and streaked onto tryptic soy agar (TSA or TSA + rifampicin) for confirmation using API 20E assays (BioMerieux Vitek, Hazelwood, MO, USA) and BioControl Assurance GDS PCR assays. Broth cultures were then stored on cryoprotect beads in glycerol at -80°C until needed.

Each bacterial strain was activated individually by transferring a single cryogenically frozen bead into either TSB containing 0.1 g/L rifampicin (rif; Sigma-Aldrich, St. Louis, MO, USA) stock solution (TSB+rif for the rifampicin-resistant STEC-7 and surrogates) or TSB (for *Salmonella* serovars) and incubated at 37°C for 24 hours. Rifampicin stock solution (rif) was prepared by dissolving 0.1 g rifampicin in 5 mL methanol (Fisher Chemical, Fair Lawn, NJ, USA) followed by filtering through a 0.22 μm sterile filter. Activated *Salmonella* strains were individually transferred into 45 mL TSB, surrogates into 45 mL TSB+rif, and STEC-7 strains into 32 mL TSB+rif and incubated at 37°C for 24 hours. After incubation, each culture strain was individually plated onto either TSA plates containing 0.1 g/L rif (TSA+rif) for rif-resistant STEC-7 and surrogates or xylose lysine deoxycholate (XLD; Difco, Becton Dickinson, Sparks, NJ, USA) agar for *Salmonella* spp. to determine concentration. Culture strains within the three target bacterial strain groups were combined into 220 mL mixed cocktails to be centrifuged at 5,520 x *g* for 15 minutes at -4°C. Centrifuged pellets were refrigerated overnight at 4°C and re-hydrated in 60 mL phosphate buffered saline (PBS) prior to use.

4.1.2.2 Antimicrobial water solutions

All test solutions were generated by Cesco-Bauer (Bellingham, WA) and ground shipped to Kansas State University in sealed 5 L plastic containers for inoculated laboratory benchtop trials. Cesco-Bauer water [i.e. municipal water that is continuously chlorinated by infusion of concentrated chlorine produced through an electrolyzed (EO) water process, acidified by introduction of CO₂ gas, and then passed through a patented nanobubble generator (U.S. Patent No. 8454837)] solutions containing 4 levels of free available chlorine [FAC; zero, low (2.91±0.45 ppm), medium (7.27±0.36 ppm), and high (11.94±0.97 ppm)], infused by addition of

EO water, were evaluated either with or without nanobubbles and at both acidic (5) and neutral (7) pH levels. Control treatments containing no FAC and no nanobubbles at pH 5 and 7 were evaluated for comparison. Nanobubbles are generated utilizing cavitation chambers and shear planes to initiate an endothermic reaction, thus, producing a high concentration of paramagnetic oxygen nanobubbles with a mean particle size between 50 and 100 nm (“Bauer Nanobubbles,” 2016). Each shipment of solutions was evaluated within 5 days of arrival at the Kansas State University Food Safety & Defense Laboratory and within 12 days of generation. FAC, pH, and ORP were measured for each sample collected using a portable photometer (HI96711 Portable Photometer, Hanna Instruments, Woonsocket, RI, USA; PT3 and PT4 pens, Myron L Company, Carlsbad, CA, USA). It should be noted that Cesco-Bauer nanobubble solutions were provided for this research, and were generated at the commercial site according to proprietary methodology. Through company experience, specific ORP readings at the plant was used as an indicator of presence and concentration of suspended nanobubbles; however, other than reading the ORP upon receipt and at the time of experimental trial, the K-State laboratory had no method of confirming the actual presence of nanobubbles. However, elevated ORP readings in nanobubble water were similar in the laboratory compared to the commercial plant.

4.1.2.3 Application of chlorinated water treatments to pure cultures

Each of the 32 total treatment combinations of chlorine level, acidity, and presence/absence of nanobubbles were evaluated for their bactericidal effect on separate multi-strain cocktails of the three target bacterial populations (Table 4.1). Aliquots (24.75 mL) of each Cesco-Bauer solution were equilibrated to either 1.7 °C or 5.6 °C in 100-mL glass beakers and were agitated with a small sterile stir-bar at 600 rpm (Isotemp, Fischer Scientific, Dubuque, IA, USA). Solutions were inoculated with 0.25 mL (~9.7 log CFU/mL) of the three rehydrated culture cocktails individually—with consideration of the dilution factor due to addition to test solutions, the level of total organisms in solution was ~7.7 log CFU/mL—and each exposed for 1 minute before neutralizing directly with 25 mL double-strength DE Neutralizing Broth (Difco, Becton, Dickinson and Co., Sparks, MD, USA).

Table 4.1 Parameters Evaluated in Cesco-Bauer Water During Determination of Minimum Bactericidal Concentrations of Chlorinated Waters in Pure Cultures Experiment

Temperature (°C)	pH	Chlorine (ppm)	Nanobubbles
1.6	5	Zero (0)	Presence
5.6	7	Low (2.91 ± 0.45)	Absence
		Medium (7.27 ± 0.36)	
		High (11.94 ± 0.97)	

4.1.2.4 Microbial analysis

Surviving populations of inoculum cocktails were determined by immediately plating serial dilutions of each neutralized sample onto injury recovery and selective media agar plates. Serial dilutions were prepared in Phosphate Buffered Saline (PBS; AMRESCO, LLC., Solon, OH, USA) blanks. STEC-7 and surrogates were enumerated by spread plating on TSA+rif and *Salmonella* spp. was enumerated on XLD agar, each incubated for 24 hours at 37°C. To quantify sublethally injured cells, samples were also spread plated onto non-selective TSA, incubated for 6 hours at 37°C, overlaid with 10 mL TSA+rif or XLD to select for STEC-7 and/or surrogates and *Salmonella* spp., respectively, and incubated for 12-18 additional hours at 37°C.

4.1.2.5 Statistical analysis

Statistical analysis was performed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). A split-split-split-plot treatment structure was assumed with chlorine level as the whole-plot treatment factor arranged in an incomplete block design with day as the blocking factor, temperature as the subplot factor with all other treatment factors (combinations of acidity, presence of nanobubbles, and target organism tested) in the sub-subplot structure, and media type (selective or injury recovery) as the sub-sub-sub plot factor. Type 3 tests of fixed effects were evaluated to determine significance of interactions and/or main effects based on a significance level of $\alpha = 0.05$.

4.1.3 Results and Discussion

The efficacy of chlorine to inactivate pathogens is dependent on concentration, pH level, contact time, temperature, and bacterial strains—all factors which were evaluated in this study along with the presence/absence of suspended nanobubbles in solution. Although post-treatment recovery of the 3 bacterial populations was variable across replications, notable reductions ranging from 3.3-7.0 log CFU/mL were observed across all three (low, medium, and high) FAC levels. Analysis of the Type 3 Fixed Effects (Table 4.2) indicate there was a significant 5-way interaction between type of organism, temperature, pH, presence of nanobubbles, and media (selective or injury recovery; $P \leq 0.05$).

Table 4.2 Type 3 Fixed Effects of Split-Split-Split Plot Analysis

Effect	Num. DF	Denom. DF	F Value	P Value
Chlorine	3	12.659	251.984	1.53842E-11
Organism	2	272.543	14.890	7.29284E-07
Chlorine*Organism	6	272.513	8.853	7.8893E-09
Temperature	1	16.021	0.023	0.882
Chlorine*Temperature	3	16.407	0.123	0.945
Organism*Temperature	2	272.425	1.338	0.264
Chlorine*Organism*Temperature	6	272.408	0.425	0.862
pH	1	272.365	1.173	0.280
Chlorine*pH	3	272.352	3.622	0.014
Organism*pH	2	272.543	2.107	0.124
Chlorine*Organism*pH	6	272.514	0.543	0.775
Temperature*pH	1	272.605	0.054	0.816
Chlorine*Temperature*pH	3	272.572	0.048	0.986
Organism*Temperature*pH	2	272.425	0.632	0.533
Chlorine*Organism*Temperature*pH	6	272.407	3.048	0.007
Nanobubbles	1	283.021	1.345	0.247
Chlorine*Nanobubbles	3	278.431	0.229	0.876
Organism*Nanobubbles	2	272.425	0.900	0.408
Chlorine*Organism*Nanobubbles	6	272.407	0.648	0.691
Temperature*Nanobubbles	1	283.257	0.209	0.648
Chlorine*Temperature*Nanobubbles	3	262.998	0.643	0.588
Organism*Temperature*Nanobubbles	2	272.543	1.511	0.223

Chlorine*Organism*Temperature*Nanobubbles	6	272.514	2.274	0.037
pH*Nanobubbles	1	272.605	0.428	0.514
Chlorine*pH*Nanobubbles	3	272.572	0.650	0.583
Organism*pH*Nanobubbles	2	272.425	0.938	0.393
Chlorine*Organism*pH*Nanobubbles	6	272.408	1.554	0.161
Temperature*pH*Nanobubbles	1	272.365	0.205	0.651
Chlorine*Temperature*pH*Nanobubbles	3	272.352	0.667	0.573
Organism*Temperature*pH*Nanobubbles	2	272.543	0.637	0.530
Chlorine*Organism*Temperature*pH* Nanobubbles	6	272.513	0.301	0.936
Media	1	297.113	187.070	2.30E-33
Chlorine*Media	3	297.115	7.152	<0.0001
Organism*Media	2	297.113	7.353	0.001
Chlorine*Organism*Media	6	297.115	3.445	0.003
Temperature*Media	1	297.113	2.227	0.137
Chlorine*Temperature*Media	3	297.115	0.625	0.600
Organism*Temperature*Media	2	297.113	0.319	0.727
Chlorine*Organism*Temperature*Media	6	297.115	1.564	0.157
pH*Media	1	297.113	0.970	0.325
Chlorine*pH*Media	3	297.115	0.937	0.423
Organism*pH*Media	2	297.113	0.299	0.741
Chlorine*Organism*pH*Media	6	297.115	1.359	0.231
Temperature*pH*Media	1	297.113	0.324	0.570
Chlorine*Temperature*pH*Media	3	297.115	0.759	0.518
Organism*Temperature*pH*Media	2	297.113	0.443	0.642
Chlorine*Organism*Temperature*pH*Media	6	297.115	0.790	0.578
Nanobubbles*Media	1	297.113	0.275	0.600
Chlorine*Nanobubbles*Media	3	297.115	0.085	0.968
Organism*Nanobubbles*Media	2	297.113	0.473	0.624
Chlorine*Organism*Nanobubbles*Media	6	297.115	2.282	0.036
Temperature*Nanobubbles*Media	1	297.113	0.424	0.515
Chlorine*Temperature*Nanobubbles*Media	3	297.115	0.872	0.456
Organism*Temperature*Nanobubbles*Media	2	297.113	0.083	0.920
Chlorine*Organism*Temperature*Nanobubbles* Media	6	297.115	0.336	0.918
pH*Nanobubbles*Media	1	297.113	1.742	0.188
Chlorine*pH*Nanobubbles*Media	3	297.115	4.894	0.002
Organism*pH*Nanobubbles*Media	2	297.113	3.499	0.031
Chlorine*Organism*pH*Nanobubbles*Media	6	297.115	1.183	0.315
Temperature*pH*Nanobubbles*Media	1	297.113	2.294	0.131
Chlorine*Temperature*pH*Nanobubbles* Media	3	297.115	2.501	0.060

Organism*Temperature*pH*Nanobubbles* Media	2	297.113	4.784	0.009
Chlorine*Organism*Temperature*pH* Nanobubbles*Media	6	297.115	1.123	0.348

* Blue highlights indicate significant interactions and effects ($P < 0.05$)

In Figures 4.1, 4.3, and 4.4, parallel lines indicate no interaction between factors or levels of factors whereas non-parallel or crossed lines indicate an interaction.

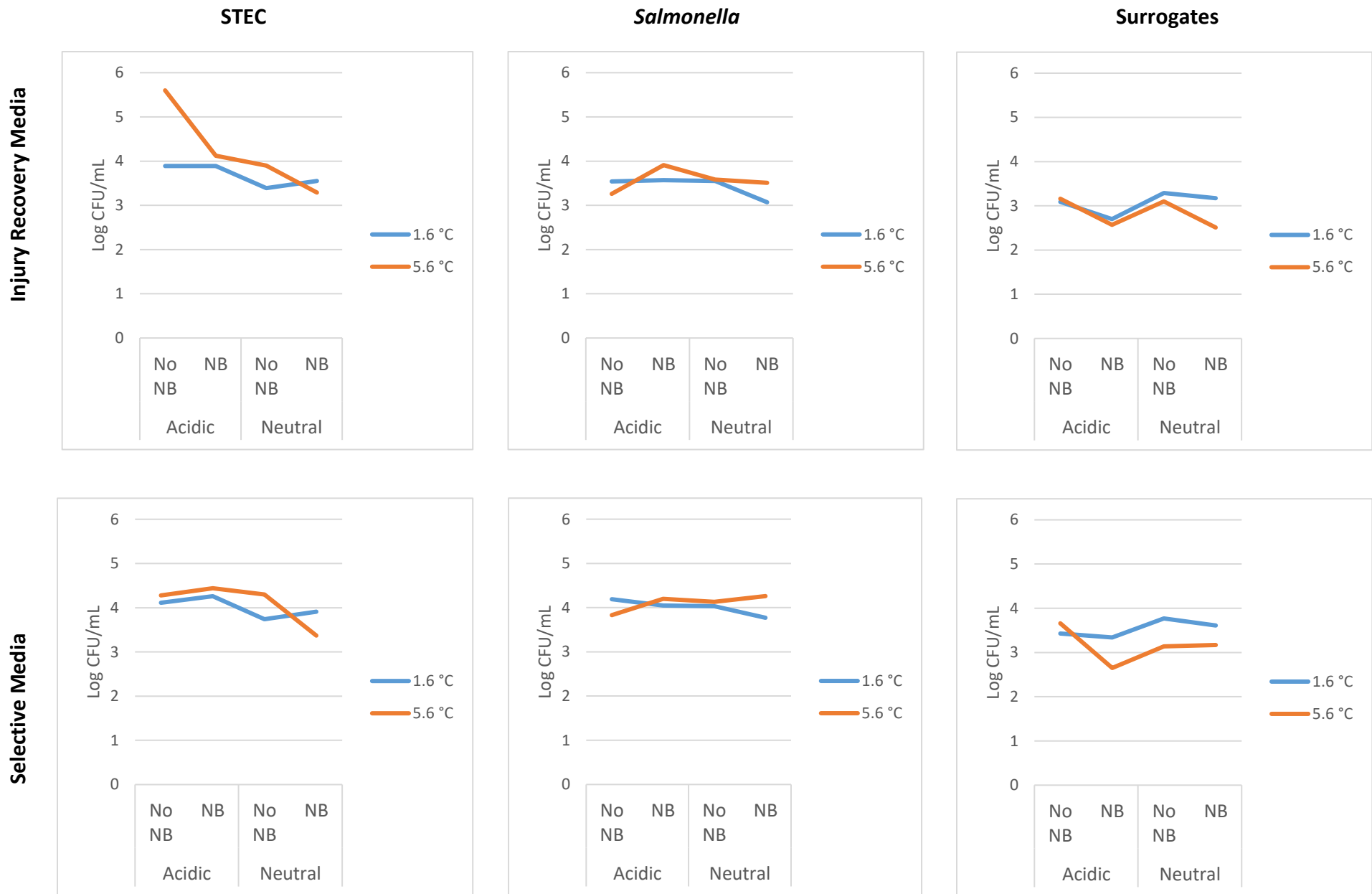


Figure 4.1 Profile Plot of 5-Way Interaction between Organism, Temperature, pH (Acidic or Neutral), Presence of Nanobubbles (NB), and Media Type. Least Square Means (Avg. Log CFU/mL) of Log CFU/mL reductions are reported for each combination.

The effect of media type (selective or injury recovery) was dependent on the interactions between type of organism, temperature, pH, and presence of nanobubbles regardless of chlorine level. Level of chlorine was found to be a significant contributor ($P \leq 0.05$), as indicated in Table 4.2, in four 4-way interactions; however, two of these interactions are more important to analyze as they show the effect of chlorine on media type, which is a factor in our highest order interaction. The first interaction, Chlorine*pH*Nanobubbles*Media, indicates that the effect of media type was dependent on the interactions between pH, presence of nanobubbles, and level of chlorine (Figure 4.3). The second interaction, Chlorine*Organism*Nanobubbles*Media, indicates the effect of media type was also dependent on the interactions between level of chlorine, type of organism, and presence of nanobubbles (Figure 4.4). As the highest order, these significant ($P \leq 0.05$) 4- and 5- way interactions must be considered. First order main effects show that the individual components of greatest importance, i.e. type of media (Figure 4.2), level of chlorine (Figure 4.6), and type of organism (Figure 4.5), are significantly different within their levels ($P \leq 0.05$) averaged across levels of all other factors, whereas there are no significant ($P > 0.05$) differences between the two exposure temperatures or the presence of nanobubbles averaged across levels of all other factors (Table 4.2).

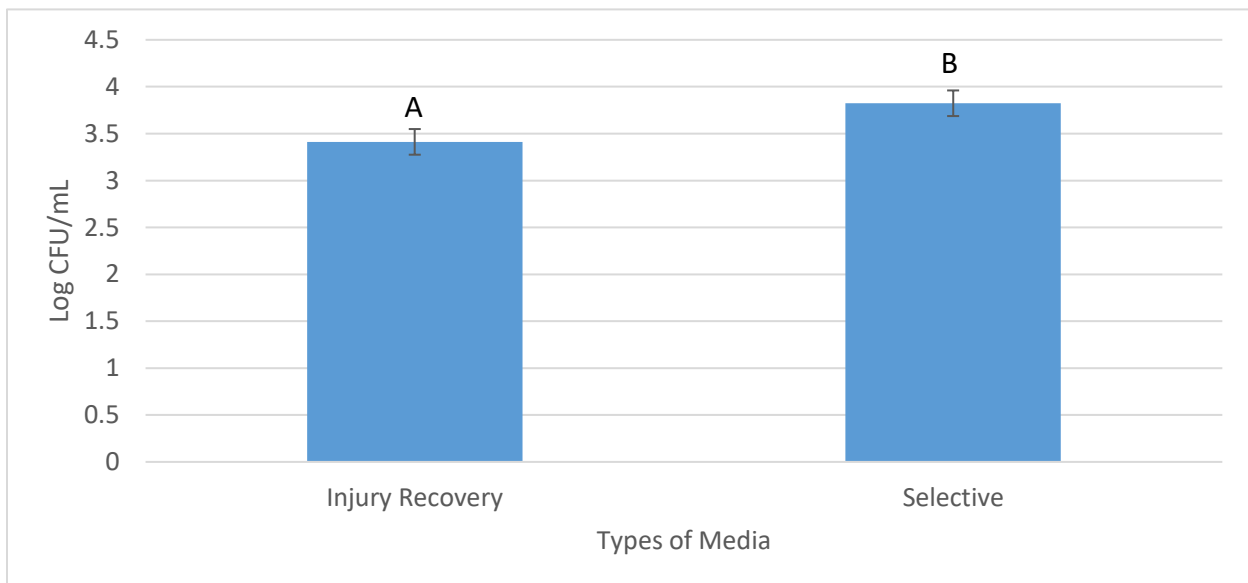


Figure 4.2 Average Log CFU/mL Reductions Based on Type of Media; A-B different letters indicate significant differences ($P \leq 0.05$)

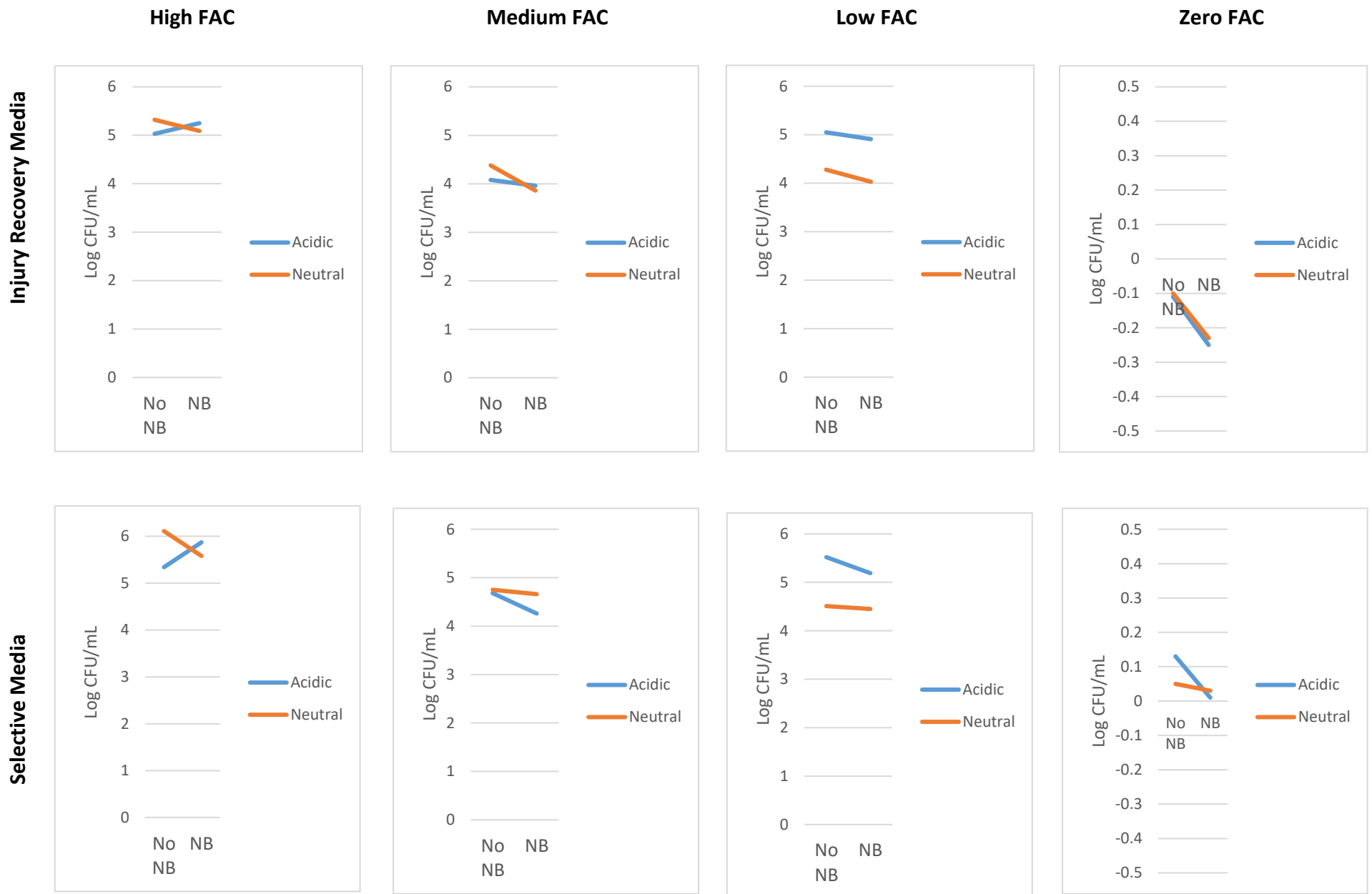


Figure 4.3 Profile Plot of 4-Way Interaction between Level of Chlorine (high, medium, low, zero FAC), pH (Acidic or Neutral), Presence of Nanobubbles (NB), and Media Type. Least Square Means (Avg. Log CFU/mL) of Log CFU/mL reductions are reported for each combination.

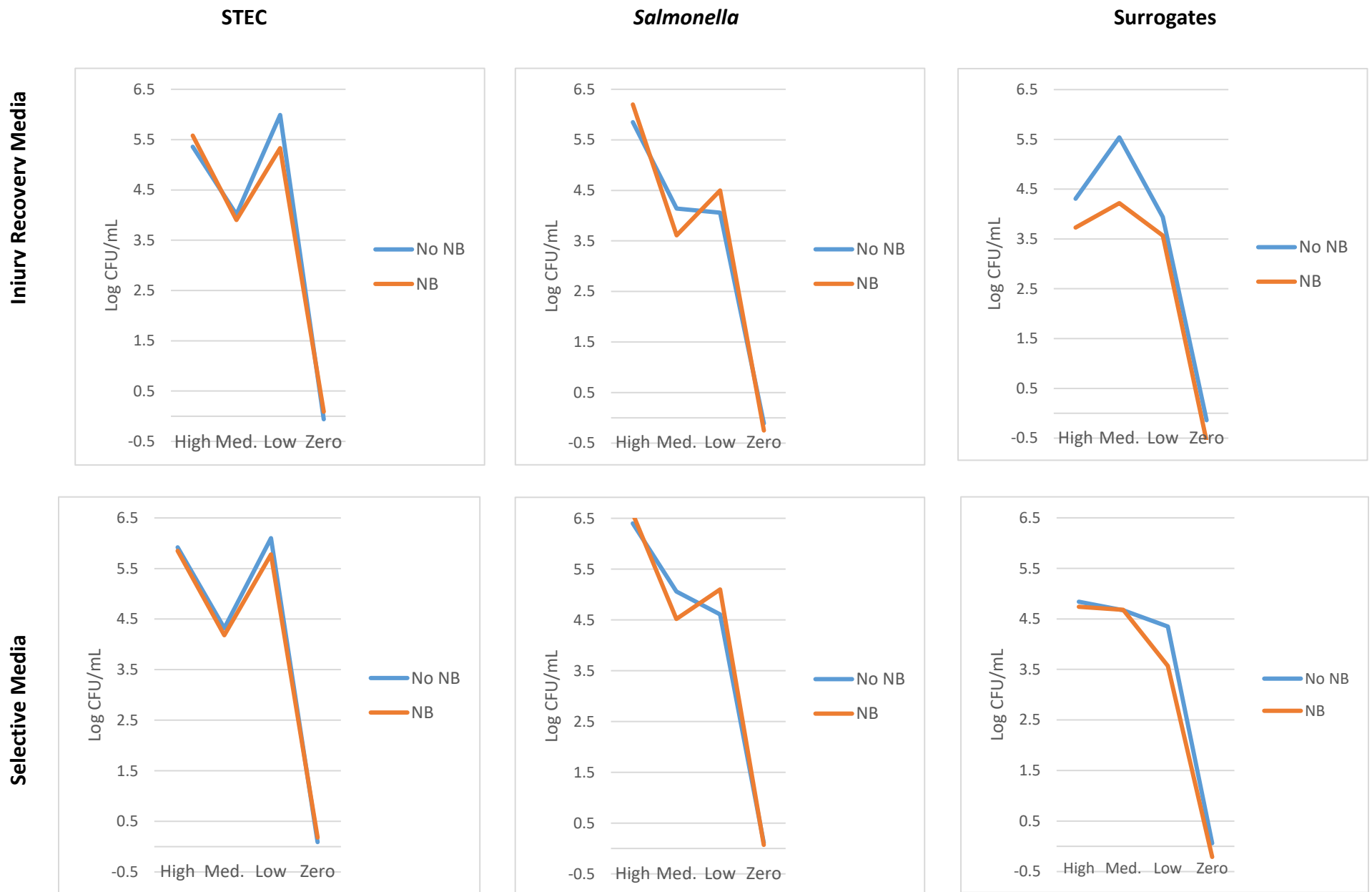


Figure 4.4 Profile Plot of 4-Way Interaction between Level of Chlorine (high, med., low, zero), Type of Organism, Presence of Nanobubbles (NB), and Media Type. Least Square Means (Avg. Log CFU/mL) of Log CFU/mL reductions are reported for each combination.

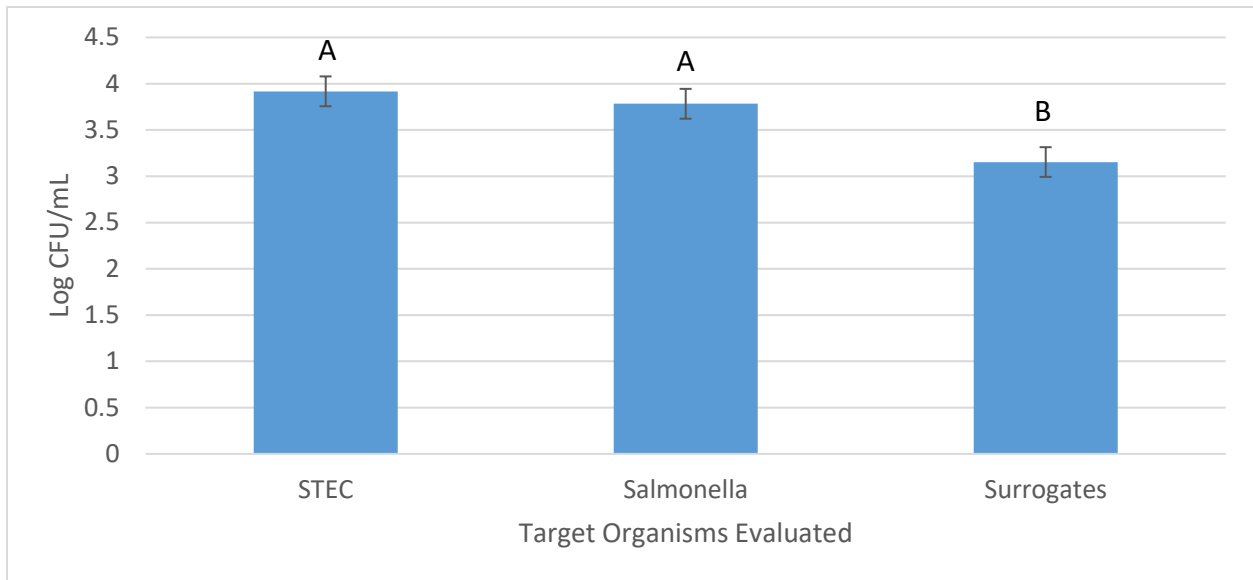


Figure 4.5 Average Log CFU/mL Reductions Based on Type of Organism; A-B different letters indicate significant differences ($P \leq 0.05$)

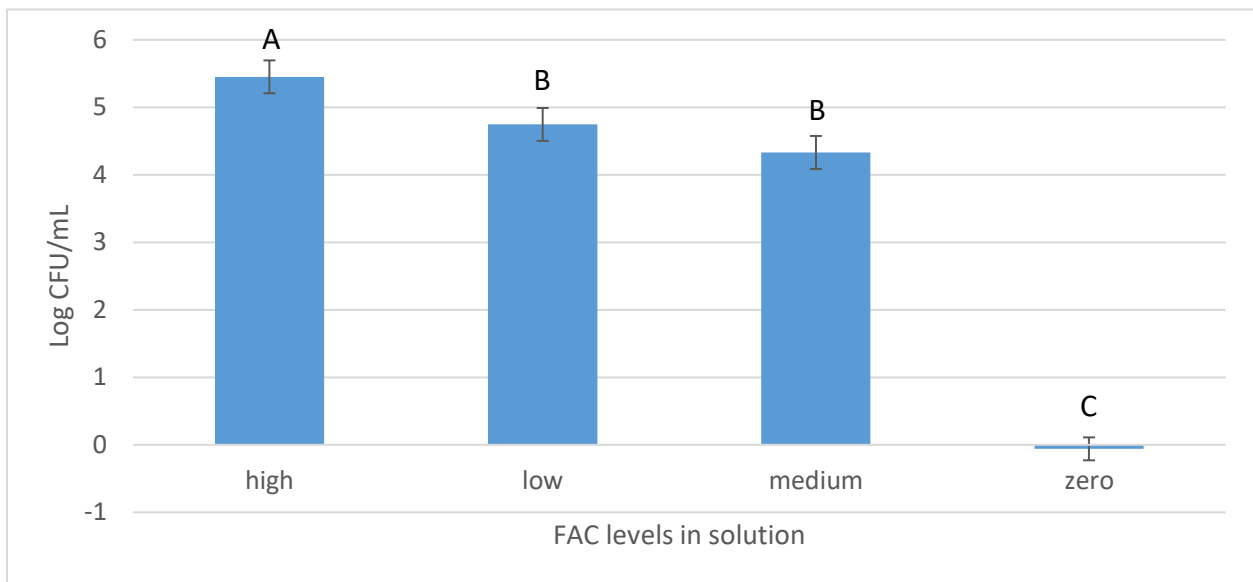


Figure 4.6 Average Log CFU/mL Reductions Based on Level of FAC; A-C different letters indicate significant differences ($P \leq 0.05$)

Figure 4.4 reports differences in log CFU/mL reductions between the injury recovery and selective media types; injury recovery reductions were 0.42 log CFU/mL lower ($P \leq 0.05$). This corresponds to higher recovery counts on injury recovery media. When conducting further experiments, researchers can use this information to save time, supplies, financial and human

resources, to justify spread plating and/or reporting results obtained using injury recovery media only. If reviewing injury recovery media data only, having lower overall reductions will lead researchers to make more conservative decisions when evaluating the success of chlorinated nanobubble antimicrobials in future studies (Chapters 4.2 and 4.3).

Similarly, when observing differences between target organism cocktail populations, the same logic applies. Surrogate *E. coli* demonstrated significantly greater resistance to the chlorinated solutions; population reductions ranged from 3.4-5.5 log CFU/mL with only slightly increased reductions at the higher FAC level. Figure 4.5 depicts significant differences between STEC-7 and surrogates, and likewise between *Salmonella* and surrogates, with surrogate reductions being ~0.70 log CFU/mL lower ($P \leq 0.05$). The lower average population reduction (i.e., higher viable cell recovery) indicates that the 5-strain surrogate cocktail is a good predictor for both STEC-7 and *Salmonella* behavior when evaluating chilled chlorinated nanobubble antimicrobial solutions, particularly for commercial in-plant validation studies. In this benchtop study, STEC-7 reductions were the most variable ranging from 3.3-7.0 log CFU/mL; whereas, *Salmonella* populations were notably reduced (4.9-7.1 log CFU/mL) by the high FAC concentrations. No definitive impacts of nanobubble inclusion or acidic pH were observed for any of the three target bacterial cocktails in pure solution testing.

Differences between the levels of FAC in solution were observed (Figure 4.6), with high FAC being more effective ($P \leq 0.05$) in reducing organism populations (by an average of 5.4 log CFU/mL) than the low, medium, or zero FAC levels. No differences ($P > 0.05$) in microbial reductions were detected between low and medium FAC levels, both reducing populations by ~4.5 log CFU/mL. All levels of chlorinated test solutions (low, medium and high) reduced target organism populations more effectively ($P \leq 0.05$) than the zero FAC control solutions.

No lethality of STEC-7, surrogates, or *Salmonella* cocktail populations was observed in acidic or neutral pH solutions with or without nanobubble technology at 0 ppm FAC at either 1.7 °C or 5.6 °C. This indicated that nanobubbles and/or pH alone were not the main contributor to microbial lethality (Figure 4.6). Park, Hung, & Chung (2004) stated that the pH may not be important to the antimicrobial efficacy of EO water, an observation which was upheld in this preliminary study. Other studies have shown >7 log cycle reductions in *E. coli*

O157:H7 and *Listeria monocytogenes* in EO water at 1 ppm FAC and pH 5 (Park et al., 2004). While EO water was the antimicrobial chlorine source in the nanobubble solutions tested in the current study, it was only present in small amounts, thus likely accounting for differences in effectiveness reported from previous research.

4.1.4 Conclusion

As the first part of a 3-part benchtop study, the goal of this experiment was to determine the minimum concentration of chlorine in combination with pH and nanobubbles as an effective antimicrobial against pure culture biological targets. This work demonstrated that chilled water containing FAC levels of approximately 12 ppm are highly effective as antimicrobials in the absence of organic loading, and that surrogates can be used as appropriate indicator organisms for STEC and *Salmonella* in chlorinated nanobubble solution applications. Low levels (approximately 3 ppm, the lowest level evaluated) of FAC in solution was still effective in reducing target organism populations in pure culture. Even lower levels of FAC could have been evaluated but were not due to the proposed applications of this antimicrobial technology in beef processing environments with high levels of organics present.

Although slightly acidifying the water to pH 5 and including nanobubbles did not definitively impact microbial reductions in pure culture, there is a possibility that these factors in conjunction with FAC will contribute to a less variable microbial kill in recirculating wash solutions and/or in the presence of organics (i.e., red water; evaluated in part 2) and beef lean and fat tissue (evaluated in part 3). Additionally, higher levels of FAC may be evaluated in nanobubble solutions depending on results from these subsequent studies.

4.2 Determining Lethality of Pathogens and Surrogates in the Presence of Red Water

4.2.1 Introduction

Chlorine is a long-standing and effective antimicrobial and sanitizing agent in the food industry; however, free available chlorine (FAC) level in solution, the indicator of sanitizing power, is dramatically affected by organic matter. This presents an interesting dilemma when considering applications of chlorine-based applications in a meat processing environment. Studies regarding the effect of chlorine on pathogen inactivation in the presence of organic materials (e.g., red water) is limited (Zhou et al., 2015). Understanding the lethality of antimicrobial components, especially chlorine, in organically-loaded water systems provides realistic insight into common food processing scenarios such as poultry chillers, produce wash solutions, and in this case, recirculating process waters used in a proprietary ground beef manufacturing process.

The two goals of this experiment were to 1) determine maximum beef purge level in Cesco-Bauer solutions whereby all free available chlorine in the nanobubble solutions is depleted, and 2) determine the effectiveness of chlorinated nanobubble solutions with added beef purge (i.e., red water) to reduce Shiga toxin-producing *E. coli* (STEC), *Salmonella* spp., and non-pathogenic surrogates over time.

4.2.2 Materials and Methods

4.2.2.1 Generation of red water solutions

Cesco-Bauer water solutions were manufactured in Bellingham, WA and ground shipped to Kansas State University. All combinations of solutions components (FAC level, pH 5 or 7, and presence/absence of nanobubbles) were generated to serve as test treatments, as defined in section 4.2.2.2 and 4.2.2.4). Chlorinated solutions formulated to contain nanobubbles were generated using municipal water infused with Aquaox 5000™ (Aquaox LL, Dillsburg, PA, USA) to attain the target FAC levels between 0 and 40 ppm. If test solutions were to be acidified to pH 5, CO₂ gas was bubbled into the water prior to nanobubble generation. Cesco-Bauer water

technology generates nanobubbles by passing water through a patented generator that uses cavitation chambers and shear planes to initiate an endothermic reaction thus producing high concentration of paramagnetic oxygen nanobubbles with a mean particle size between 50 and 100 nm (“Bauer Nanobubbles,” 2016). Each shipment of solutions was evaluated in inoculated benchtop studies within 5 days of arrival at KSU and within 12 days of generation. Beef purge collected from stored vacuum packaged beef subprimals (obtained from the Kansas State University Meat Lab, Manhattan, KS) was added by percent volume (at varying purge levels defined in sections 4.2.2.2 and 4.2.2.4) to simulate realistic red water levels likely to represent the proprietary commercial ground beef processing system evaluated in Chapter 5.

4.2.2.2 Preliminary chlorine loss determination resulting from varying levels of purge addition

To quantify the impact of beef purge loading on FAC in 16 treatment combinations, Cesco-Bauer water manufactured to contain a wide range of FAC in solutions [zero ppm control, low (~3.5 ppm FAC), medium (~4.5 ppm FAC), medium-high (7-11 ppm FAC), high (20-26 ppm FAC)] and characterized as pH 5 or 7, and presence/ absence of nanobubbles was evaluated. Beef purge was initially added at a 5% target by volume to simulate an estimated maximum organic load in red water in a beef processing dip system. Aliquots (50 mL) of each solution combination of FAC level, acidity, and presence/absence of nanobubbles were contained in 125-mL glass Erlenmeyer flasks at 4°C and continuously agitated at 140 rpm (Multi-Platform Shaker; Fisher Scientific, Pittsburgh, PA, USA). FAC was measured using a portable photometer (Model HI96711, Hanna Instruments, Woonsocket, RI) before introduction of purge and at several time points (5, 28, 35, 58, and 65 min) after the introduction of purge. These agitated red water solutions were re-infused with fresh Cesco-Bauer water of the same composition as the original treatment solution (i.e. acidic nanobubble re-infused with acidic nanobubble, acidic no nanobubble re-infused with acidic no nanobubble), after 30 and 60 minutes of exposure. This re-infusion process entailed removal of 10% ‘used’ solution immediately followed by addition of 10% fresh solution to help mimic the recirculating water in the proprietary commercial recirculating nanobubble water system.

This first organic loading experiment was conducted, only to find that the 5% purge addition completely eliminated any FAC in the Cesco-Bauer solutions. After discussion of the

actual commercial system with the meat processor, it was determined that commercial circulating process water could be maintained at lower purge levels. In a follow-up experiment, stronger FAC solutions (20-26 ppm and ~30 ppm FAC) were obtained from Cesco-Bauer; solutions with lower FAC levels were not evaluated further due to the inability to maintain any level of residual FAC in the presence of beef purge. In this follow-up study, lower levels of beef purge (0 to 0.25% by volume, with increases at 0.05% intervals) were mixed with the two high FAC solution levels of Cesco-Bauer water and evaluated. Aliquots (50 mL) of each solution combination of chlorine level, acidity, and presence/absence of nanobubbles were contained in 125-mL glass Erlenmeyer flasks at 4°C and continuously agitated at 140 rpm. FAC was measured using a portable photometer before introduction of purge and at several time points (1, 5, 25, 35, 45, or 65 minutes) after the introduction of purge. No re-infusions were done in order to determine how long residual FAC levels persisted in Cesco-Bauer red water solutions.

4.2.2.3 Bacterial cultures and inoculum preparation

After gaining an understanding of the impact of varying levels of purge loading in the Cesco-Bauer solutions (detailed in 4.2.2.2), an inoculated study was conducted to evaluate the antimicrobial effectiveness of organically loaded Cesco-Bauer waters. Rifampicin-resistant *E. coli* surrogates, rifampicin-resistant Shiga toxin-producing *E. coli*, and *Salmonella* serovars used in this study were propagated and prepared as described section 4.1.2.1.

4.2.2.4 Application of chlorinated water treatments to reduce target bacterial populations in the presence of 0.1% red water

Aliquots (49.5 mL) of 33-40 ppm FAC, pH 5 Cesco-Bauer solutions representing each combination of presence/absence of nanobubbles were contained in 125-mL glass Erlenmeyer flasks at 4±3°C and agitated at 140 rpm (Multi-Platform Shaker; Fisher Scientific, Pittsburgh, PA). Cesco-Bauer solutions were spiked with beef purge at a 0.1% by volume level to create red water. After approximately 1 min, red water solutions were inoculated with 0.5 mL (~9.7 log CFU/mL) of the 24-h culture cocktails individually—adjusting for the dilution effect of the initial test solution, the level of total organisms in solution was ~7.7 log CFU/mL. Cultures were exposed in the various solutions for 60 minutes with continuous shaking. Samples (5 mL) were

taken after 1, 25, and 60 minutes (25 mL) of exposure to the red water/Cesco-Bauer solution and neutralized immediately with double-strength DE Neutralizing Broth. Red water solutions were re-infused with fresh Cesco-Bauer water, of the same composition as the original treatment solution, after 30 minutes of exposure by removing 10% of the 'used' solution and adding 10% of fresh solution to Two replications of the experiment were completed.

4.2.2.5 Microbial analysis

Surviving populations of each target inoculum group were determined by plating serial dilutions in phosphate buffered saline (PBS) of each neutralized sample onto injury recovery and selective media agar plates as described in section 4.1.2.4. In cases where no viable cells were recovered, 5-7 mL of the original neutralized sample were transferred to either 100 mL TSB+rif or Rappaport-Vassiliadis Broth (RV; Difco, Becton, Dickinson and Company, Sparks, MD, USA) to enrich for STEC-7 or surrogates and *Salmonella* spp., respectively. All enrichments were incubated at 37°C for 24 h and subsequently streaked onto either TSA+rif or XLD for qualitative detection of surviving organisms below the direct plating detection limit (0.3 log CFU/mL).

4.2.3 Results and Discussion

It is important to note that this section consists of data that has not been statistically analyzed and therefore should be considered preliminary. The purpose of this evaluation and the findings therein was to provide insight into the impact of organic materials (beef purge) on FAC levels in the Cesco-Bauer water treatments in order to plan for the subsequent in-plant validation studies (Chapter 5).

4.2.3.1 Preliminary evaluation of chlorine loss

In the presence of any organic material, free chlorine in solution is lost rapidly. Yang et al. (2012) showed that an initial level of 35 ppm FAC is reduced to zero after only four lettuce dip washes; replenishing the solution with the same amount of NaClO as originally used only resulted in FAC restoration to levels between 7.2 and 17 ppm indicating that higher levels of NaClO is needed over time to maintain FAC in solution. Measuring level of organic matter in recirculating water solutions is difficult based on inconsistency of initial organic loading on product and constant changes in water properties.

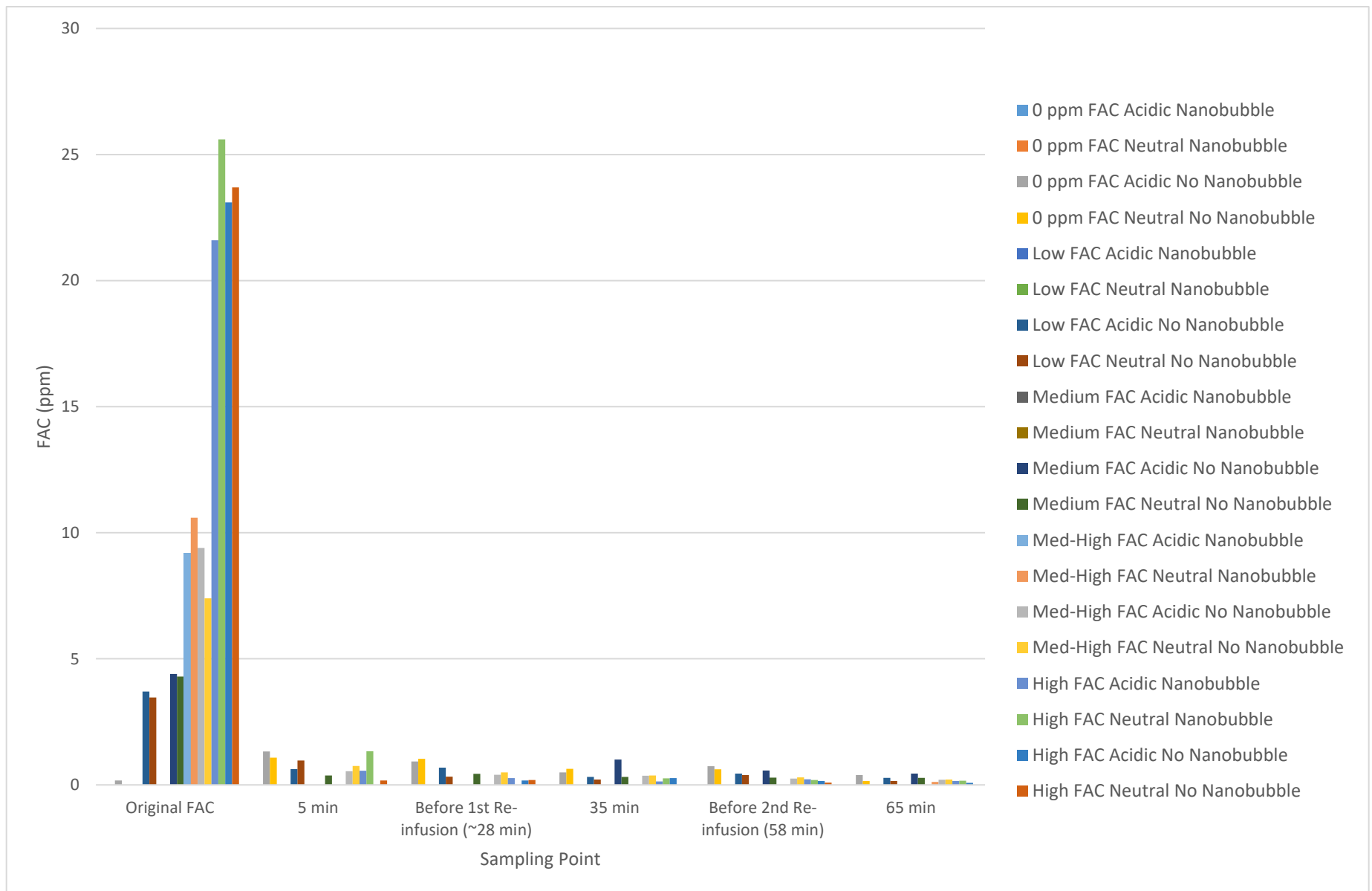


Figure 4.7 Residual Free Available Chlorine after Addition of 5% Purge to Cesco-Bauer Water Solutions

During the preliminary trial, purge was initially added at 5% by volume to Cesco-Bauer solutions as the estimated maximum level of anticipated purge-to-water in a novel beef processing dip/immersion system to be evaluated in subsequent commercial in-plant studies. It was determined quickly that 5% purge completely inactivated all FAC present in a wide range of solutions [zero, low (~3.5 ppm FAC), medium (~4.5 ppm FAC), medium-high (7-11 ppm FAC), high (20-26 ppm FAC)] even with reinfusion of new Cesco-Bauer water (Figure 4.7). Once it became apparent that the research team could not maintain levels of FAC even at the high initial levels, stronger solutions (i.e. 30 ppm FAC) were obtained for subsequent studies. The first experiment (depicted in Figure 4.7) demonstrated that chlorine solutions with initially low levels of FAC could not be maintained; only high FAC solutions were used in subsequent chlorine demand experiments.

To determine the maximum level of purge that could be added before completely inactivating free chlorine in the Cesco-Bauer solutions, lower concentrations of purge were added to high FAC Cesco-Bauer water solutions (24.66 ± 0.97 ppm) and monitored for a total of six minutes without re-infusion (Figure 4.8). From these results, it was determined that 0.1% beef purge red water solutions or lower should be evaluated in the microbial inactivation study, as FAC was bound at all purge levels higher. This test also indicated that FAC is bound very quickly thus prompting a second test of evaluating FAC levels over 1 hour to determine the extent of residual chlorine levels in solution with no re-infusions (Figure 4.9). A control (0% purge) was also included.

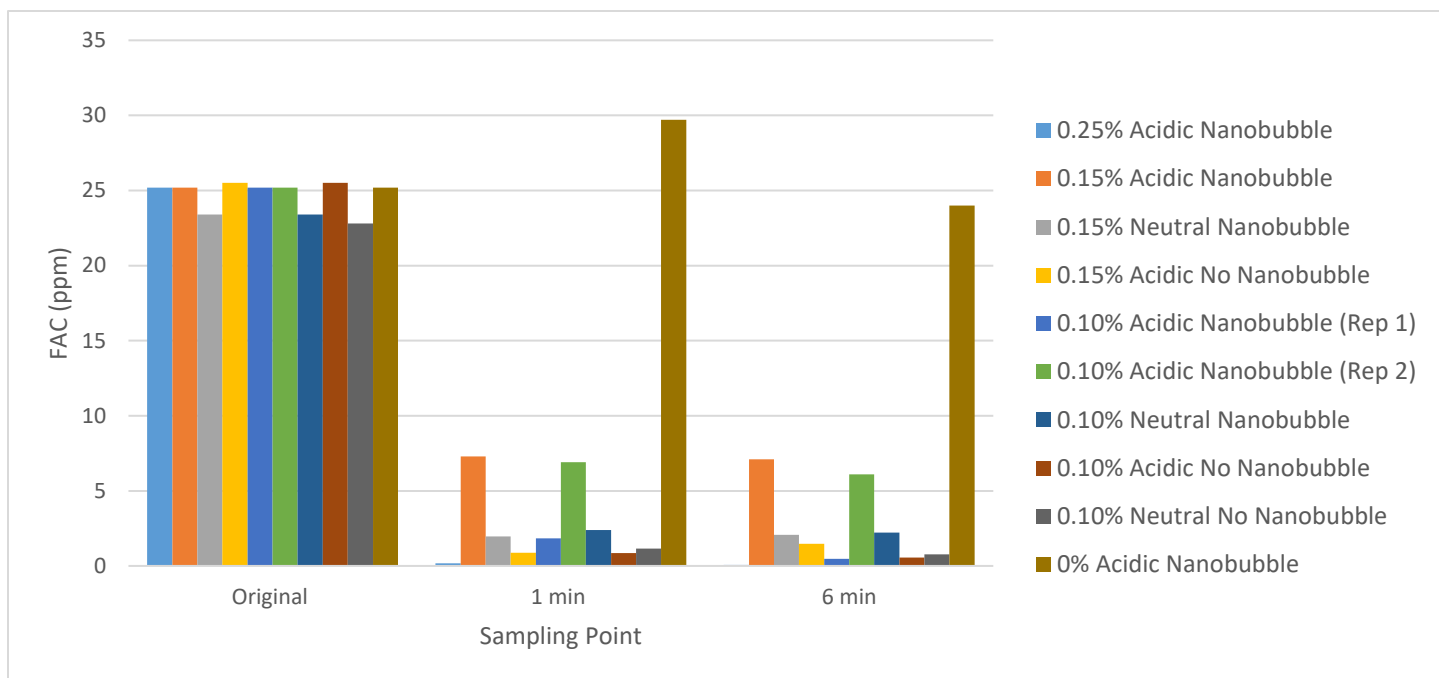


Figure 4.8 Residual Free Available Chlorine after Purge Addition (0-0.25%) to 25 ppm FAC Cesco-Bauer Water Solutions

Eventually, ~30 ppm FAC Cesco-Bauer water solutions were generated in Bellingham, WA, shipped, and received by the Kansas State University Food Safety and Defense Laboratory. To determine how long residual FAC lingered in solution, 0.05 and 0.1% beef purge was added to ~30 ppm FAC and monitored for 60 minutes. It was determined that a maximum level of 0.05% purge with an initial level of 27.78 ± 2.31 ppm free chlorine creates a red water solution that maintains residual levels of chlorine (~1 ppm) after 1 hour of exposure without re-infusion (Figure 4.9).

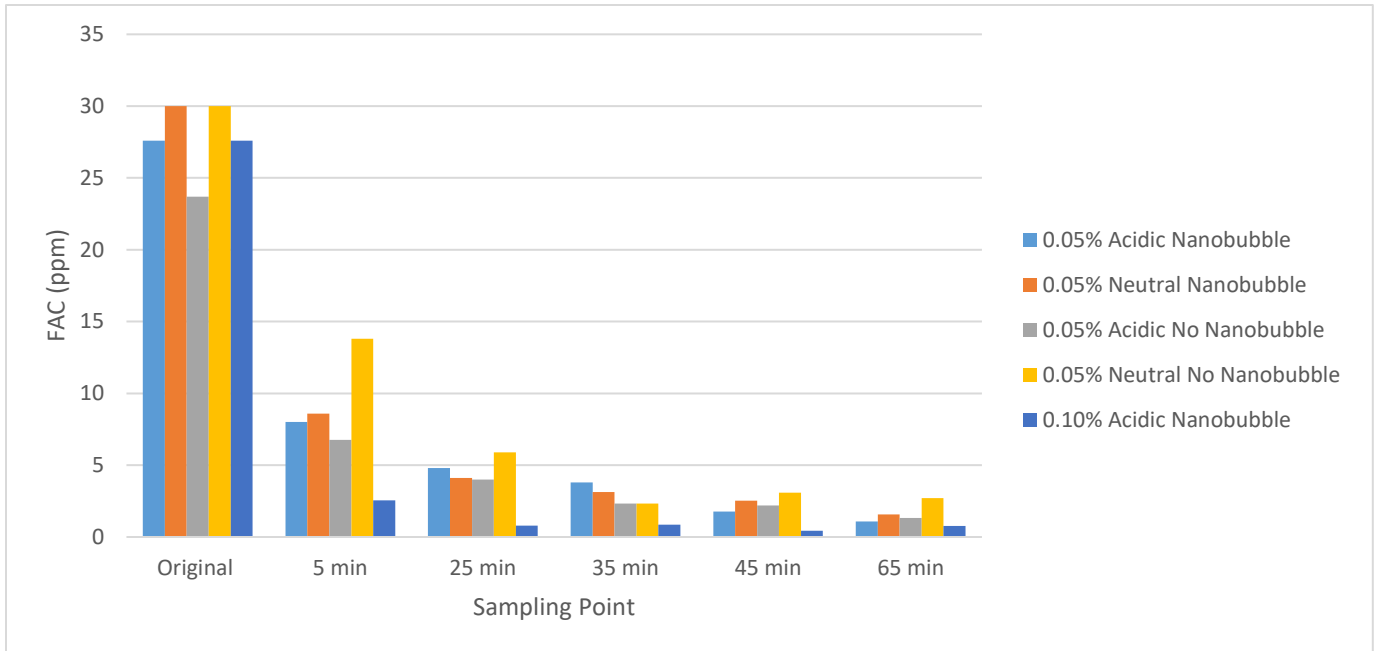


Figure 4.9 Residual Free Available Chlorine after Purge Addition (0.05-0.10%) to 23-30 ppm FAC Cesco-Bauer Water Solutions with No Re-infusion of Fresh Solution

Understanding the loading and effect of organic matter on chlorine loss in solution allows processors to determine recirculating water requirements and levels of chlorine-based antimicrobial needed to consistently maintain bactericidal levels in process water.

4.2.3.2 Pathogen survival

Based on the results from the chlorine demand experiments, higher levels FAC in initial solutions were obtained (~35 ppm FAC) and utilized in this experiment. Only acidic (pH 5) solutions were evaluated. Due to the use of a slightly stronger FAC Cesco-Bauer water, 0.1% purge was evaluated instead of 0.05% purge to mimic a ‘worst-case-scenario’ organically loaded red water. Zhou et al. (2015) determined that a minimum FAC level of 3.66 ppm at 5.12 pH in a recirculating produce wash water system, similar to red water, was sufficient to reduce *Salmonella*, *E. coli*, and *L. monocytogenes* by 6 log cycles after a 30 second contact time. Lethal levels of FAC were present in both the nanobubble and no nanobubble red water solutions during the initial exposure (1 min); however, levels of FAC were slightly higher in the no nanobubble solutions (Table 4.3), although there was no apparent difference in lethality of

target organisms in nanobubble versus no nanobubble solutions. In the presence of 0.1% purge red water solutions, the surrogates, STEC-7, and *Salmonella* spp. were reduced by ~5 log CFU/mL, ~4.8 log CFU/mL, and ~4 log CFU/mL, respectively after 1 minute. After 60 minutes exposure to Cesco-Bauer solutions, and one re-infusion at 35 minutes, STEC-7 populations were completely eliminated as determined by enrichment, whereas surrogates and *Salmonella* exhibited slightly higher resistance and were still recovered at 1.3 log CFU/mL or less. It has been argued that time exposure does not necessarily enhance the reduction of target organisms (Stopforth et al., 2008). However, this study indicates that the populations of target organisms decreased over time. As predicted, no lethality was observed in control solutions containing no nanobubbles and 0 ppm FAC over 60 minutes, with a solution re-infusion at 35 minutes.

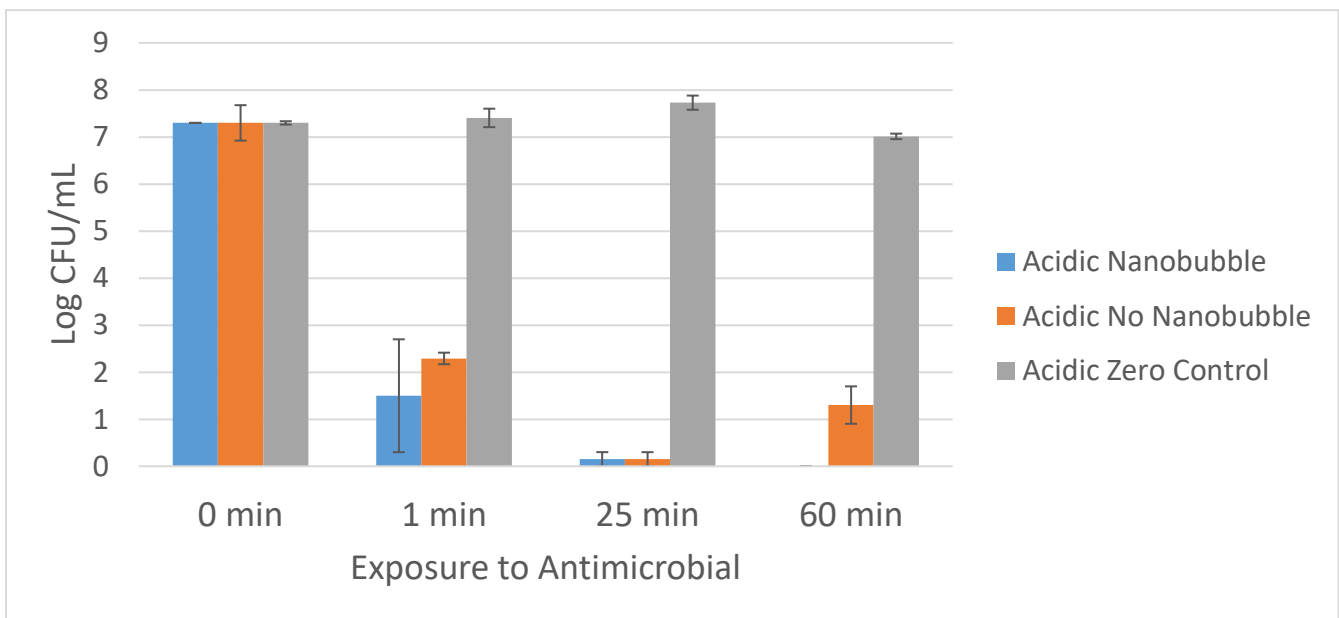


Figure 4.10 Average Recovery of Surrogates After Exposure to Red Water for 60 min in the Presence of 0.1% Purge on Injury Recovery Media

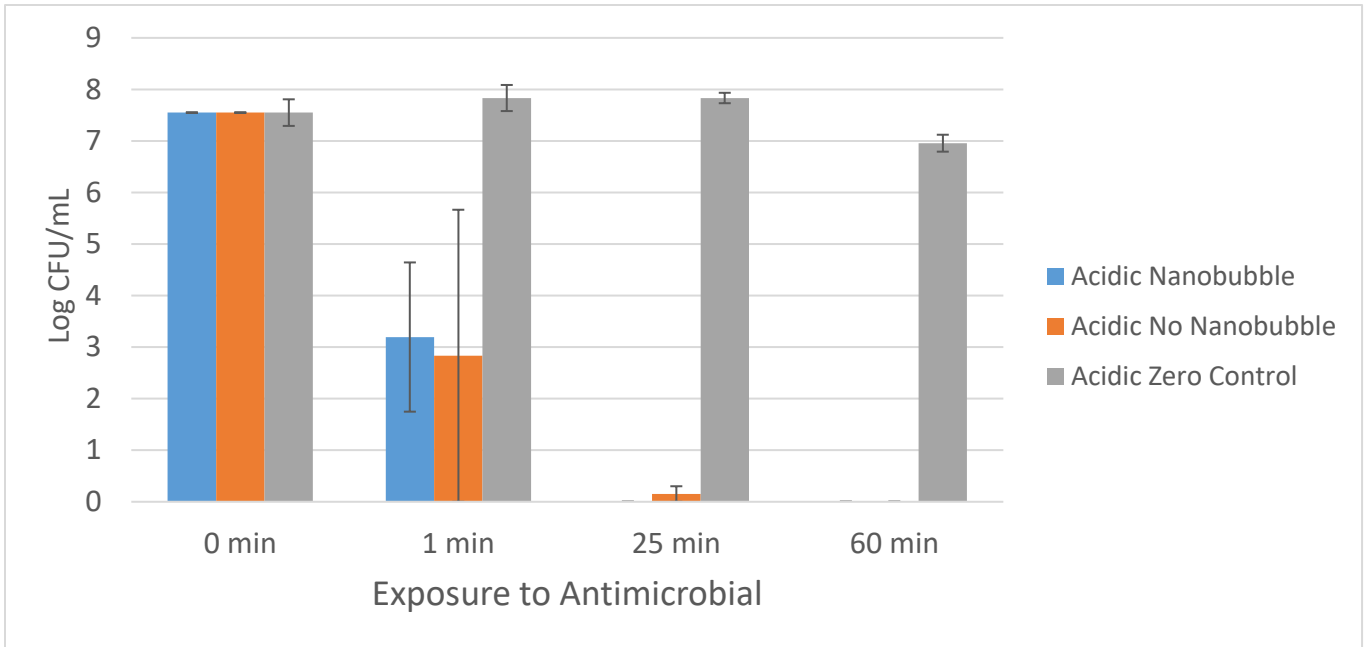


Figure 4.11 Average Recovery of STEC-7 After Exposure to Red Water for 60 min in the Presence of 0.1% Purge on Injury Recovery Media

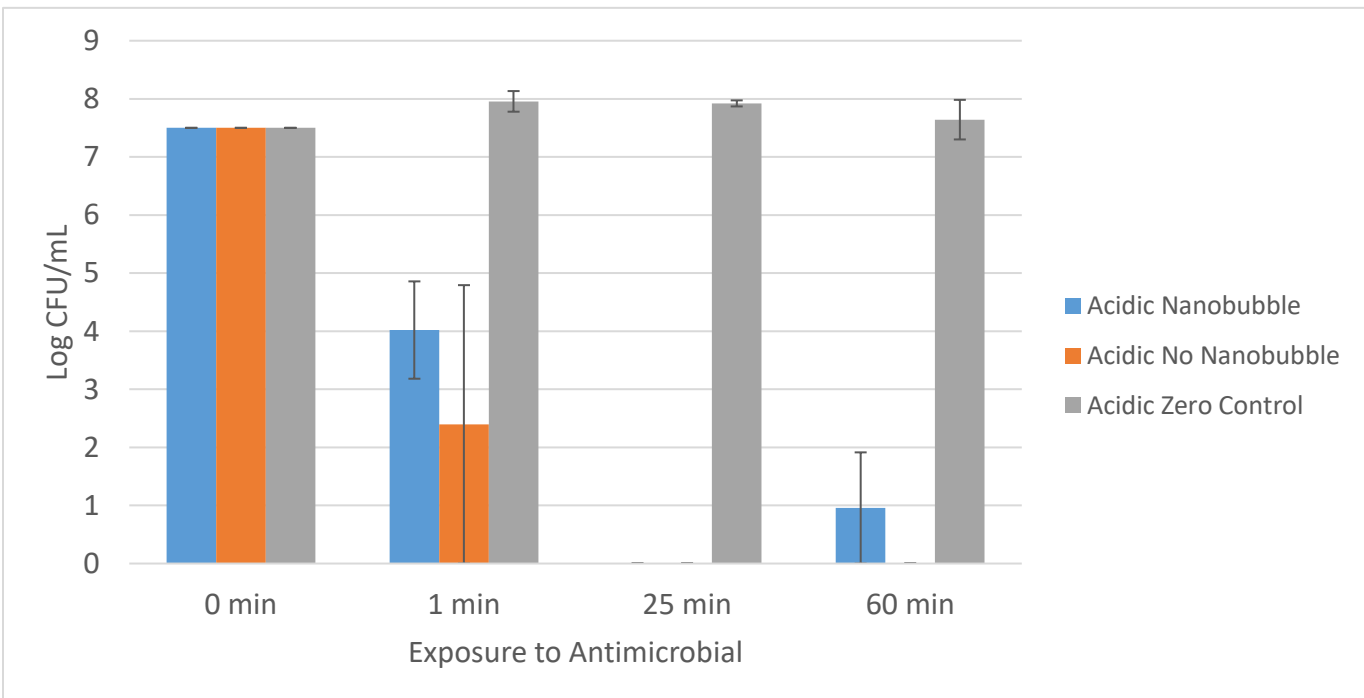


Figure 4.12 Average Recovery of *Salmonella* After Exposure to Red Water for 60 min in the Presence of 0.1% Purge on Injury Recovery Media

Table 4.3 Level of Free Available Chlorine (ppm) in Each Solution During 0.1% Purge Experiment at Each Sampling Point

		Nanobubble	No Nanobubble	Control
STEC	Initial Level	33.75±1.25	40.25±0.75	0
	1 min	5.64±1.56	10.19±6.01	0
	25 min	0.535±0.225	0.94±0.40	0
	35 min (after re-infusion)	1.4±0.77	2.7±1.50	0
	60 min	0.84±0.52	1.655±0.98	0
	Surrogates	Initial Level	33.75±1.25	40.25±0.75
1 min		4.91±1.21	11.03±5.17	0
25 min		0.34±0.05	0.855±0.45	0
35 min (after re-infusion)		1.335±0.35	2.205±0.35	0
60 min		0.57±0.29	1.435±0.71	0
Salmonella		Initial Level	33.75±1.25	40.25±0.75
	1 min	3.09±0.45	9.26±2.34	0
	25 min	0.19±0.09	0.565±0.15	0
	35 min (after re-infusion)	0.635±0.21	2.555±0.48	0
	60 min	0.385±0.12	0.85±0.12	0

4.2.4 Conclusion

When the target organisms were exposed to 3.09-11.03 ppm FAC solutions for 60 seconds, populations were notably reduced by 4-5 log CFU/mL in 0.1% red water. Although statistical analysis was not completed for this preliminary research, we can determine that the surrogate organisms act similarly to STEC-7 and *Salmonella* spp. in the presence red water, thus confirming the 5-strain surrogate cocktail to be an appropriate indicator for both target pathogens.

4.3 Efficacy of Chlorinated Nanobubble Solutions on Beef Lean and Fat in the Presence of Red Water

4.3.1 Introduction

Chlorine has proven to be an effective antimicrobial and sanitizing agent in the food industry; however, the level of free available chlorine (FAC), the indicator of sanitizing power, is dramatically affected by the presence of organic matter. This presents an interesting dilemma in a beef processing environment. Little research has been reported with chlorine on beef tissues, especially in a processing water dip scenario. Previous benchtop research reported in sections 4.1 and 4.1 determined the effectiveness of low levels of FAC in chlorinated nanobubble red waters against target organisms, but has not evaluate the effectiveness of these solutions for reducing microbial population levels on the surface of lean and fat tissues.

The primary goal of this experiment was to characterize the lethality of chlorinated nanobubble (i.e. Cesco-Bauer solution) red water against Shiga toxin-producing *E. coli* (STEC), select *Salmonella* serovars, and non-pathogenic surrogates on the surface of “shattered” lean and fat beef tissue (the preparation of “shattered” lean and fat is described in Chapter 5). The secondary goals of this experiment were to 1) determine the level of water contamination and length of time pathogens persist in recirculating ground beef processing water (mimicking the proprietary commercial processing system), and 2) determine the level of contamination picked up by non-inoculated meat entering the system following inoculated meat exposure. The experiment consisted of one red water container per organism cocktail held and treated for 6 continuous days to simulate the operational parameters of a novel proprietary ground beef manufacturing process (profiled in Chapter 5).

4.3.2 Materials and Methods

4.3.2.1 Meat source

Fifty pounds each of ‘shattered’ lean and fat pieces were obtained from the commercial beef processor (Clackamas, OR, USA) and frozen at 0°C until use. Approximately 500 g of lean or fat was thawed daily at 4°C 18 to 24 hours prior to use. The ‘shattered’ lean and fat pieces

are created by crust-freezing course ground (through a 3/4" inch plate) 50/50 lean/fat in a liquid-nitrogen tunnel, and passing the crust frozen meat through two smooth metal rollers that "shatter" the meat into smaller pieces (see detailed process description in Chapter 5).

4.3.2.2 *Bacterial cultures and inoculum preparation*

The rifampicin-resistant *E. coli* surrogates, rifampicin-resistant STEC strains, and *Salmonella* serovars used in this study were propagated and prepared as described in section 4.1.2.1; except the centrifuged culture cocktails were rehydrated in 90 mL of phosphate buffered saline (PBS).

Samples (100 g) of lean and fat tissues were mist-inoculated with 5-7 mL of rehydrated target culture cocktails (~7.5 log CFU/mL) individually and allowed to attach at 4°C overnight (approximately 24 hours) before use. The same culture cocktails were used to inoculate lean and fat pieces throughout the experiment. Rehydrated cocktails were stored at 4°C throughout the duration of the experiment.

4.3.2.3 *Antimicrobial water solutions*

Cesco-Bauer water adjusted to pH 5 and 38.5 ppm FAC was generated in Bellingham, WA and was ground shipped to the Kansas State University Food Safety and Defense Laboratory. This shipment was used within 18 days of production, and utilized throughout the 6-day experiment as the initial water solution to prepare red water, and for solution re-infusions throughout the experiment. pH was manually adjusted at the K-State laboratory by bubbling small amounts of CO₂ gas into the stored Cesco-Bauer solutions at the beginning and middle of the experiment (Day 1 and Day 4) to maintain pH 5 in the original Cesco-Bauer water.

4.3.2.4 *Experimental procedure*

One liter of 38.5 ppm FAC, pH 5 Cesco-Bauer water was added to three sterilized, sealable plastic containers (Rubbermaid, Atlanta, GA) individually—one container for each organism cocktail—and stored in a 4°C walk-in cooler for the duration of the 6-day experiment. 0.1% by volume beef purge was added to each container to create red water. Every 6 hours, red water solutions were re-infused with fresh 38.5 ppm, pH 5 Cesco-Bauer water; 10% 'used' solution was removed and 10% fresh solution was added to mimic recirculating water in the

commercial processing system. The red water solutions were also re-infused following each introduction of non-inoculated meat twice daily.

Refrigerated lean (25 g) and fat (25 g) portions that had been inoculated 24 h previously were combined and dropped into each container at the beginning of the day, every other day—Days 1, 3, and 5. Each container was manually agitated to stimulate a laminar flow scenario. Lean and fat tissues were exposed to the red water antimicrobial solutions for ~60 seconds and then removed with a sterilized metal strainer. The lean and fat tissues were separated from each other using sterilized metal spoons, with components weighed into separate filtered stomacher bags (Seward, United Kingdom) containing 100 mL DE broth. Refrigerated non-inoculated lean (25 g) and fat (25 g) was similarly introduced into the container, exposed for ~60 seconds, and removed (as described above) twice daily, 15 and 45 minutes after the inoculated meat removal, to determine pick-up of surviving organisms. Non-inoculated meat was added again to the containers to determine pick-up of surviving organisms 24 hours later—Days 2, 4, and 6.

Red water samples (5 mL) were collected each time meat was introduced into the container, both inoculated and non-inoculated, and every 6 hours prior to re-infusion of new solution to determine surviving levels of target organisms in solution. FAC, pH, and ORP was measured for each water sample collected (HI96711 Portable Photometer, Hanna Instruments, Woonsocket, RI, USA; PT3 and PT4 pens, Myron L Company, Carlsbad, CA, USA). Due to the nature of a continuously re-infused solution, replications are differentiated by inoculation day with reps defined as Day 1 and 2, Day 3 and 4, and Day 5 and 6 for a total of three replications.

4.3.2.5 *Microbial analysis*

Meat Samples—Portions (25 g) of lean or fat were added to filtered stomacher bags containing 100 mL DE broth, stomached (Smasher™, bioMerieux, Hazelwood, MO, USA) for 60 seconds, and serially diluted with PBS. To enumerate surviving *Salmonella* populations, dilutions were spread plated on XLD agar incubated for 24 hours at 37°C, or on TSA that was incubated for 6 hours at 37°C and followed by overlaying with XLD with additional incubation for 12-18 h at 37°C. To enumerate rifampicin-resistant surrogates and STEC-7 on selective media, 10 mL of the original homogenized sample (25 g meat + 100 mL DE broth) was removed from the original

bag and added to 0.1 g/L rif and serially diluted using PBS blanks containing 0.1 g/L rif (PBS+rif). Dilutions were plated on *E.coli*/coliform Petrifilm (ECC; 3M Corporation, Saint Paul, MN, USA), and incubated at 37°C for 24 hours. To determine injury recovery of rif-resistant surrogates and STEC-7, samples were diluted with PBS blanks, spread plated on TSA and incubated for 6 hours at 37°C, overlaid with TSA+rif, and incubated for 12-18 additional hours at 37°C.

Water Samples— Red water aliquots (5 mL) were pipetted directly into a test tube containing 5 mL DE broth, manually mixed for 60 seconds, serially diluted with PBS, and spread plated on XLD agar that was incubated for 24 hours at 37°C to detect *Salmonella* spp. Additionally, to detect injured *Salmonella* cells, the dilutions were spread plated onto TSA incubated for 6 hours at 37°C, subsequently overlaid with XLD, and incubated for 12-18 additional hours at 37°C. To enumerate rifampicin-resistant surrogate population and STEC-7 on selective media, 2 mL of the original homogenized water sample (5 mL water + 5 mL DE broth) was removed from the tube and added to 0.1 g/L rif, serially diluted with PBS blanks containing 0.1 g/L rif (PBS+rif), plated on *E.coli*/coliform Petrifilm (ECC; 3M Corporation, Saint Paul, MN, USA), and incubated at 37°C for 24 hours. To enumerate injured populations of rif-resistant surrogates and STEC-7, samples were diluted using PBS blanks, spread plated on TSA, incubated for 6 hours at 37°C, overlaid with TSA+rif, and incubated for 12-18 additional hours at 37°C.

Due to time and labor constraints, enrichments were not completed on any samples that tested negative by direct plating protocol (detection limit for water samples 0.3 log CFU/mL, meat samples 0.7 log CFU/g).

4.3.3 Results and Discussion

It is important to note that this section consists of data that has not been statistically analyzed and therefore should be considered preliminary. The purpose of this evaluation and the findings therein was to provide insight into the effectiveness of Cesco-Bauer solutions loaded with 0.1% beef purge (i.e., red water) treatments to help plan for the subsequent in-plant commercial validation studies described in Chapter 5.

4.3.3.1 Free available chlorine, pH, and ORP of red water solutions

The original Cesco-Bauer water consisted of 38.5 ppm FAC, 5.03 pH, and 833 mV ORP. The FAC in the solutions dropped considerably to 13.87 ± 1.09 ppm after the introduction of 0.1% by volume beef purge; pH and ORP did not change. However, once meat entered the system 15 minutes after purge addition, the properties of the water changed dramatically; FAC levels dropped below 1 ppm (Figure 4.13), pH increased to 5.7 (Figure 4.14), and ORP decreased to 350 mV (Figure 4.15) and stayed at these levels throughout the duration of the experiment despite re-infusion of fresh Cesco-Bauer water solution every 6 hours.

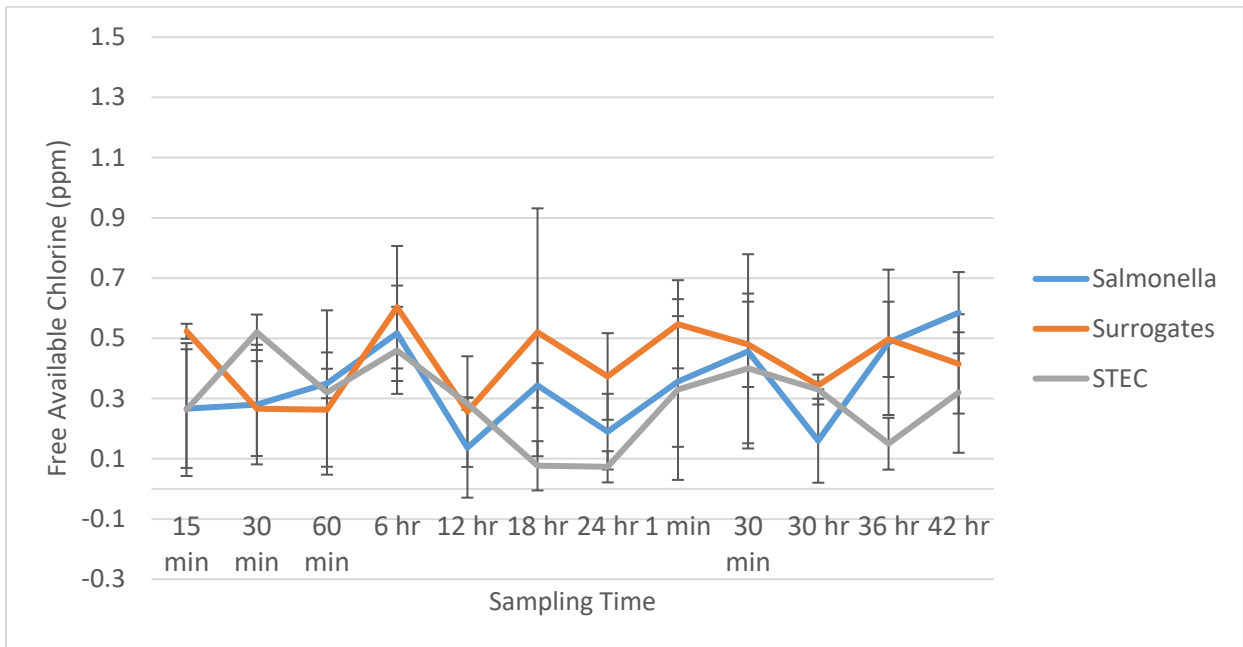


Figure 4.13 Average Free Available Chlorine in Red Water over 42 hours

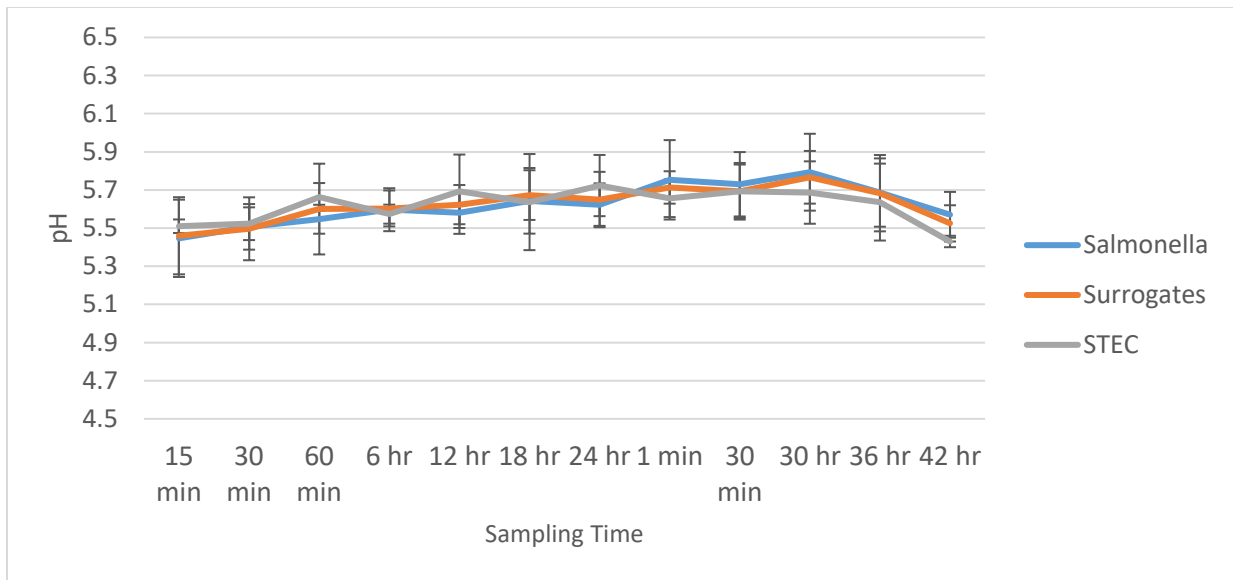


Figure 4.14 Average pH of Red Water over 42 hours

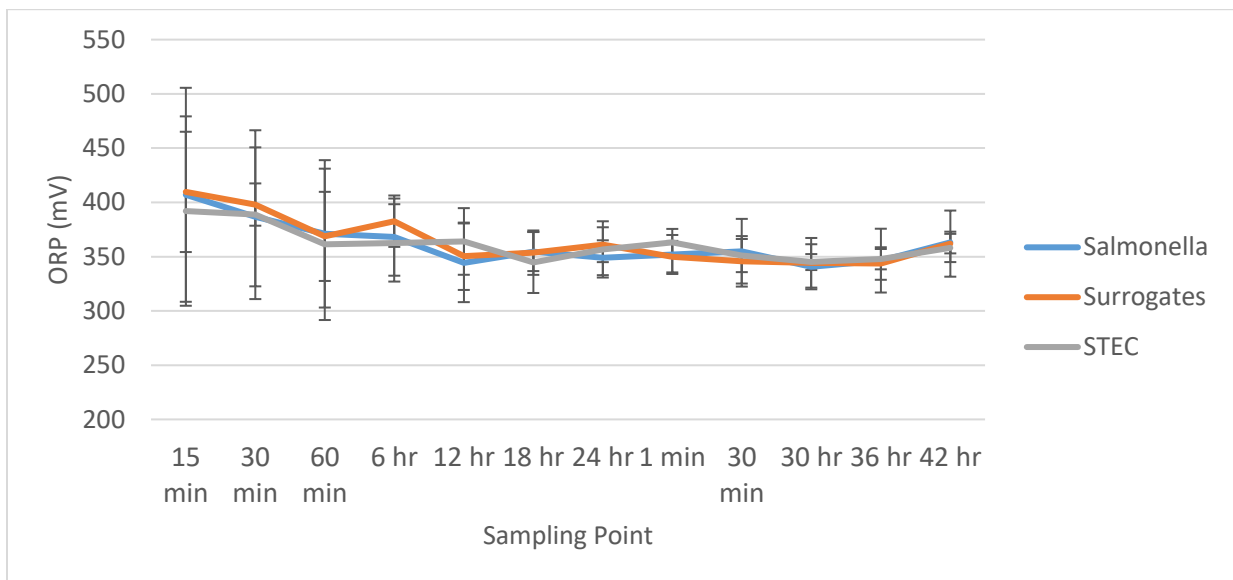


Figure 4.15 Average ORP of Red Water over 42 Hours

4.3.3.2 Pathogen recovery in water samples

Zhou et al. (2015) determined that a minimum FAC level of 3.66 ppm at pH 5.12 to 6.97 in a recirculating produce wash water system was sufficient to reduce *Salmonella*, *E. coli*, and *Listeria monocytogenes* by 6 log cycles after a 30 second contact time. As reported in section 4.3.3.1, the levels of FAC and ORP were not effectively maintained to have an antimicrobial

effect on STEC-7, *Salmonella*, or surrogates within the 60 seconds of initial exposure. However, in the presence of proteins, chlorine will form chloramines and retain some level of antimicrobial effect even when FAC is reduced to unmeasurable concentrations. Block (1991) reported 100% reductions of *Salmonella pullorum* in a 130 ppm hypochlorite solution with 5% organic matter, although there was no measurable level of FAC. This demonstrated the sanitizing capacity of chloramines. It is likely that the initial (38.5 ppm) level of chlorine did not form chloramines at a bactericidal level in this experiment. pH was maintained at an appropriate level to facilitate formation of almost exclusively hypochlorous acid. The re-infusion of fresh solution contributed to the reduction and eventual elimination of surrogates and STEC-7 after 24 hours and *Salmonella* after 42 hours. Gradual reductions during the first hour of sampling may be attributed to a washing effect of organisms attached to non-inoculated meat, which was subsequently removed from the red water for lab testing. Of the target organisms evaluated, the *Salmonella* serovars examined appear to have higher resistance to the Cesco-Bauer chlorinated nanobubble solutions, surviving longer than the rif-resistant surrogates and STEC-7 strains in red water (Figure 4.16).

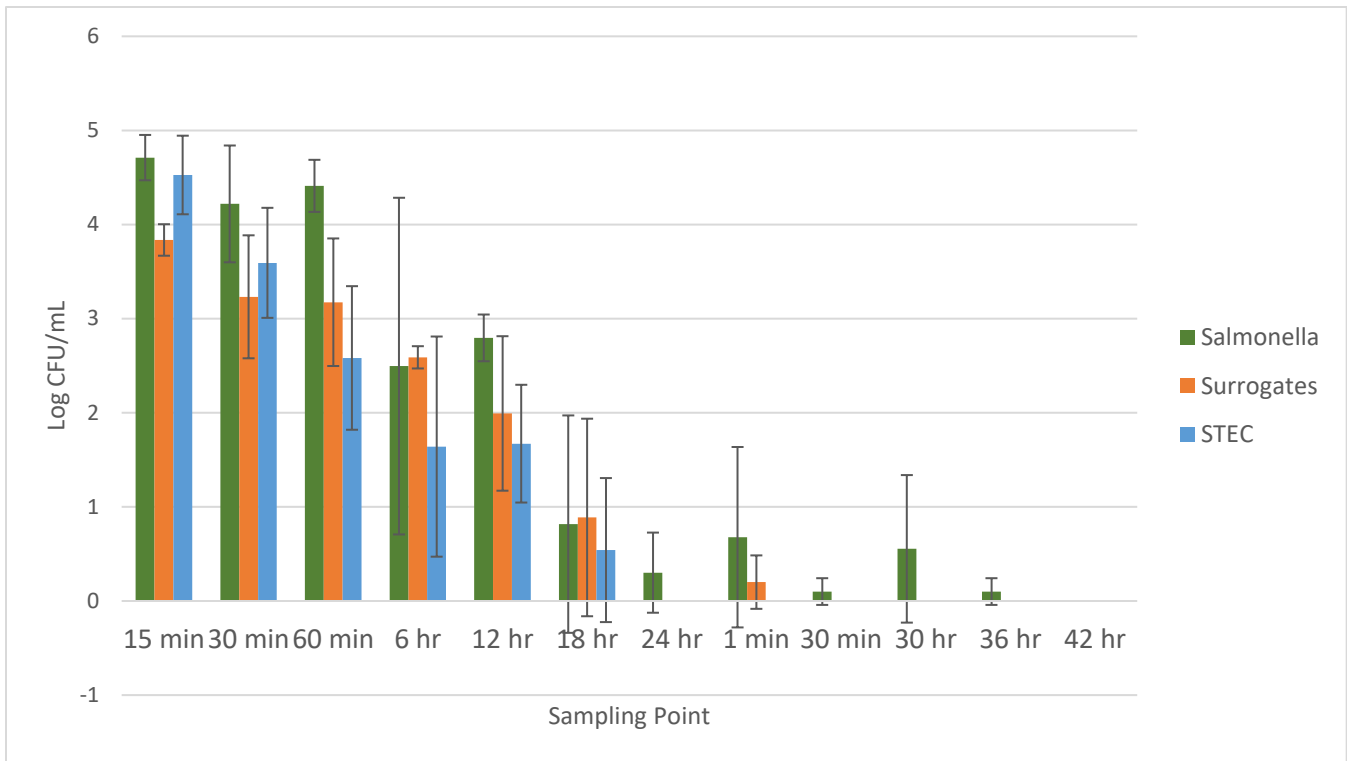


Figure 4.16 Average Recovery of Organisms in Red Water over 42 Hours on Injury Recovery Media

4.3.3.3 Pathogen recovery on lean and fat

Little to no reduction of target organisms occurred on the surface of either inoculated lean or fat tissues (Figure 4.17). This can be attributed to non-lethal levels of FAC present in the red water solution at the time the meat was introduced (4.13). All target organisms were recovered at 3-4 log CFU/g on non-inoculated lean and fat added to the containers 15 and 45 minutes after inoculated meat was dropped into the red water and subsequently removed (Figure 4.18). This corresponds to the level of organisms recovered in water at the same time points (30 and 60 minutes as depicted in Figure 4.16). Target organisms were still recovered on non-inoculated meat dropped into the system 24 hours after inoculated meat was introduced to the system (Figure 4.19); these levels do not necessarily correlate to the levels of organisms present in the red water. STEC-7 and surrogates were recovered at lower levels (≤ 0.73 log CFU/g) than *Salmonella* spp. (1.0-1.6 log CFU/g).

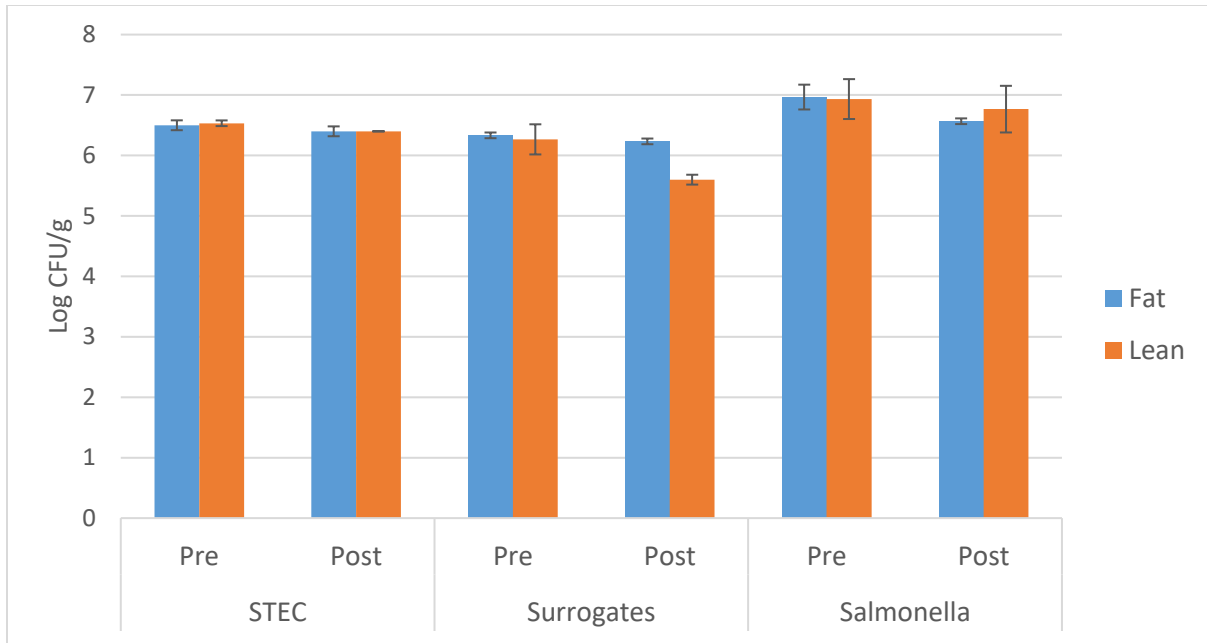


Figure 4.17 Average Recovery of Organisms on Inoculated Meat Before and After ~60 second Exposure to the Antimicrobial Red Water on Injury Recovery Media

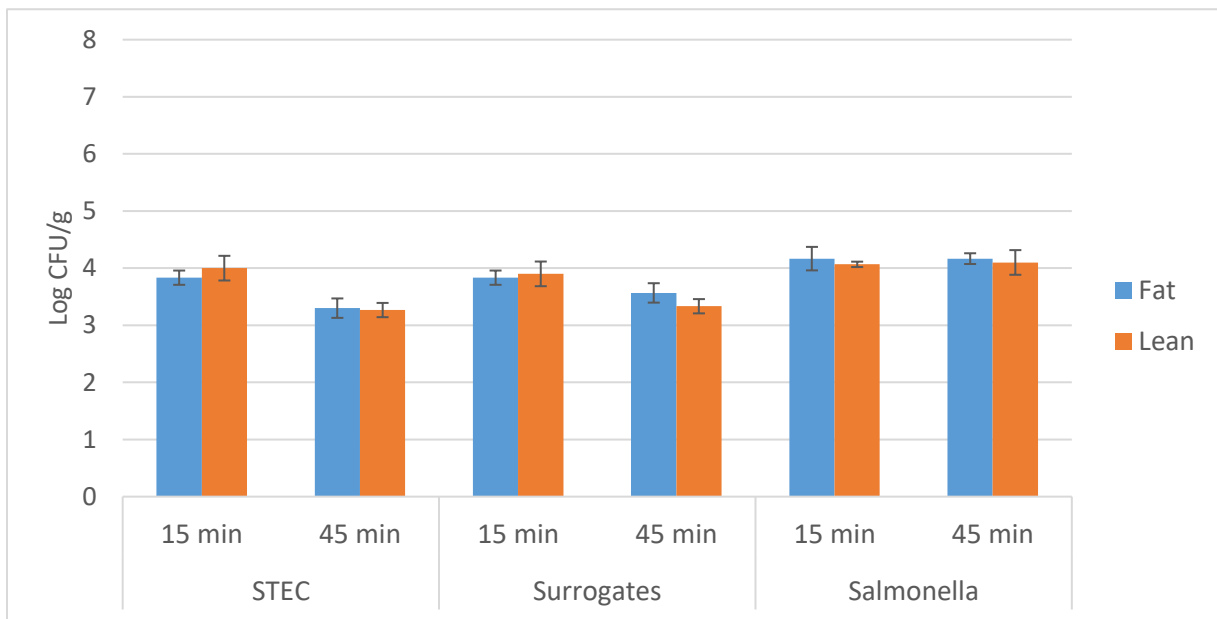


Figure 4.18 Average Recovery on Non-Inoculated Pick-Up Meat Introduced into the System 15 and 45 minutes after Inoculated Meat on Injury Recovery Media

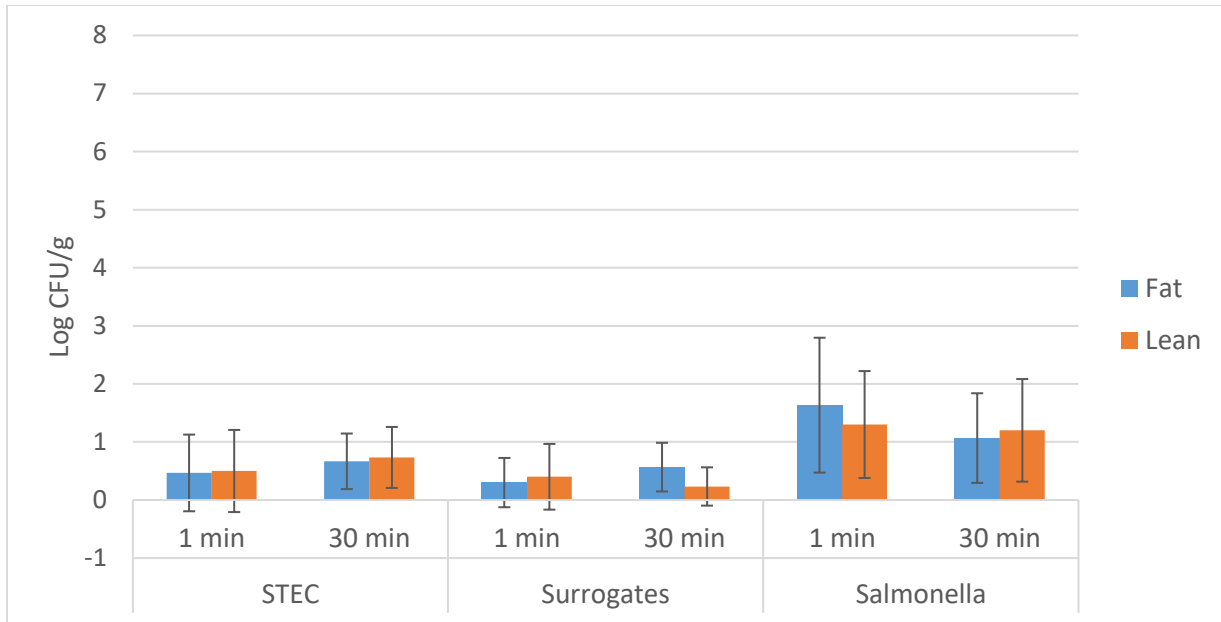


Figure 4.19 Average Recovery of Target Organisms on Non-Inoculated Meat Introduced to Red Water 24 hours after Inoculated Meat on Injury Recovery Media

4.3.4 Conclusion

Ultimately, FAC levels were extremely low and did not contribute much to lethality of STEC-7, surrogates, or *Salmonella* on the surface of inoculated lean and fat. *Salmonella* spp. persisted in the red water for 18 hours longer than STEC-7 and the surrogates and was recovered at higher levels than STEC-7 and surrogates on non-inoculated meat 24 hours after the introduction of inoculated meat. This could be due to the slightly higher inoculation load (~0.5 log CFU/g) on the lean and fat entering the system for *Salmonella*, or could be a result of higher resistance of *Salmonella* to the Cesco-Bauer water. Statistical analysis was not completed for this particular experiment therefore making judgements on differences difficult.

This experiment provided insight into the estimated number of organisms introduced into the Cesco-Bauer water system from inoculated meat and how long organisms persist in red water. Further research should be conducted to determine reduction of target organisms on inoculated lean and fat when dipped into a 5 ppm FAC red water solution, matching the USDA regulatory limit and target in-plant commercial validation study FAC parameters. Additional experiments should evaluate the effect of re-infusing solutions at different levels and/or

different lengths of time compared to no re-infusions. The primary goal of this benchtop experiment was to approximate the parameters of a proprietary ground beef system in preparation to conduct an in-plant study validating a recirculating 5 ppm FAC, pH 5 Cesco-Bauer water enhanced using nanobubble technology as an antimicrobial step. Several parameters were hard to duplicate closely and could be re-evaluated in potential future benchtop experiments. These parameters include 1) a nitrogen crust freeze of meat to better represent beef tissues being treated in the commercial system, 2) continuous recirculation and reinfusion of Cesco-Bauer of water, 3) filtering of red water through a 50 μm particle filter during recirculation to remove some organics, and 4) maintaining lethal levels of FAC (approximately 5 ppm) in red water constantly.

Chapter 5 - Evaluation of a Chlorinated Nanobubble Water System to Control Shiga Toxin-producing *E. coli* Surrogates in a Novel Commercial Ground Beef Manufacturing Process

5.1 Introduction

Beef products, specifically ground beef, have been associated with human illness, disease outbreaks, and product recalls due to contamination from Shiga toxin-producing *E. coli* (STEC) and *Salmonella enterica*, both cattle harbored foodborne pathogens. Although not declared adulterants in beef, *Salmonella* has been associated with 35% of ground beef outbreaks from 2002-2011 (Laufer et al., 2015). The United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) has declared seven serotypes of enterohemorrhagic STEC to be adulterants in raw, non-intact beef—O26, O45, O103, O111, O121, O145, and O157: H7—due to the severity of illness caused after human consumption (CDC, 2012). STEC infection traditionally induces hemorrhagic colitis and, in severe cases, hemolytic uremic syndrome (HUS) in high-risk populations such as children. Similar to *E. coli*, *Salmonella* infections are characterized by gastrointestinal illness; however, *Salmonella* illness usually is self-limiting within 72 hours, except in severe cases death can occur from complications related to the illness, usually dehydration (FDA, 2012). *Salmonella* infections cause an estimated one million illnesses and 19,000 hospitalizations in the US annually (CDC, 2016), and are a major risk factor in a wide variety of raw and processed food products.

Meat processing establishments are required to implement Hazard Analysis and Critical Control Point (HACCP) plans which specifically mandate identification of biological, chemical, and/or physical hazards and critical operating parameters to control identified hazards, such as STEC and *Salmonella* in beef (FSIS, 1996). These critical operating parameters must be based on scientific evidence. If sufficient data is not available in the scientific literature, an in-plant validation study should be conducted (USDA FSIS, 2015). Validation of in-plant antimicrobial processes against select pathogens is critical; however, actual pathogens cannot be reasonably brought into food processing environments and laboratory research, while valuable as a reference, is not a substitute for actual in-plant validation (Niebuhr et al., 2008). Therefore, the

use of appropriate non-pathogenic indicator organisms, otherwise known as surrogates, can be valuable when evaluating effectiveness of individual plant processes against pathogens such as STEC and *Salmonella*.

Chlorinated water is often used as a critical control point (CCP) for controlling pathogens in poultry and produce washing processes; however, processes are not often scientifically validated during commercial process operations (Zhou et al., 2015). Electrolytically generated hypochlorous acid, one form of a chlorine antimicrobial, is allowed for use in meat and poultry processing operations as processing water and recirculated red water at levels not exceeding 5 ppm free available chlorine (FAC), and in poultry chiller water at a maximum of 50 ppm FAC (USDA FSIS, 2016). These treatments have been found to effectively reduce pathogens in wash water (Zhou et al., 2015) and on the surface of poultry carcasses and produce (Najjar & Meng, 2009; Sohaib et al., 2016; Stopforth et al., 2008; Yang et al., 2012). Little research has been conducted with chlorinated water solutions as an antimicrobial on beef tissues, especially in a recirculating processing water dip scenario.

A relatively new concept in the food industry, nanobubble technology shows promise to aid in the development of improved food safety interventions. There is interest in nanobubbles due their proposed surfactant abilities or cleaning effect. Small particles in water can be effectively removed by introducing micro- or nanobubbles of opposing charge and zeta potential, which is controlled by the pH of the solution (Tsuge, 2014). Nanobubbles also provide increased surface area-to-volume ratio per mass as compared to standard water or other aqueous solutions ("Bauer Nanobubbles," 2016), which theoretically, enhances the efficiency of any dissolved or suspended antimicrobial components in solution. Nanobubble treatment, as a sanitation method, has been evaluated against norovirus surrogates in oyster bodies and was found to inactivate more than 99% of active virus after 12 hours (Tsuge, 2014).

The antimicrobial intervention for a novel ground beef manufacturing process (Patent No. 9167843) was evaluated at a non-inspected commercial-scale pilot plant facility, with all manufactured beef products during the period of the inoculated studies being sent to inedible rendering operations. Briefly, this patented system utilizes higher-fat commercial beef trimmings to separate predominantly lean tissues from predominantly fatty tissues while the

trimmings are being transported through a recirculating chilled antimicrobial fluid conduit system. Through proprietary operating conditions, lean tissues are recovered for further processing into raw ground beef demonstrating typical quality characteristics of traditionally manufactured product. An added benefit of this trim processing approach is the submersion of all beef tissue surfaces (fat and lean) in the recirculating fluid, which if adequately antimicrobial, provides a unique pathogen control opportunity in the raw ground beef manufacturing process. The portion of the trim treatment process utilizing the recirculating fluid is envisioned as operating over a multi-day period with continuous re-infusion of fresh make-up antimicrobial solution at defined points and intervals. Thus, such a system must be capable of inherent disinfection to prevent microbial build-up over the extended processing period.

Two inoculated in-plant studies were conducted using USDA-approved non-pathogenic surrogate cultures to evaluate the effectiveness of the recirculating antimicrobial fluid—an acidic (pH 5) Cesco-Bauer water [i.e. municipal water that is continuously chlorinated by infusion of concentrated chlorine produced through an electrolyzed (EO) water process, acidified by introduction of CO₂ gas, and then passed through a patented nanobubble generator (Patent No. 8454837)]. The first study was conducted to determine preliminary inefficiencies in operational variability/stability of the novel commercial scale ground beef system over a determined 6-day continuous production run and to optimize inoculation, sampling and testing protocols. The second study incorporated necessary processing system modifications to enable characterization of lethality of the antimicrobial process water on inoculated beef trimmings and equipment components included in the recirculating conduit system that support multi-day continuous processing. The optimized study will be used by the ground beef manufacturing company as scientific validation of this novel system for controlling enteric pathogens such as STEC and *Salmonella* spp. in their future commercial ground beef operations.

5.2 Unit Components of the Novel Ground Beef Processing System

The performance of a recirculating nanobubble water solution (i.e. Cesco-Bauer water) at pH 5 and that was continuously infused with concentrated chlorine [maximum free available chlorine (FAC) level of 5 ppm] was evaluated in a commercial-scale ground beef processing

environment using a 6-day continuous run schedule. The processing flow is described below and depicted in Figure 5.1.

Receiving Beef Trim—Beef trim is received at the facility in approximately 2,000-lb commercial combo totes and stored between 3 and 5°C until processing.

Grinding—The refrigerated combos are dumped into a commercial grinder and the beef trim is course ground through a 3/4" plate onto a conveyor belt.

Freezing Tunnel—Meat is delivered to a liquid-nitrogen tunnel by the conveyor belt. The meat is exposed to a rapid 8-minute freeze on a separate stainless-steel switchback belt.

Bond Breaker—Crust-frozen meat drops from the freeze tunnel belt through two smooth metal rollers that “shatter” the meat into smaller pieces, beginning the fat and lean separation process of the proprietary system.

Vortex—From the bond breaker, the crust frozen ‘shattered’ meat falls directly into the antimicrobial chlorinated nanobubble process water at a vortex. This is the beginning point of a recirculating process water system comprised of multiple machinery components subsequently listed. The recirculating process water is described below. The vortex connects directly to the manifold.

Bulk Tank Chlorinated Cesco-Bauer Nanobubble Water—Bulk chlorinated (5 ppm FAC), acidified (pH 5) nanobubble water is generated onsite using a Cesco-Bauer process (in the current studies, a mobile generation plant on a trailer was utilized), which is then stored in a ~19,000 L bulk tank. This water is chilled to approximately 4 °C and used to initially fill the entire recirculating water system prior to beginning meat processing. Additionally, Cesco-Bauer water from this tank is infused at an approximate rate of 10% percent per hour immediately at the “Y” in the manifold, as described below, during meat processing operations to maintain proper processing water quality and replace water losses.

Manifold—The antimicrobial process water carries meat through a series of stainless steel pipes for 30 to 90 seconds. From a separate holding tank, fresh 5 ppm FAC, pH 5 Cesco-Bauer water is also introduced at 30-52 L/min at the “Y” section in the manifold. A majority of the predominantly lean meat tissue sinks to the bottom of the manifold pipes and subsequently drops down through a series of drop-ports into a collection tank that directly feeds to a

dewatering centrifuge. Predominantly fat tissue floats through the manifold and into the agitated flotation tank.

Agitated Flotation Tank—Meat enters the ~18,000 L flotation tank at the front end (A) of the ~6 m long tank. A series of slow-moving rotating stainless steel paddles at the top of the tank facilitate agitation of the water and movement of the meat to either the bottom of the tank (mostly lean-type tissue) or the top of the tank (mostly high-fat tissue). Meat at the bottom of the tank is extracted through a series of ports and is pumped back to the collection tank mentioned above that feeds the dewatering centrifuge. Any meat floating in the tank or adhered to the walls of the tank is removed by the rotating paddles at the front end (A) of the tank (Figure 5.1). To maintain the target level of 5 ppm FAC in the flotation tank, 50 ppm chlorine solution derived by diluting 5000 ppm Aquaox 5000™ (Aquaox LLC, Dillsburg, PA, USA) is added into the back end (B) of the tank at 80-685 mL/minute (Figure 5.1). The movement of the rotating paddles helps uniformly distribute the chlorine throughout the flotation tank.

*Note: The addition of 50 ppm chlorine into the flotation tank to raise FAC in the presence of organic material in the tank water was only conducted during the optimized study.

Dewatering Centrifuge—The meat from four drop-ports along the ~6.7 m length of the manifold conduit is combined with lean meat that settles to the bottom of the flotation tank in the collection tank that supplies the dewatering centrifuge. This meat is centrifuged (P-3000 Sharples, Alfa-Laval, Warminster, PA) at 795.2 x g to remove excess process water from the final lean meat product.

Final Product—Final beef products are gathered in two places: 1) Lean meat dropped from the dewatering centrifuge into a sanitized plastic bin or onto a collection belt; and 2) Fat scraped from the top of the agitated floatation tank and collected into a large plastic bin for further usage applications (not evaluated in the current study).

Particle Filter—The recirculating process water accumulates organic build-up over time from beef tissues introduced into the system. The antimicrobial process water is continuously pumped at a rate of ~20 L/min from the back end of the flotation tank through a 20 or 50-micron filter (Tequatic Plus SS-17 and SS-22 filters; Dow Chemical Company, Midland, MI, USA) to remove particles from the recirculating system.

Chlorine Infusion—After the particle filtration, concentrated chlorine is continuously re-infused back into the recirculating system using full strength Aquaox 5000™ (5,000 ppm FAC; Aquaox LLC, Dillsburg, PA), which is commercially generated via electrolyzed water technology, to a target of 5 ppm FAC in the processing “red water” as it returns to the beginning vortex point of the process. Infusion is accomplished using a pump that delivers 40-162 mL/min into the conduit of the recirculating water stream.

Carbon Dioxide Infusion—Carbon dioxide gas is bubbled into the conduit containing the recirculating water immediately after particulate filtration to maintain the target pH 5.

Nanobubble Generation— In-line equipment generated new nanobubbles into the reinfused water system. The re-introduction of nanobubbles assists in stabilizing chlorine and pH levels in an aqueous solution with high organic levels, while also maintaining a higher ORP reading (an indirect measurement of nanobubble concentration).

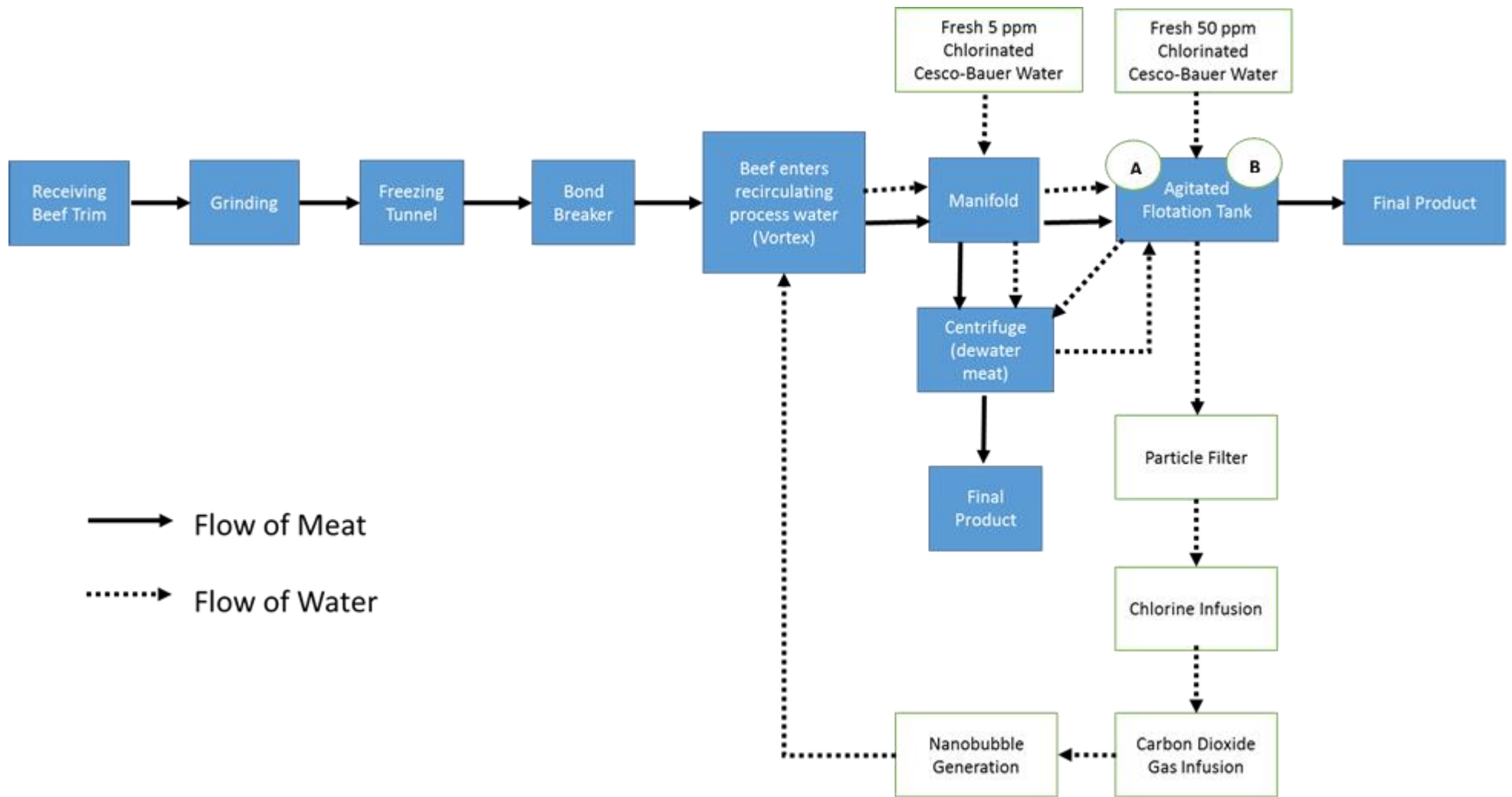


Figure 5.1 Novel Ground Beef Manufacturing System Utilizing Chlorinated Nanobubble Process Water
 Point A indicates the front end of the agitated flotation tank whereas Point B indicates the back end of the tank.

5.3 Materials and Methods

5.3.1 Preliminary Validation Study

5.3.1.1 Antimicrobial treatment water source

The total volume of the recirculating process water system was ~23,000 L. On the day prior to initiating meat processing studies, the system was filled with Cesco-Bauer nanobubble water (pH 5, 5 ppm FAC), generated onsite by technical staff from Cesco Solutions, Inc. (Bellingham, WA) using a mobile generation system, and chilled to 4.5°C. Additional Cesco-Bauer water was generated and stored in a separate ~19,000 L bulk tank which entered the system at the 'Y' in the manifold as described above. Nanobubbles are generated by passing water through a patented (Patent No. 8454837) generator utilizing cavitation chambers and shear planes to initiate an endothermic reaction thus producing a high concentration of paramagnetic oxygen nanobubbles with a mean particle size between 50 and 100 nm ("Bauer Nanobubbles," 2016).

The recirculating filtered Cesco-Bauer water solution ('red water') was continuously re-infused with a small volume (40-162 mL/min) of concentrated Aquaox 5000™ to boost processing water FAC levels back to 5 ppm, CO₂ gas was metered into the conduit (1-2 times daily at 1 min/ft³ for approximately 15 min) to maintain 5 ± 0.5 pH to increase the proportion of hypochlorous acid in the water while restricting chlorine off-gassing, and nanobubbles were generated by passing through a patented in-line generator as described above. This filtered, re-infused Cesco-Bauer water at 5 ppm FAC and pH 5 then reentered the opening of the vortex to treat in-coming crust frozen beef trim.

5.3.1.2 Meat source

Six 2,000-lb combo totes of 25/75 (% lean to fat) commercial beef trim were obtained from a large beef processor approximately 7 days following production. These plastic covered totes were held in the processing room of the pilot facility at 8-17°C for the 6-day study, with one combo tote used on each processing day. On the day of use of each combo of beef trim, the microbiological baseline quality of the product prior to inoculation was determined by

collecting 15 mL of purge and conducting an analysis to estimate the total aerobic bacterial population and to determine the presence of any naturally present rifampicin-resistant bacterial populations.

5.3.1.3 Bacterial cultures and inoculum preparation

Five strains of rifampicin-resistant non-pathogenic surrogate *Escherichia coli* (ATCC BAA-1427 P1, BAA-1428 P3, BAA-1429 P8, BAA-1430 P14, and BAA-1431 P68), trained to be rifampicin resistant (0.1 g/L) were obtained from Dr. Gary Acuff (Texas A&M University, College Station, TX) for use in this study (Laster et al., 2012). These strains are rifampicin-resistant progeny of USDA FSIS-approved cultures deposited at the American Type Culture Collection (Manassas, VA) for use as STEC surrogates for in-plant validation studies. Each strain was activated individually by transferring cryogenically frozen beads into Tryptic Soy Broth (TSB; Bacto, Becton, Dickinson and Co., Sparks, MD, USA) containing 0.1 g/L rifampicin (TSB+rif; Sigma-Aldrich, St. Louis, MO, USA) stock solution and incubated at 37°C for 24 hours. Rifampicin stock solution (Rif) was prepared by dissolving 0.1 g rifampicin in 5 mL methanol (Fisher Chemical, Fair Lawn, NJ, USA) followed by filtering through a 0.22 μm sterile filter. Each culture was verified to not contain any virulence characteristics by latex agglutination (*E. coli* non-O157 identification kit, Prolex, Round Rock, TX, USA) and by Assurance GDS PCR assay (BioControl, United Kingdom).

Meat—To prepare inoculum, 0.1 mL of each activated surrogate culture was transferred individually into 50 mL of TSB+rif and incubated at 37°C for 24 hours. After incubation of the six flasks, 1 mL of each surrogate strain was combined into a 10-mL tube, mixed, serially diluted in phosphate buffered saline containing rifampicin, and plated on *E.coli*/coliform Petrifilm (ECC; 3M Corporation, Saint Paul, MN) that was incubated at 35°C for 24 hours to confirm overall surrogate cocktail concentration. The remaining 49 mL of each culture were combined into a large sterile bottle (total of 245 mL) to use as a 5-strain surrogate cocktail inoculum. Two sets of surrogate cocktails were grown up to inoculate two separate batches of beef trim. Two 25 ± 5 lb batches of course-ground (3/4" grinder plate) beef trim obtained from a single combo to be used in the following day's studies were inoculated with 245 mL of the surrogate cocktail. In a disinfected plastic bin, the inoculum was evenly pipetted across the beef contained in each bin

and mixed manually with latex-gloved hands to achieve uniform distribution. The inoculated beef in each batch was covered with plastic and held at ~4°C for 24 hours before use.

Water—A preliminary process-water only (no meat added to the recirculating system) study, defined in section 5.3.1.4 below, was conducted. To prepare surrogate inoculum for this study, 0.5 mL of each culture was added individually to 0.8 L of TSB+rif and incubated at 35°C for 24 hours. Aliquots (1 mL each) from each 24-h culture bottle were mixed in a single 10 mL tube and enumerated as previously described to confirm overall surrogate cocktail concentration. The remaining portions of the individual cultures were combined into a surrogate cocktail inoculum (4 L total volume, referred to as the inoculum “slug”).

5.3.1.4 Preliminary water-only study inoculation study parameters

Due to the large volume of water in the recirculating processing system, a preliminary study was conducted to evaluate a worst-case scenario of contaminated water. The ~23,000 L system was filled with pH 5, 5 ppm FAC Cesco-Bauer water 24 hours prior to the study, chilled to 4.5°C, and recirculated continuously at approximately 151 L/minute. Water temperature was maintained between 4 and 10°C (Appendix A). Processing room temperature varied between 8 and 17°C during the time period of the testing, thus, influencing processing water temperature (Appendix A).

The addition of the concentrated surrogate inoculum “slug” into the Cesco-Bauer process water system was performed to achieve three primary objectives; 1) To determine the sensitivity of an Iso-Grid filtration method to enumerate surrogate populations diluted within the large make-up volume of the recirculating loop of the process, and 2) To determine the ability of a daily 4-h disinfection step (i.e., 5 ppm FAC chlorinated nanobubble water) in the continuous-run process to inactivate a high level of microbial contamination at the end of a day’s processing run.

The 4 L slug of surrogate cocktail in TSB (at ~9 log CFU/mL) was introduced into the system at the vortex. The second goal of this preliminary study was to determine if recirculating the Cesco-Bauer water for 4 hours in an empty system (i.e., no meat) would reduce any remaining surrogate organisms to undetectable levels. Water samples were collected from four locations: the manifold at the drop-port, the flotation tank, and after the

particle filter (before re-infusion of Aquaox 5000™ concentrated chlorine solution and CO₂), and the vortex (after re-infusion, where meat would enter the recirculating system). After the inoculum slug introduction, a process water sample was immediately (within 30 seconds, a time established by visually observing the amber inoculum solution passing by a window in the manifold conduit) drawn from the manifold drop-port; whereas, process water samples from the flotation tank, after the particle filter, and at the vortex return were taken after one hour of circulation to ensure a majority of inoculum passed through all parts of the processing system. The flotation tank holds ~75% of the total system's water and completely exchanges its volume after 90 minutes of total system recirculation. Approximately 2 L of water was collected from each sample port into a gallon-sized Ziplock bag (Johnson and Johnson Co., Racine, WI, USA), and samples were taken to the on-site laboratory for immediate processing and analysis.

5.3.1.5 Parameters for the preliminary inoculated meat processing study

The preliminary inoculated meat study began 48 hours after the completion of the preliminary water-only study. Approximately 12 hours following the preliminary water-only study, the company processed ~3000 lbs of non-inoculated beef trim (25/75 lean to fat) over a 4-h period to calibrate and equilibrate the system in preparation for beginning the preliminary inoculated beef study.

Each day before meat processing began, it was confirmed that the Cesco-Bauer processing water was equilibrated to 5 ppm FAC and pH 5 throughout the recirculating water. Each day 771 kg (1700 lbs) of beef was processed at 192-204 kg/hour over a 4-h period. At the beginning of each test day after 30 minutes of non-inoculated trim processing, the first 25-lb lug of surrogate-inoculated coarse ground beef was introduced onto the processing belt immediately after the grinder head, and this inoculated beef trim then passed through the nitrogen tunnel becoming crust frozen, and subsequently dropping through the bond breaker into the vortex. After approximately 2 hours of processing (the middle of production), the second 25-lb lug of inoculated ground trim was similarly introduced into the system.

Meat samples were collected at five successive sampling points in the process—the vortex (post-freezing but pre-introduction into the antimicrobial water solution), the manifold drop-port, after the dewatering centrifuge (final lean product), from the bottom of the flotation

tank (representing secondarily recovered lean), and from the top of the flotation tank (representing fat)—using a sanitized wire mesh food strainer (Good Cook Touch, Rancho Cucamonga, CA) and transferred aseptically into Ziplock bags. The frozen shattered meat sample collected at the vortex was timed to ensure actual sampling of inoculated meat, as opposed to the continuously introduced non-inoculated ground trim from the grinder head, by timing the belt speed through the nitrogen tunnel. Meat samples at the other points were collected at 30-90 sec time intervals (dependent on valve openings) successively as the meat progressed through the recirculation system. Additionally, a matching set of meat samples were collected 20-45 minutes after the inoculated meat had exited the system to evaluate the level of surrogate organisms picked-up by non-inoculated beef product.

The ~23,000 L system filled with Cesco-Bauer water was recirculated continuously at approximately 151 L/minute. Water temperature was maintained between 4 and 10°C during a 4-h meat production run, and between 10 and 15°C (Appendix A) during the 4-h period of recirculating water-only (water containing 5 ppm FAC at pH 5, but no meat entering the system) and overnight. Room temperature was maintained between 8 and 17°C (Appendix A). Processing water samples were collected three times daily: 10 minutes after inoculated meat entered the system at the beginning and middle of the day, and after the 4-hour disinfection period. Water samples were collected from four locations: the manifold at the drop-port, the flotation tank, after the particle filter (before re-infusion of Aquaox 5000™ concentrated chlorine solution and CO₂), and at the vortex (after re-infusion where meat would enter the recirculating system) as described above.

Due to the nature of a recirculating solution system, replications are differentiated by day with each day being an experimental replication (for a total of 6 replications). Each replication (day) was characterized by a new combo tote of beef trim, newly prepared and administered surrogate inoculum cocktail, and a production break consisting of the 4-h disinfection period plus overnight recirculation of processing water through the conduits and its associated equipment.

5.3.1.6 Pre-operation and environmental sponges

Sterile sampling sponges (Nasco, Fort Atkinson, WI, USA) rehydrated in 25 mL of DE Neutralizing Broth (DE broth; Difco, Becton, Dickinson and Co., Sparks, MD, USA) were used to swab major pieces of equipment—the grinder conveyor belt, the bond breaker, and the inside lid and drop chute of the dewatering centrifuge— prior to production starting on Day 3. Using rehydrated sponges, environmental samples were also collected to determine if rif-resistant organisms were present outside of the beef processing system on items such as door handles, lab coats, floors, etc.

5.3.1.7 Chemical analysis of process water

pH and oxidation-reduction potential (ORP) of the recirculating Cesco-Bauer water were monitored continuously with in-line probes (M300; Mettler Toledo, Columbus, OH). Free available chlorine (FAC) and total chlorine (TC) levels were determined by amperometry (Chlorosense, Palintest, Erlanger, KY, USA). These readings were recorded three times per day on water samples within 5 min of collection. Independent readings of the FAC level (Service Complete Kit; Taylor Technologies, Sparks, MD, USA), pH, and ORP were taken by Cesco Solutions technicians to determine an overall profile of system operations and to continuously adjust the processing water to 5 ppm FAC and pH 5.

5.3.1.8 Microbiological analysis

Meat Samples—Twenty-five gram portions of each meat sample were added to a Whirl-Pak bag (Nasco, Fort Atkinson, WI, USA) containing 100 mL DE broth within 2 min of collection from the processing system. Bags were stomached (Stomacher 400 Lab Blender, Seward Laboratory Systems Inc., FL, USA) at 230 RPM for 60 seconds and plated on Aerobic Plate Count Petrifilm (APC; 3M Corporation, Saint Paul, MN, USA) to determine overall microbial populations. To determine recovery of rifampicin-resistant surrogates, 10 mL of the original homogenized sample in DE broth was removed from the original bag and added to 0.1 g/L rif and plated onto *E. coli*/Coliform (ECC) Petrifilm using Phosphate Buffered Saline (PBS; AMRESCO, LLC., Solon, OH, USA) blanks containing 0.1 g/L rif (PBS+rif). All ECC and APC Petrifilm were incubated at 35°C for 24 hours.

Water Samples—A 500 mL aliquot of each water sample was immediately pre-filtered through a No. 1 Whatman filter (United Scientific Supplies, Waukegan, IL, USA) using a sanitized plastic or ceramic Buchner funnel (Sigma-Aldrich, Darmstadt, Germany) fitted onto a sanitized 1-L side-armed flask connected to a mechanical vacuum pump to remove suspended organic particles. Portions of the pre-filtered water sample were subsequently filtered through two 1600-square hydrophobic grid membranes (Neogrid; Neogen, Lansing, MI, USA), separately filtering 60-250 mL (dictated by how fast the filter became loaded) through each membrane filter under vacuum. One membrane was washed with a 5-mL aliquot of DE broth to remove any residual chlorine and placed onto a Sorbitol MacConkey Agar plate containing 0.1 g/L rifampicin (SMAC-R) to enumerate surviving surrogate populations; the other membrane was inserted into a sterile conical tube (VWR International, Randor, PA) containing 45 mL TSB+rif for enrichment and subsequent qualitative detection of viable surrogate populations present in process water but below the direct plating detection level of the Iso-Grid procedure (1 CFU/60-250 mL filtered). Both the membrane grids on SMAC-R plates and in enrichments were incubated at 37°C for 24 hours. Each water sample was also directly plated using APC Petrifilm to determine overall microbial levels, and using ECC Petrifilm diluted with PBS+rif to recover rif-resistant surrogates in the processing water.

Sponges—Environmental sponges in their respective Whirl-pak bags were hand massaged for 1 min. To recover rifampicin-resistant surrogates, 10 mL of the original DE Neutralizing broth squeezed from the sponge was removed and added to 0.1 g/L rif and plated using PBS+rif dilution blanks onto ECC. Each sample was also directly plated onto ECC. All ECC Petrifilm plates were incubated at 35°C for 24 hours.

5.3.2 Optimized Validation Study to Evaluate the Effectiveness of the Cesco-Bauer Recycling Process Water System for Controlling STEC Surrogates during a 6-Day Processing Scenario

5.3.2.1 Antimicrobial process water source

The production and application of the chlorinated Cesco-Bauer nanobubble water in the proprietary ground beef manufacturing system are described in section 5.3.1.1.

5.3.2.2 *Meat source*

Six tons of 25/75 % (lean/fat) beef trim was obtained from a large beef processor approximately 7 days following production, as described in section 5.3.1.2. Upon receipt, the meat was held on a refrigerated truck at -1°C for the duration of the study.

5.3.2.3 *Bacterial cultures and inoculum preparation*

USDA-approved *E. coli* surrogate cultures, preparation of inoculum, and the inoculation procedure of 25-lb lugs of beef trim are described in section 5.3.1.2.

5.3.2.4 *System sanitation and disinfection*

When this novel ground beef manufacturing system is actually utilized to commercially process beef trim into finished ground beef product, it is envisioned that the production schedule would be 20 hours of continuous separation and treatment of lean and fat from combo beef trim, followed by a 4-hour running of the recirculated water with all processed meat removed from the system and no new meat introduced. During this 4-h period, the recirculating Cesco-Bauer water would be infused with concentrated chlorine (Aquaox 5000) to elevate the FAC level to 50 ppm for a 30-min period as a total system disinfection step, with a decline in FAC back to 5 ppm by the end of the 4-hour period, at which time the same beef trim processing schedule would be utilized. This 20-h process and 4-h disinfection rotation would be repeated daily for 6 days. On the seventh day, a total breakdown of all equipment and conduit components for cleaning and disinfection would occur.

In this surrogate-inoculated beef trim study, a 4-h disinfection period was incorporated into the study design directly following production at the end of every day, as would occur during regular commercial processing for this establishment. The Cesco-Bauer process water, now 'red water', continued to recirculate throughout the system. During the disinfection period of the recirculating process water loop, major pieces of external equipment were taken off-line and manually cleaned and disinfected, specifically, the interior lid and drop chute of the dewatering centrifuge, the bond breaker, the particle filter, and the conveyor belt at the

grinder. Equipment was rinsed with 82°C water followed by a quaternary-ammonium based foaming surfactant cleaner (Cesco Hurricane; Cesco Solutions, Bellingham, WA), manually scrubbed, re-rinsed with 82°C water, and disinfected (Cesco Avalanche Chip; Cesco Solutions, Bellingham, WA, USA). Equipment normally included within the Cesco-Bauer water processing loop (dewatering centrifuge and particulate filter) was temporarily by-passed from the recirculating solution during cleaning and disinfection. Following equipment disinfection, the FAC in the recirculating system was brought up to 50 ppm with 150-190 L Aquaox 5000™ manually added to the top of flotation with 5-gal buckets for a period of 30 minutes to disinfect the water, internal equipment parts, flotation tank fat skimming paddles, and manifold piping. This elevated level of FAC was then reduced by adding 1,200-2,000 mL of sodium thiosulfate (Cesco Antichlor No. 3, Cesco Solutions, Bellingham, WA, USA) at the end of the disinfection period to return the system to the 5 ppm FAC target operational level. In this inoculated study, after the 4-h elevated chlorine disinfection process, the process water continued recirculating overnight for 12-15 hours with 30 mL Aquaox 5000™/min being continuously added at the point of chlorine infusion.

5.3.2.5 Water-only inoculation study to evaluate effectiveness of an optimized set of operational parameters and an elevated chlorine disinfection step

The morning (~5 h) prior to initiating the inoculated meat processing study, the Cesco-Bauer chlorinated nanobubble system circulating at 151 L/min and equilibrated to 5 ppm FAC, pH 5 was inoculated with a 4-L slug of surrogate cocktail (~9 log CFU/mL in TSB) in a similar manner as described in section 5.3.1.4. Water temperature was maintained between 4 and 10°C. Room temperature was maintained between 8 and 17°C. The goal of this evaluation was to determine if the 4-h disinfection period, specifically the increase of FAC to 50 ppm for 30 minutes, would reduce the overall surrogate population in the processing water to below detectable levels. Water samples were collected from four locations—the manifold, the flotation tank, and after the particle filter (before re-infusion of electrolyzed chlorinated water and CO₂), the vortex (after re-infusion where meat would enter the recirculating system)—before and after the 50 ppm FAC spike. After the 4 L inoculum slug introduction, a sample was immediately (within 30 seconds) drawn from the manifold; whereas, water samples from the

flotation tank, after the particle filter, and at the vortex were taken after one hour to ensure passage of the inoculum through all parts of the system (conduits, valves, and processing equipment within the recirculating loop). The flotation tank holds ~75% of the system's water volume and completely exchanges its volume over a 90-min period. Approximately 2 L of water were collected from each sample port into a gallon-sized Ziplock bag, which was immediately transported to the onsite laboratory for microbiological analysis.

5.3.2.6 Inoculated meat study parameters

The inoculated meat validation study began 30 minutes after the completion of the water-only study described in section 5.3.2.5. Before initiating ground beef processing, the FAC and pH levels of the recirculating Cesco-Bauer water system were confirmed to be 5 ppm and 5, respectively, throughout the recirculation loop.

On each of the six consecutive days, 771 kg of beef trim (25/75 percent lean/fat) was processed at 192-204 kg/h over 4 hours. This protocol was similar to section 5.3.1.5 except the experimental design called for inoculated beef trim to be added to the processing flow on days 1, 3 and 5 and non-inoculated meat to be processed on days 2, 4 and 6. On the inoculated meat processing days, a 25-lb lug of inoculated course ground (3/4" plate) beef trim was dumped at the start-up of the production run in a single layer onto the conveyor belt exiting the grinder head, allowing this trim to pass through the nitrogen freeze tunnel and bond breaker into the vortex (point where trim enters the Cesco-Bauer water processing loop). A second 25-lb lug of inoculated trim was dumped once again in the middle of the 4-h production run. After each inoculated batch of trim entered the vortex, meat samples were collected as described in 5.3.1.5. Non-inoculated beef trim was coarse ground, crust frozen, shattered, and processed through the Cesco-Bauer water system after each inoculated lug dump in a continuous manner. On these days, beef component samples were collected 45 minutes after each inoculated lug had exited the recirculating system to evaluate the level of surrogate organisms picked-up by subsequently processed non-inoculated beef trim (representing a highly contaminated batch of trim going through the system to determine propensity for contamination to spread to non-contaminated product).

Production runs on days 2, 4 and 6 were conducted, whereby only non-inoculated beef trim was processed through the same manufacturing system on the day following the previous evening's 4-h disinfection process. The goal of this part of the study was to determine if *E. coli* surrogates might survive the Cesco-Bauer water disinfection protocol at points along the processing continuum and potentially contaminate the next day's production run. Lean and/or fat beef samples were collected at the beginning, middle, and end of the 4-hour beef trim processing run. For both inoculated and non-inoculated study components, meat samples were collected from 5 sampling points in the Cesco-Bauer recirculating loop as described in section 5.3.1.8.

Throughout the study, the ~23,000 L system, filled with pH 5, 5 ppm Cesco-Bauer water, was recirculated continuously at approximately 151 L/minute. Water temperature was maintained between 4 and 10°C (Appendix A) during production and between 10 and 15°C during the disinfection period and overnight (Appendix A). Room temperature was maintained between 8 and 17°C (Appendix A). Water samples for microbiological analysis were collected three times daily as described in section 5.3.2.6. On inoculated meat processing days, Cesco-Bauer process water was collected 10 minutes after inoculated meat entered the system at the beginning and middle of the day, and after the 4-hour disinfection period. On non-inoculated meat processing days, process water samples were collected at the beginning and end of beef production, and after the 4-hour disinfection period. Water samples were collected as described in water-only study (section 5.1.3.5).

Due to the nature of a recirculating solution, and whereby new meat, new inoculum, daily 4-hour disinfection occurred, three experimental replications were determined. Replication 1 was defined as production days 1 and 2, replication 2 as days 3 and 4, and replication 3 as days 5 and 6.

5.3.2.7 Pre-operation environmental sponges to identify presence of viable surrogate bacteria

To gauge the effectiveness of nightly processing room and equipment sanitation during the 6-day experiment, sterile cellulose sampling sponges rehydrated in 25 mL of DE Neutralizing Broth were used to swab major pieces of equipment—the grinder conveyor belt, the bond breaker, the inside lid and drop chute of the dewatering centrifuge—previously identified as

potential microbial harborage points in the preliminary in-plant study (sections 5.3.1.5 and 5.3.1.6 —after daily sanitation. Using rehydrated sponges, 3-7 additional environmental samples were taken on items such as door handles, lab coats, floors, etc. every other day to determine if rif-resistant organisms were present outside of the inoculated beef processing system.

5.3.2.8 Chemical analysis of Cesco-Bauer processing water samples

Free available chlorine (FAC), total chlorine, pH, and oxidation-reduction potential (ORP) readings of processing water were collected as described in 5.3.1.7. These analyses were conducted on-site using an amperometric meter (Chlorosense, Palintest, Erlanger, KY, USA) for FAC and total chlorine and in-line probes for ORP and pH.

The method used for trihalomethane analysis was a modification of Dos Santos, Martendal, & Carasek (2011) using a solid phase microextraction fiber (SPME) coupled with gas chromatography- mass spectrometry (GC-MS) operated in the SIM/SCAN mode. Process water samples for analysis were obtained before production, after production, and after the 4-hour disinfection period at the vortex and flotation tank daily. Water was collected in 120-mL glass amber bottles certified for chemical residue detection in potable water systems (Cat. No. 241-1020; Thermo Scientific, Rockwood, TN) and immediately frozen on-site. Samples were then shipped overnight for analysis at the Kansas State University Food Chemistry Laboratory directed by Dr. J. Scott Smith. Upon receipt at the lab, samples were held at -20°C until analyzed.

The frozen process water samples were removed from -20°C storage and held at room temperature for about 2 hours with occasional shaking until thawed. From each sample, a 0.75 or 1.5 mL aliquot was pipetted into a 4-mL glass vial with a silver seal cap having a PTFE/silicone liner. A solid phase microextraction fiber (75 µm CAR-PDMS, Supelco, USA) was exposed to the sample headspace for 15 minutes, withdrawn and inserted into the GC injection port for 1 minute at 280°C. GC/MS (Agilent Technology Inc., Santa Clara, CA) separation was achieved on a HP-5MS (60 m × 0.25 mm × 0.25 µm) column with a temperature program of: hold 40 °C for 4 min, increase to 180 °C at a rate of 40 °C/min, and hold at 180 °C for 4 min. The helium carrier gas was at a flow rate of 1 mL/min. The MS data was collected in either the SCAN or SIM

modes. For the SCAN mode, ions were collected between 35-600 m/z and peaks were checked for compound identification with the NIST/EPA/NIH Mass Spectral Library (version: NIST 14). Quantification of trichloromethane was with the MS operated in the SIM mode set for the major fragment ions of trichloromethane (m/z 83, 85, 118, and 120 ions). A standard curve of trichloromethane levels versus concentration was achieved by measuring integrated peak areas of the major ions of standards of 1, 25, 50, 100, and 150 ppb in the headspace vial. Reagent grade chloroform (Acros Organics, 99.8+%, stabilized with ethanol, CAS 67-66-3) was used to make the standard dilutions from a 100 ppm standard stock solution diluted with halogen-free LC/MS water (Optima, Fisher Chemicals).

5.3.2.9 Microbiological analysis of meat and process water samples

Meat Samples—Samples were processed as described above in section 5.3.1.8. For enrichment of meat samples, 10 mL of original homogenized sample was added to 90 mL TSB+rif and incubated at 37°C for 24 hours. 24-hour enrichments were subsequently streaked using sterile cotton swabs onto SMAC-R agar plates and incubated at 37°C. Results were read as positive (growth) or negative (no growth) for qualitative detection of surviving organisms below the detection limit (0.4 log CFU/mL) after 24 hours.

Water Samples—Samples were prepared and processed similar to section 5.3.1.8 with several modifications. A 500-mL aliquot of each water was immediately pre-filtered through a series of ~4 Whatman filters decreasing in size (Q5, P4; Fisher Scientific, Pittsburgh, PA, USA; #1; United Scientific Supplies, Waukegan, IL, USA) to remove particles larger than 4 μm . The used pre-filters were aseptically placed into a Whirl-pak with 100 mL of TSB+rif for enrichment using sterilized forceps to qualitatively determine if surrogates potentially were attached to larger organic particles in the process water, thus being filtered out by the pre-filter process. Of each resultant pre-filtered water sample, a 60-250 mL portion (dictated by how fast the filter became loaded) was filtered through a 1600-square hydrophobic grid membrane (Neogrid; Neogen, Lansing, MI, USA) under vacuum, subsequently washed with 5 mL of DE broth, and placed onto a SMAC-R plate to enumerate viable STEC surrogates. The Whatman pre-filter enrichments were incubated at 37°C for 24 hours. After 24 hours, pre-filter enrichments were streaked onto SMAC-R agar plates and incubated for 24 hours at 37°C. Each water sample was

also directly plated onto APC Petrifilm to determine overall microbial concentration in the process water and on ECC Petrifilm diluted with PBS+rif to enumerate rif-resistant surrogates. Sponges—Sponges were prepared and processed as described above in section 5.3.1.8.

5.3.2.10 Statistical analysis of meat sample microbiological data

There are two points in the overall beef trim processing system where pathogens can be reduced on meat tissues: 1) During the cryofreeze as shown from the inoculated meat to the vortex due to general freeze injury and possibly dehydration, and 2) From exposure to the free available chlorine in the recirculating water as shown at the post-surge tank, final lean, fat tank and final fat. Therefore, two statistical analyses were completed. Both analyzes were performed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). For each analysis, a randomized complete block design was assumed and type 3 tests of fixed effects were evaluated to determine significance of interactions and/or main effects based on a P-value of $\alpha=0.05$.

The first analysis, determining the effect of the cryofreeze, utilized Fisher's Protected LSD based on initial review of the model and graphical diagnostics, which showed two variances. For the second analysis, treatments were separated into 7 scenarios: 1) AM inoculated meat, 2) AM same-day pick-up meat, 3) PM inoculated meat, 4) PM same-day pick-up meat, 5) 24-hour pick-up meat beginning of day, 6) 24-hour pick-up meat middle of day, and 7) 24-hour pick-up meat end of day and evaluated using a Tukey-Kramer adjustment for all comparisons.

5.4 Results and Discussion

5.4.1 Water Samples

A hydrophobic grid membrane filter (HGMF) method, a common form of enumeration in water microbiology, were used in this study to improve detection limits from process water samples. Benefits of HGMF include no need for serial dilutions, reduced labor, reduced counting error due to grid-colony distinction, and the filtration of larger volumes of water than can be analyzed by direct plating (Patel, 1995). ISO-GRID HGMF consists of a 1600-grid

extended Most Probably Number (MPN) test with accuracy up to 4 log cycles of growth, reported as Most Probable Number of Growth Forming Units (MPNGU) calculated as described in Equation 1 (Patel, 1995).

5.4.1.1 Preliminary water studies

In a worst-case scenario, a high level of contamination may be present in recirculating water following a day of beef processing. To determine the sanitizing power of the recirculating water alone, without organic material in the system, inoculum was added via the 4-L concentrated inoculum slug that, through theoretical calculation, would result in a level of 1.6×10^5 CFU/mL of *E. coli* surrogates in the water if no lethality occurred. During the first preliminary water-only study that occurred before the preliminary meat processing validation study, no apparent immediate lethality was seen at the manifold; this is most likely due to an initial dilution factor of the inoculum entering the system as a ‘slug’ and subsequent immediate binding of chlorine as the slug passed through (Table 5.1). There was no recovery of surrogates after 4 hours of recirculating process water at an average 3.4 ± 1.6 ppm FAC. This indicates that simply recirculating Cesco-Bauer water during a 4-hour sanitation period (with no enhanced-level chlorine spiking) would reduce pathogen populations in the water to undetectable levels.

Table 5.1 Water-only Study Results, Preliminary Validation Study

		Manifold		Flotation Tank		Post-Particle Filter		Vortex	
		Beginning	End	Beginning	End	Beginning	End	Beginning	End
Preliminary Study	FAC	0.02	3.9	2.6	5.6	2.8	4.2	3.6	5.0
	Total Cl	-	4.8	4.8	6.2	3.7	4.7	4.8	5.0
	pH	5.67	5.69	5.69	5.69	5.65	5.53	5.55	5.55
	ORP	472	885	884	910	890	913	895	917
	CFU/mL	7.7 Log	-	235*	-	-	-	-	-

Beginning—Samples taken directly after inoculum introduced to system; Manifold-30 seconds; Flotation Tank, Post-Particle Filter, Vortex-1 hour post-inoculum introduction; End—Samples taken at the end of the 4-hr sanitation (water recirculating at 3.4 ± 1.6 ppm FAC)

* Determined by positive enrichment ISO-GRID, calculated based on detection limit

Similar results are reported for the water-only study that included the implementation of a 50 ppm FAC boost during the 4-hour sanitation period. *E. coli* surrogates were added in to

the system at the same level as previously described, but were immediately recovered at much lower levels. High levels of surrogate organisms were recovered at the manifold, albeit slightly lower than the previous 5 ppm FAC water-only study, indicating that aside from an initial dilution factor, free chlorine had an initial impact before being completely bound (Table 5.2). A low level of surrogates was still detectable at the beginning of the 50 ppm chlorine boost; however, surrogates were not recovered anywhere in the system after the 4-hour elevated chlorine disinfection period.

Table 5.2 Water-only Study Results, Optimized Validation Study

		Manifold		Flotation Tank		Post-Particle Filter		Vortex	
		Beginning	End	Beginning*	End	Beginning*	End	Beginning	End
Optimized Validation Study	FAC	0.14	4.1	27.5	4.4	41.5	3.9	43*	5.1
	Total Cl	3.9	4.1	29	4.9	41.5	4.7	43	5.8
	pH	5.64	4.88	5.68	4.92	5.64	4.92	5.04	4.93
	ORP	850	926	967	945	706	636	956	834
	CFU/mL	~4 logs¥	-	0.028	-	-	-	-	-

* 1-hr sampling point occurred during beginning of 50 ppm chlorine boost to system; ¥ ISO-GRID overgrown, no growth on ECC direct plate, estimated approximately 4 logs MPNGU in water due to ISO-GRID sensitivity

Equation 1: Most Probable Number of Growth Forming Units (MPNGU) CFU/mL reported for samples with growth on 1600 MPN grids calculated as:

$$\left(-1600 * \text{LOG} \frac{1600 - \# \text{ of positive grids}}{1600}\right) \div \# \text{ of mLs passed through grid}$$

5.4.1.2 Chlorine demand and surrogate recovery during meat processing

A major limitation of chlorine in a meat processing system is that it is easily bound and deactivated by organic matter (Sohaib et al., 2016). Although the particle filter removes coarse debris from the recirculating water solution, soluble organic matter continues to accumulate over time which accelerates free chlorine depletion (Yang et al., 2012; Zhou et al., 2015). Chlorine dosing, especially when done manually as was performed during this study, can be difficult to determine and, therefore, difficult to maintain consistent free chlorine levels. Yang et al. (2012) showed that an initial level of 35 ppm FAC was reduced to 0 ppm after only 4

lettuce dip washes; replenishing the solution with the same amount of NaClO as originally used only resulted in FAC restoration levels between 7.2 and 17 ppm. This observation indicated that higher levels of NaClO were needed over time to maintain FAC in solution. When levels of FAC are low in a recirculating solution, pathogens have a potential opportunity to survive and cross-contaminate meat in the system. However, Zhou et al. (2015) determined that organic loading and initial chlorine concentration do not directly affect chlorine efficacy in solution, contrary to prior belief. Chlorine demand in protein-containing (i.e. beef and poultry) solutions is quite high compared to other food matrices. In a study conducted by Waters and Hung (2014), up to 82.5% and 75-92.5% of total free chlorine was lost in beef and turkey solutions, respectively, compared to relatively low chlorine loss observed in starch, fat, and mineral solutions. Zhou et al. (2015) determined that a minimum FAC level of 3.66 ppm at pH 5.12 to 6.97 and an ORP above 850 mV in a recirculating produce wash water system was sufficient to reduce *Salmonella enterica*, *E. coli* O157:H7, and *Listeria monocytogenes* by 6 log cycles after a 30 second contact time independent of organic loading and initial chlorine concentration.

During the optimized study, the average FAC and ORP across the system was lower than the 3.66 ppm and 850 mV observed by Zhou et al. (2015) to be successful for pathogen reduction in produce wash water: Vortex 3.31±0.92 ppm, Manifold 1.31±1.39 ppm, Flotation Tank 1.74±1.26 ppm, Post-Particle Filter 1.62±1.23 ppm and an average ORP of 715±161 mV as recorded by KSU personnel. However, no viable organisms were recovered on any ISO-GRID. A few of the Whatman filter enrichments (Table 5.4) were found to be positive following introduction of inoculated meat into the system, indicating that surrogates were still in the recirculating red water at low levels, but were attached to filterable organic material. Whereas, no pre-filter enrichments were found to be positive following the 4-h disinfection period after production. It is likely that there were antimicrobial effects from chloramine formation—which was not measured in this study. In the presence of proteins, chlorine will form chloramines and retain residual antimicrobial effects even after depletion of free available chlorine. Block (1991) reported 100% reductions of *Salmonella pullorum* in a 130 ppm hypochlorite solution with 5% organic matter although there was no measurable level of FAC, showing the sanitizing capacity of chloramines.

In the preliminary meat processing study, Whatman pre-filters were not enriched to determine presence of surrogates attached to filterable particulates. However, similar results were observed overall compared to the optimized beef processing study. The average FAC at the manifold, flotation tank, and post-particle filter were relatively lower in the preliminary meat processing study than in the optimized study—Vortex 3.47 ± 0.91 ppm, Manifold 0.66 ± 0.92 ppm, Flotation Tank 0.24 ± 0.30 ppm, Post-Particle Filter 0.26 ± 0.33 ppm—but, the ORP throughout the system was similar between studies, with an average ORP of 717 ± 164 mV. During the preliminary study, several ISO-GRID membranes recovered rif-resistant surrogates: 0 of 18 Vortex samples, 6 of 18 Manifold samples (including 1 after the 4-hr water recirculation disinfection period), 1 of 18 Flotation Tank samples, and 2 of 18 Post-Particle Filter samples. This is most likely due to decreased free chlorine levels in the manifold, flotation tank, and after the particle filter as compared to the optimized study. Based on the results from the preliminary study and knowing chlorine would bind with the organic material from the meat and in the water almost immediately, the goal of the optimized study was to maintain a level of 5 ppm chlorine, or at least residual chlorine, throughout the system in order to continuously allow for chlorine to contact the meat surface and reduce the level of surrogate organisms recovered in the water. This was an effective strategy. In the future, in-line chlorine meters should be installed and used to operate the mechanical injection of Aquaox 5000™ into the system to maintain better control of free chlorine levels throughout the recirculating water. In processing environments, chlorine is extremely effective at controlling pathogen levels in wash water, yet minimally impacting the levels of pathogens on the surface of produce or poultry (Zhou et al., 2015), thus supporting the minimal levels of rif-resistant surrogates recovered in both studies.

Table 5.3 Free Available Chlorine (FAC), Total Chlorine, pH, ORP of Recirculating Water during Preliminary Study

		Vortex			Manifold			Flotation Tank			Post-Particle Filter		
		A	B	C	A	B	C	A	B	C	A	B	C
Day 1	FAC	1.19	3.3	4.0	0.18	0.06	0.49	0.20	0.03	0.11	0.04	0.06	0.11
	Total Cl	9.0	15.0	10.5	6.4	6.3	4.6	6.1	4.2	5.9	5.9	5.6	5.9
	pH	5.15	4.69	4.93	5.03	4.8	5.03	5.08	5.12	4.72	5.15	5.20	4.90
	ORP	826	836	878	843	618	915	783	570	841	515	423	537
	CFU/mL	-	-	-	0.014	-	-	-	-	-	-	-	-
			A	B	C	A	B	C	A	B	C	A	B
Day 2	FAC	2.5	2.3	3.3	0.04	0.16	0.62	0.04	0.02	0.49	0.48	0.06	0.05
	Total Cl	9.8	12.2	12.3	6.3	7.8	10.0	5.4	5.5	8.5	5.6	6.6	9.1
	pH	5.38	5.42	5.51	5.11	5.15	5.49	4.91	5.08	4.04	5.33	5.39	5.18
	ORP	833	828	811	761	640	903	735	592	920	441	399	538
	CFU/mL	-	-	-	0.092	0.026	-	-	-	-	-	-	-
			A	B	C	A	B	C	A	B	C	A	B
Day 3	FAC	4.1	4.2	3.4	0.04	0.04	2.4	0.07	0.16	0.31	0.06	0.05	0.32
	Total Cl	10.2	14.6	13.7	6.7	7.9	11.0	7.7	7.2	9.5	7.0	6.4	9.3
	pH	5.49	5.49	5.83	5.15	5.18	5.96	4.84	4.94	4.92	5.38	5.39	5.64
	ORP	861	870	800	774	671	870	785	629	875	496	399	538
	CFU/mL	-	-	-	0.002	0.051	0.004	-	-	-	0.002	-	-
			A	B	C	A	B	C	A	B	C	A	B
Day 4	FAC	3.9	3.7	3.6	1.51	0.06	2.9	0.12	0.04	0.82	0.06	0.84	0.89
	Total Cl	13.7	11.2	12.3	10.1	7.5	9.5	8.1	8.5	7.4	9.3	8.3	7.0
	pH	5.47	5.46	5.68	5.21	5.19	5.83	4.76	4.9	4.57	5.28	5.33	5.39
	ORP	873	867	817	831	747	885	827	641	904	468	376	633
	CFU/mL	-	-	-	-	-	-	-	-	-	0.005	-	-
			A	B	C	A	B	C	A	B	C	A	B
Day 5	FAC	3.0	4.5	4.2	0.07	0.16	2.3	0.18	0.21	1.17	0.89	0.04	1.12
	Total Cl	9.9	17.9	10.0	5.9	8.4	7.8	5.2	4.9	6.9	5.3	5.6	6.6
	pH	5.52	5.54	5.49	5.29	5.31	5.56	4.88	4.97	4.58	5.37	5.41	5.25
	ORP	852	866	848	751	725	910	763	676	927	433	388	674
	CFU/mL	-	-	-	-	-	-	0.006	-	-	-	-	-
			A	B	C	A	B	C	A	B	C	A	B
Day 6	FAC	5.3	3.3	2.7	0.07	0.11	0.66	0.05	0.02	0.2	0.19	0.03	0.18
	Total Cl	12.4	12.9	12.9	5.6	6.8	9.5	5.7	6.2	9.9	4.9	6.1	8.2
	pH	5.58	5.69	6.3	5.37	5.47	6.41	4.95	5.13	5.4	5.37	5.51	6.00
	ORP	846	837	743	793	731	830	786	688	815	469	400	416
	CFU/mL	-	-	-	-	-	-	-	-	-	-	-	-
			A	B	C	A	B	C	A	B	C	A	B

A – Water collected after inoculated meat at beginning of production run, B—Water collected within 10 minutes of inoculated meat during middle of production run, C—Water collected after 4-hr sanitation; Average pH 5.27 ± 0.38 ; Average ORP 717 ± 164 mV; Average FAC: Vortex 3.47 ± 0.91 ppm, Manifold 0.66 ± 0.92 ppm, Flotation Tank 0.24 ± 0.30 ppm, Post-Particle Filter 0.26 ± 0.33 ppm

Table 5.4 Free Available Chlorine (FAC), Total Chlorine, pH, ORP of Recirculating Water during Optimized Study

		Vortex			Manifold			Flotation Tank			Post-Particle Filter		
		A	B*	C	A*	B*	C	A	B	C	A*	B	C
Rep 1	FAC	4.3	2.1	4.3	0.67	0.68	3.5	1.28	0.62	0.44	1.19	0.50	0.45
	Total Cl	8.2	11.5	11.4	4.7	8.2	9.6	5.9	9.5	8.5	5.1	9.5	8.7
	pH	5.13	5.35	5.24	4.96	5.15	5.12	5.08	5.18	5.01	5.04	5.20	5.20
	ORP	796	716	703	914	763	890	908	888	828	558	484	376
		D	E	F	D	E	F	D	E	F	D	E	F
	FAC	4.3	2.9	2.2	0.39	0.2	3.3	3.8	0.76	3	4.2	0.6	3.4
	Total Cl	10.7	14.2	7.6	7.8	13.5	9.8	8.7	13.7	9.3	9.7	11.0	11.0
	ORP	724	687	669	900	759	882	865	846	915	505	357	518
Rep 2		A	B	C	A*	B*	C	A	B	C	A	B*	C
	FAC	2.7	2.7	2.8	0.88	0.18	2.7	0.69	0.75	2.3	0.46	0.70	2.2
	Total Cl	10.5	9.5	10.7	9.3	9.2	9.5	9.7	11.0	9.6	8.1	11.5	9.4
	pH	5.53	5.59	5.41	5.27	5.24	4.95	5.34	5.23	4.98	5.48	5.54	5.42
	ORP	705	710	667	779	856	904	803	845	902	556	491	518
		D	E	F	D	E	F	D	E	F	D	E	F
	FAC	2.2	3.4	4.1	0.15	0.29	3.5	1.16	2.5	3.3	2.2	1.13	1.94
	Total Cl	9.8	13.0	9.6	7.5	7.7	8.5	9.2	13.0	10.0	7.4	13.5	8.8
pH	5.48	5.50	5.24	5.13	5.16	4.59	5.30	5.21	4.94	5.47	5.49	5.26	
ORP	701	701	686	827	720	874	850	877	925	491	518	585	
Rep 3		A	B	C	A*	B*	C	A*	B	C	A	B	C
	FAC	2.8	2.2	4.3	0.24	0.14	3.2	0.55	0.26	2.4	0.58	0.21	2.4
	Total Cl	10.0	14.5	12.9	7.8	11.3	10.8	9.9	14.3	11.6	10.0	11.7	10.9
	pH	5.39	5.51	5.40	4.94	5.02	4.56	5.29	5.34	5.00	5.38	5.49	5.40
	ORP	694	670	681	801	785	817	831	791	912	388	319	566
		D	E	F	D	E	F	D	E	F	D	E	F
	FAC	5.3	3.4	3.5	0.22	0.1	3.3	4.4	0.56	2.5	3.3	0.52	2.2
	Total Cl	13.5	17.8	12.4	9.7	9.7	11.9	11.5	13.0	12.3	12.2	12.8	10.9
pH	5.50	5.75	5.55	4.98	5.08	4.95	5.32	5.40	5.17	5.49	5.72	5.31	
ORP	727	661	715	753	699	797	879	831	899	618	413	513	

* Positive samples following Whatman filter enrichment; Samples A-C collected on Day 1 (inoculation days), D-F collected on Day 2 (24-hr pick-up days); A – Water collected after inoculated meat at beginning of production run, B—Water collected within 10 minutes of inoculated meat

during middle of production run, C—Water collected after 4-hr sanitation, D—Water collected within 10 minutes of pick-up meat at beginning of production run, E—Water collected within 10 minutes of pick-up meat at end of production run, F—Water collected after 4-hr sanitation; Average pH 5.26 ± 0.24 ; Average ORP 715 ± 161 mV; Average FAC: Vortex 3.31 ± 0.92 ppm, Manifold 1.31 ± 1.39 ppm, Flotation Tank 1.74 ± 1.26 ppm, Post-Particle Filter 1.62 ± 1.23 ppm

5.4.1.1 Chlorine by-product results in recycled processing water

The values reported (Table 5.5) were obtained with the MS operated in the SIM mode. Many samples were diluted with halogen-free water in order to fit on the standard curve. The method used was unable to identify other organohalogen compounds, such as dichloromethane, when analyzed with the MS in the SCAN mode and, is not suitable for the detection of trihaloacetic acids. All of the water samples analyzed report levels of THMs higher than 0.08 mg/L or 80 ppb (Table 5.5), the maximum residual disinfectant level for potable water in the United States (EPA, 2001). This is interesting because levels of chlorine are much higher in commercial poultry establishments yet yield much lower levels of disinfection by-products, generally below 0.08 mg/L (Najjar & Meng, 2009; Vizzier-Thaxton et al., 2010). These by-products are volatile and reported to dissipate in an open environments, especially in agitated chill tanks (Vizzier-Thaxton et al., 2010), often leading to non-hazardous levels within water.

Risk assessments have been conducted evaluating levels of residual disinfection by-products on the surface of chicken tissues; chicken skin and fat exposed to a traditional chlorinated chill tank water system (50 ppm FAC). Results from these assessments concluded that either no or extremely low levels (< 4.5 ppm) of THMs were present on the surface of exposed poultry tissues (Najjar & Meng, 2009; Vizzier-Thaxton et al., 2010). Poultry chiller water maintained at 50 ppm chlorine accounts for 0.3-1% of human THM exposure, thus not signifying a significant risk for cancer or other health conditions from consuming poultry products (Najjar & Meng, 2009). The meat in this novel ground beef manufacturing system is only exposed to a maximum level of 5 ppm FAC for a matter of minutes. Despite the level of THMs in the water, there should not be a health hazard from consuming the lean beef recovered in the final product. However, the potential health hazards caused by extended exposure due to constant chlorine reinfusion over 6 days should be investigated.

Future experiments should be conducted to 1) replicate the results of the THMs recovered during the optimized validation study and 2) evaluate the levels of chlorine disinfection by-products on the final meat product to determine if there is a health hazard.

Table 5.5 Trichloromethane Analysis of Recirculating Water during Optimized Validation Study

Day	Time	Location	mg/L
1	Before Study	Vortex	0.1-0.3*
		Flotation	
1	Before Study	tank	0.293
	After Preliminary		
1	Water Study	Vortex	0.211
	After Preliminary	Flotation	
1	Water Study	tank	0.1-0.3*
1	After Production	Vortex	0.1-0.3*
		Flotation	
1	After Production	tank	0.188
	Before		
2	Production	Vortex	0.1-0.3*
	Before	Flotation	
2	Production	tank	0.1-0.3*
2	After Production	Vortex	0.24
		Flotation	
2	After Production	tank	0.221
2	After Sanitation	Vortex	0.176
		Flotation	
2	After Sanitation	tank	0.201
	Before		
3	Production	Vortex	0.109
	Before	Flotation	
3	Production	tank	0.091
6	After Production	Vortex	0.226
		Flotation	
6	After Production	tank	0.198
6	After Sanitation	Vortex	0.205

* Samples higher than the standard curve levels and thus can only be estimated between 100-300 ppb; only 1 repetition of data

5.4.2 Pre-Operation and Environmental Samples

Pre-Operation samples targeting rif-resistant surrogates were taken daily, starting on the third day of the production run when the research team learned the plant operations staff had not been cleaning equipment daily between each day. Due to a lack of cleaning the dewatering centrifuge and bond breaker were opened and noticeable build-up was present. Although major pieces of equipment, including the grinder belt, dewatering centrifuge lid and drop chute, and the bond breaker, were cleaned daily, low levels of contamination were still present at the bond breaker which continued to contaminate non-inoculated meat entering the system at the beginning of every day as indicated by pre-inoculated vortex and manifold meat samples (Table 5.6, Table 5.7).

Table 5.6 Preliminary Study Pre-Operation Equipment Samples

		ECC Log CFU/cm ²			
Day		3*	4	5	6
	Grinder Conveyor Belt	0.002	0.0	0.0	0.0
	Centrifuge Drop Chute	0.002	0.0	0.0	0.0
	Bond Breaker	1.8	0.10	0.009	0.011
Sampling Location	Centrifuge Lid	-	0.0	-	-
	Manifold Sample Pipe	0.0	0.0	-	-
	Crytotunnel Conveyor Belt	0.015	-	-	-
	FFT sample pipe	0.0	-	-	-
	Centrifuge conveyor belt	0.001	-	-	-

* Daily cleaning of equipment began after Day 3

The build-up within the centrifuge was highly contaminated (Table 5.7), thus acting as a secondary inoculating point during processing if not thoroughly cleaned at least daily. Aside from the build-up within the centrifuge lid, the high levels of contamination present on non-inoculated meat exiting the centrifuge as a final product 24 hours following introduction of

inoculated meat into the system indicates that 1) the inner-workings of the centrifuge are not being effectively disinfected by the 5 ppm FAC recirculating solution overnight (~20 hours), and 2) the centrifuge currently in place is not cleanable within the anticipated 4-hour sanitation period and may need to be replaced with more cleanable equipment.

Table 5.7 Preliminary Study Indicators of Equipment Cleanliness

		Meat Samples (ECC Log CFU/g)			
Day		3*	4	5	6
	Centrifuge Lid build-up	3.0	4.2	-	-
	Particle Filter build-up	0.0	0.0	-	-
Sampling Location	Pre-Inoculated Meat at Vortex	0.0	0.0	0.0	2.2
	Pre-Inoculated Meat at Manifold	1.0	0.0	2.4	0.7
	Pre-Inoculated Meat at Centrifuge	1.9	3.1	1.8	2.2

* Daily cleaning of equipment began after Day 3

Table 5.8 Preliminary Study Environmental Samples

Sampling Point	ECC Log CFU/cm²
Edible bin	0.0
Technician sleeves	0.0
Operator gloves	0.0
Doorknob	0.0
Manifold sampling knob	0.0
Pipet used to filter water samples	0.0

Production and laboratory environment was evaluated for rif-resistant surrogate contamination on the fifth production day. No level of surrogates was found in any of the samples collected.

The results from these pre-operation equipment and indicator meat samples prompted the implementation of better equipment cleaning and water sanitation practices

during the optimized study, as described in 5.3.2.4.

Pre-Operation samples targeting both rif-resistant and total aerobic bacteria were collected daily during the optimized validation study. Starting on the fourth day of production, equipment samples were taken at the end of each following cleaning in preparation for the following day's production. No surrogates were recovered on the equipment and very

low levels of aerobic bacteria were recovered (Table 5.9) indicating much improved sanitation practices compared to the preliminary study. This directly correlates to essentially no surrogates recovered in non-inoculated meat samples prior to introduction of inoculum (Table 5.10) thus indicating the necessity of proper equipment and recirculating red water sanitation. Unlike, the preliminary study, inoculated meat was introduced every other day, thus pre-inoculum non-inoculated samples were taken every other day. On the fifth production day, it was noticed that fat was building up within the water exit ports of the centrifuge; the build-up was collected and enumerated revealing 2.6 Log CFU/g rif-resistant surrogates if not addressed (Table 5.9).

Table 5.9 Optimized Study Pre-Operation Equipment Samples

		Log CFU/cm ²						
		ECC						
Day		1	2	3	4*	5	6	End of 6-Day Run
Sampling Location	Grinder Conveyor Belt	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Bond Breaker	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Centrifuge drop Chute	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Centrifuge Lid	-	0.0	0.0	0.0	0.0	0.0	0.0
	Grinder Belt Conveyor 'fins'	0.0	-	0.0	0.0	0.0	0.0	0.0
			APC					
Sampling Location	Grinder Conveyor Belt	0.0005	0.0	0.0007	0.0004	0.0	-	-
	Bond Breaker	0.0	0.0	0.0	0.0196	0.0124	0.0	0.0
	Centrifuge drop Chute	0.0	1.37	0.0034	0.0038	0.0022	0.0023	0.0043
	Centrifuge Lid	-	0.0	0.0017	0.0018	0.0	0.0	0.0
	Grinder Belt Conveyor 'fins'	2.54	-	-	-	-	-	-

* Collecting samples in evening after sanitation instead of morning before production

The centrifuge exit ports were immediately cleaned in preparation for the last production day. A sample was taken from the same port following the last day of production and no surrogates were recovered; this could indicate that the 2.6 Log CFU/g recovered the previously day was partially due to 5-days build-up over the course of the study. It is possible that this could be indicative of a day's worth of surrogate build-up that would be reduced during the 50 ppm chlorine boost that recirculates through the centrifuge.

Table 5.10 Optimized Study Indicators of Equipment Cleanliness

Meat Samples (Log CFU/g)							
ECC							
Day		1	2	3	4	5	6
	Centrifuge build-up	-	-	-	-	2.6	0.0
Sampling Location	Pre-Inoculated Meat at Vortex	0.0	-	0.0	-	0.0	-
	Pre-Inoculated Meat at Manifold	0.0	-	0.0	-	0.0	-
	Pre-Inoculated Meat at Centrifuge	0.0	-	0.0	-	0.0	-
APC							
	Centrifuge build-up	-	-	-	-	5.7	3.7
Sampling Location	Pre-Inoculated Meat at Vortex	5.8	-	6.7	-	4.6	-
	Pre-Inoculated Meat at Manifold	5.3	-	6.5	-	4.4	-
	Pre-Inoculated Meat at Centrifuge	5.0	-	6.1	-	4.3	-

Environmental samples (Table 5.11) were collected every other day, with the exception of a positive control sample taken on Day 5 of gloves used to inoculate beef trim for Day 6 inoculation. Rif-resistant surrogates were only recovered in one floor sample, indicating that

relatively little environmental contamination occurred throughout the study. APC counts across samples were extremely low indicating good manufacturing practices and cleanliness was maintained throughout the production floor and the laboratory areas.

Table 5.11 Optimized Study Environmental Samples

(Log CFU/cm²)			
	Sampling Point	ECC	APC
Day 2	Doorknob	0.0	0.0
	Vortex Port Handle	0.0	0.1
	Edible Bin	0.0	0.0
Day 4	Fat drop chute	0.0	0.00068
	Grinder Drop Chute	0.0	0.0016
	Flotation Tank Port Handle	0.0	0.0
	Lab Stomacher	0.0	0.0
	Lab Pipetter	0.0	0.0
Day 5	Inoculated Glove (Positive Control)	4.98	5.25
Day 6	Lab Benchtop	0.0	0.0
	Technician jacket sleeves	0.0	0.022
	Drain	0.0	0.023
	Worker Boots	0.0	0.076
	Plastic buchner funnel post-sanitation	0.0	0.091
Day 6	Blue Tote, holds Ziplock Water Samples	0.0	0.022
	Red Squeegee	0.0	0.0
	Floor by Centrifuge	0.0065	0.23
	Worker Glove	0.0	1.11

5.4.3 Meat Samples

Inoculated Meat—There are two points where pathogens can be reduced in the system: 1) During the cryofreeze as shown from the inoculated meat to the vortex and 2) From exposure

to the chlorine in the recirculating water as shown at the post-surge tank, final lean, fat tank, and final fat.

Parameters from the preliminary study to the optimized study did not change between the introduction of inoculated meat to the vortex, therefore we can accurately describe a reduction in surrogate organisms due to freezing from 18 total observations. Due to the nature of sampling and limited effect of freezing on *E. coli* and surrogate organisms, variation is expected to be higher in vortex samples thus requiring the use of Fisher's Protected LSD for statistical analysis. Freezing alone results in a significant 0.4 log CFU/g reduction ($P \leq 0.05$) as described in Figure 5.2.

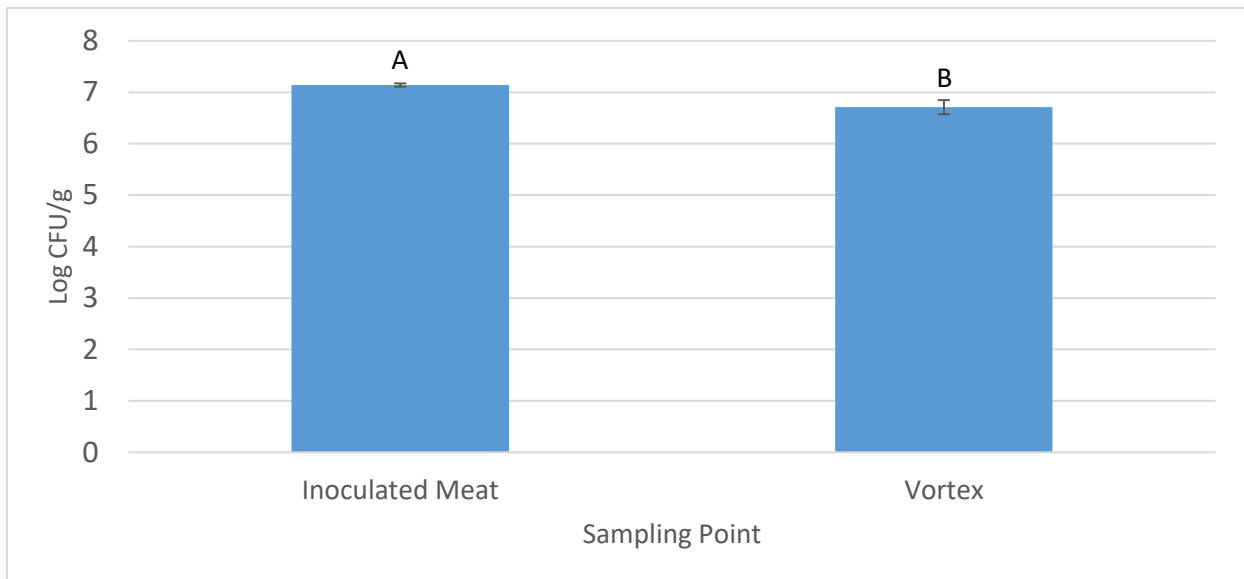


Figure 5.2 Recovery of Surrogates (ECC) Before and After the Cryofreeze Tunnel; A-B different letters indicate significant differences ($P \leq 0.01$).

During the optimized study, an average 1.2 Log CFU/g reduction of surrogates from the vortex to all points exposed to chlorine solution (i.e. the combination of the Post-Surge Tank, Final Lean, FFT lean/Fat Tank, and Final Fat) was observed. This totals to an average 1.6 Log CFU/g reduction of surrogates on the inoculated meat across the system whereas, an average total 1.0 Log CFU/g reduction of surrogates on inoculated meat samples across the system was observed in the preliminary study (Figure 5.3). Statistical analysis of the optimized study

revealed that, while we had significant kill, there was no difference between the individual sampling points ($P > 0.05$) as described in Figure 5.4.

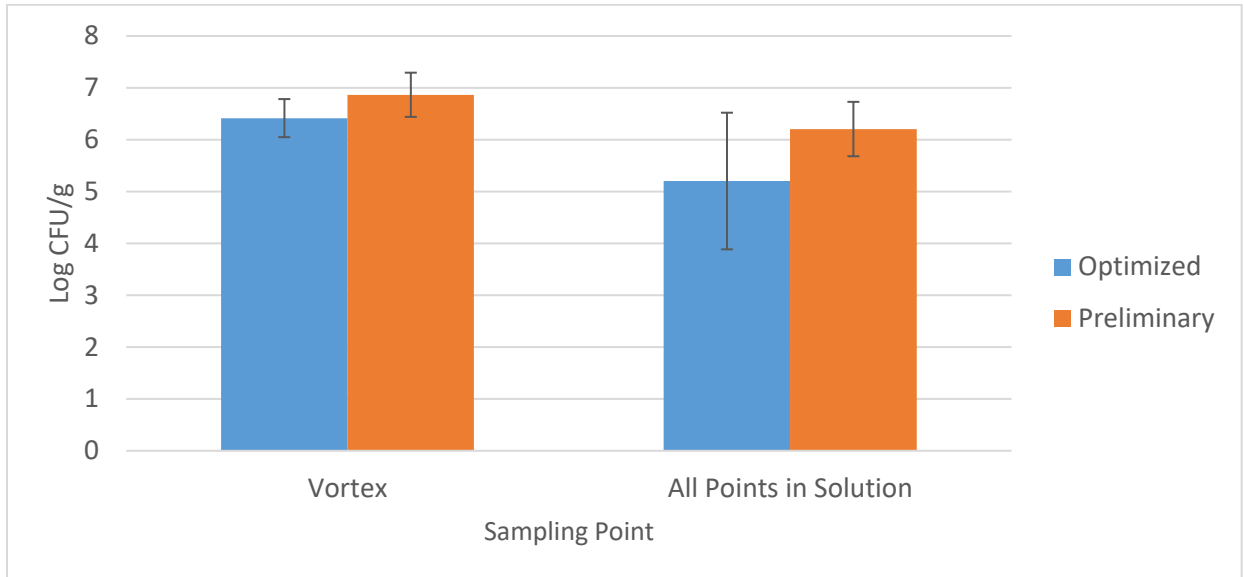


Figure 5.3 Recovery of Surrogates (ECC) Averaged Across All Sampling Points in Contact with Chlorinated Nanobubble Process Water

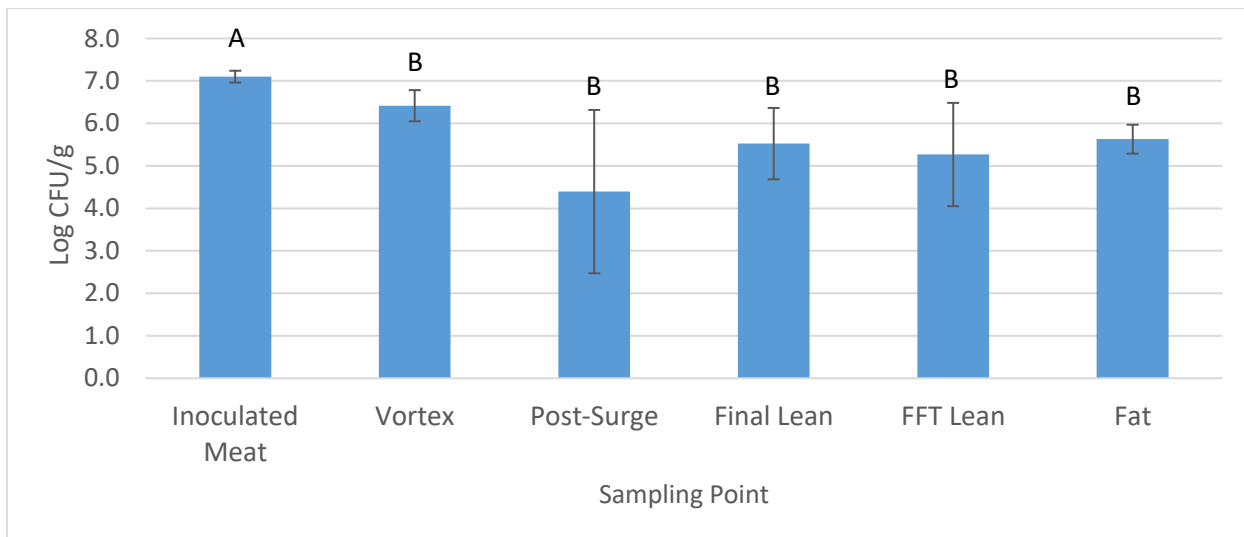


Figure 5.4 Average Recovery of Surrogates (ECC) on Inoculated Meat at Different Sampling Points Throughout the System, A-B different letters indicate significant differences ($P \leq 0.05$)

Same-day Pick-up Meat—Similar to the preliminary study, approximately 2.7 Log CFU/g of the *E. coli* surrogates were picked up on non-inoculated meat between the grinder conveyor belt

and the bond breaker and introduced into the recirculating antimicrobial solution during the optimized study. From the vortex to the final lean product, an additional 1.5 Log CFU/g is picked up, most likely from harborage of organisms in the centrifuge. While >2.9 Log CFU/g was collected on the non-inoculated meat, relatively little contamination is acquired from the recirculating solution, however, contamination picked-up is not necessarily reduced by the average 3.3 ppm FAC chlorine in solution before the final lean or final fat products. This indicates that any level of contamination, especially a high level, may be carried over into previously 'clean' meat and the recirculating solution due to dirty equipment. There are no significant difference across sampling points ($P > 0.05$); however, the same-day pick-up samples are significantly lower than the inoculated meat samples ($P \leq 0.05$).

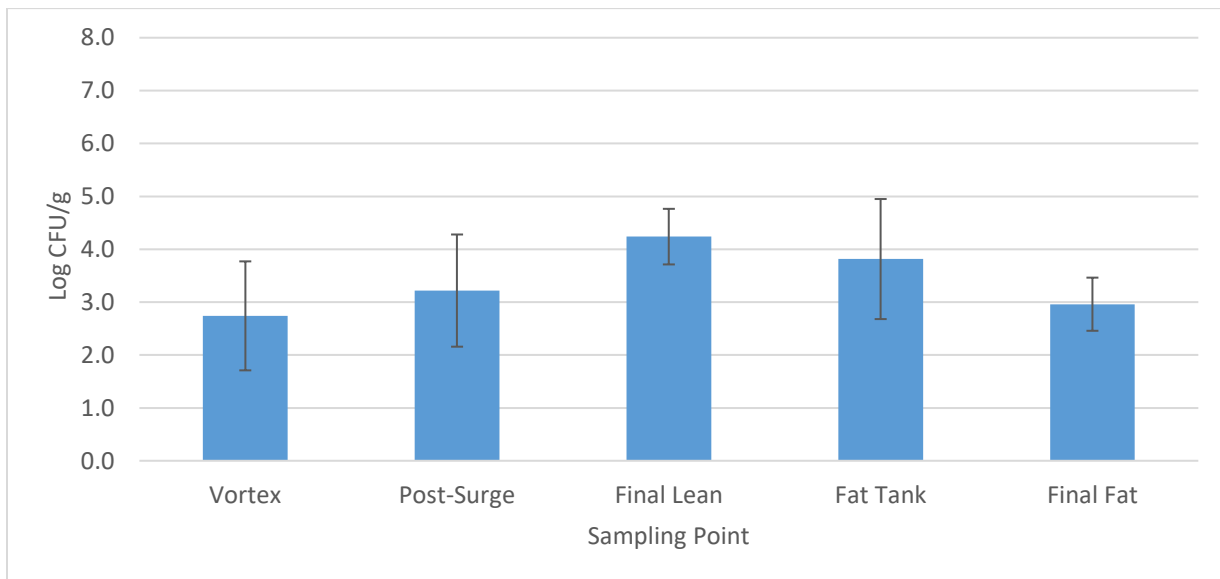


Figure 5.5 Average Recovery of Surrogates (ECC) on Same-Day Pick-Up Meat at Different Sampling Points Throughout the System, A-B different letters indicate significant differences ($P \leq 0.05$)

24-hour Pick-up Meat— During the optimized study, meat was inoculated every other day. The results from the 24-hr pick up meat indicate that there is a small level of surrogate contamination carrying over onto non-inoculated meat, which is mostly likely from bacteria harbored on equipment before the vortex (i.e. grinder conveyor belt, cryofreeze tunnel, and/or

bond breaker) and in the centrifuge. The presence of surrogates in meat samples could also be due to biofilm build-up within the manifold, fluid transport pipes, or flotation tank; although, these areas are not accessible except during full system breakdown and were not sampled. However, much of this contamination was found by enrichment and indicates that surrogates were present in levels less than the detectable limit of 0.4 Log CFU/g. As few as 1 cell would cause a positive enrichment but, this is important because only one STEC or *Salmonella* cell can cause illness in high risk human populations. It should be noted that any contamination present at or before the vortex clears out of the system over the course of a 4-hour production day however (Table 5.10), contamination is still present in the centrifuge. No significant differences were found ($P > 0.05$) between time of day (beginning, middle, or end) in 24-hour pick-up samples.

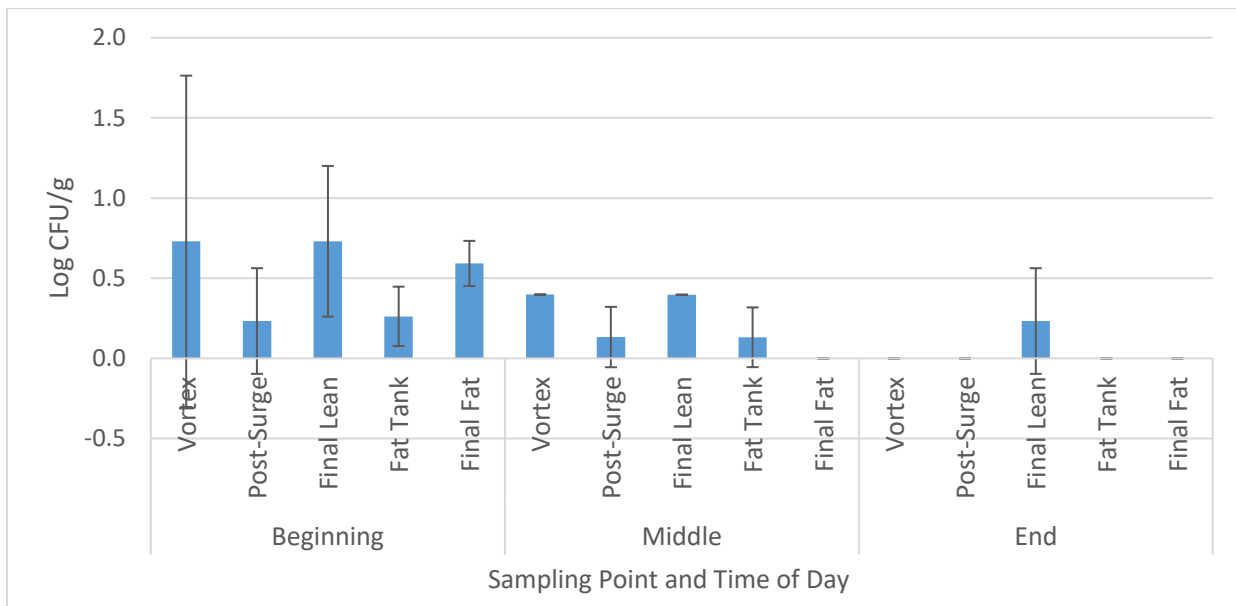


Figure 5.6 Average Recovery of Surrogates (ECC) on 24-hour Pick-Up Meat at Different Sampling Points Throughout the System at the Beginning, Middle, and End of the production Day

A significant interaction ($P \leq 0.05$) was observed between treatment and sampling point. There are also slightly significant ($P \leq 0.05$) sample and treatment effects. Most notably, the significant differences between combined inoculated meat samples and same-day pick-up meat samples ($P \leq 0.05$), and same-day pick-up samples and 24-hour pick-up ($P \leq 0.05$)

samples show a strong decreasing trend in recovery of organisms over time. These results support the use of the chlorinated nanobubble recirculating solution as an antimicrobial in this system and the implementation of equipment cleaning (albeit this still needs to be addressed due to the positive samples) and the 50 ppm FAC boost to the system for 30 minutes during the sanitation period.

It is often recommended that surrogate organisms marked with antimicrobial resistance genes, such as the surrogates used in this study, should be avoided and are unnecessary due to the unnaturally high level of controlled inoculum that is added in validation studies (USDA FSIS, 2015); however, in this study, the Aerobic Plate Counts (APC) were higher than in a standard meat processing environment due to the age of the meat obtained and length of storage. In addition, no statistical difference was observed between sampling groups (Inoculated Meat, Same-Day Pick-Up Meat, and 24-hour Pick-Up Meat) and the APC were not reduced significantly ($p > 0.05$) by freezing or by exposure to chlorine in the recirculating water and, therefore, are not a good indicator of process efficiency at any point in this system (Appendix C). Had non-rif-resistant organisms been used, the lethality contributions of the system would not have been accurately characterized.

5.5 Conclusion

Zhou et al. (2015) described chlorine as having a limited capacity to inactivate pathogens on the surface of products but extremely effective at controlling pathogen levels in wash water. Most commercially available chlorine based sanitizers only reduce pathogens on the surface of produce by 1-2 log cycles (Yang et al., 2012). This phenomenon was evident in the results from the water and meat samples in this study.

Relative to the novel ground beef commercial manufacturing system evaluated in this research, the optimized system that utilized chlorinated Cesco-Bauer nanobubble water showed excellent potential for success in reducing contamination present in beef trim in a 6-day continuous run processing scenario. The combined reduction from freezing (average of 0.4 log CFU/g) and chlorine exposure (average of 1.2 log CFU/g) on inoculated meat provides a total process reduction of 1.6 log CFU/g of final ground beef. The implementation of nightly manual

equipment cleaning and a 50 ppm chlorine spike reduced levels of target organisms > 6 log CFU/g picked up on meat 24-hours after inoculated meat entered the system. No surrogate organisms were recovered in Cesco-Bauer process water samples indicating the sanitizing properties of the water. However, surrogates were recovered on Whatman filter enrichments indicating the organisms were attaching to small organic particles within the recirculating water. Thus, during meat processing operations, low levels of viable surrogates were present in recirculating water, but the 4-hour elevated chlorine period at the end of each production day eliminated this contamination, thereby managing the risk of contamination carrying over into the next production day.

The cleaning protocols utilized during the optimized study should be included, strictly outlined in Sanitation Standard Operating Procedures (SSOPs), and implemented daily during production, paying special attention to the centrifuge and all pieces of equipment before the vortex (i.e. bond breaker, grinder conveyor belt, grinder, open areas of the cryofreeze belt). The nightly 50 ppm chlorine boost provides a thorough disinfection of the recirculating red water and must be implemented to establish a break in the multi-day continuous run process (i.e., defining each day's meat production as a manufacturing lot. Precautions should be taken to ensure employee safety and compliance with OSHA regulations during the chlorine boost period.

References

- Agarwal, A., Ng, W. J., & Liu, Y. (2011). Principle and applications of microbubble and nanobubble technology for water treatment. *Chemosphere*, *84*(9), 1175–1180.
<https://doi.org/10.1016/j.chemosphere.2011.05.054>
- Astridge, K., Craig, D., Conlan, J., Daughtry, B., Dias-Wanigasekera, B., Jin, H., ... Yates, A. (2013). *Shiga toxin-producing E. coli (STEC)*. Retrieved from
<http://www.foodstandards.gov.au/publications/Documents/Shiga%20toxin-producing%20Escherichia%20coli%20%28STEC%29.pdf>
- Barkocy-Gallagher, G., Arthur, T. M., Rivera-Betancourt, M., Nou, X. N., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2003). Seasonal Prevalance of Shiga toxin-producing *Escherichia coli* O157:H7 and Non-O157 Serotypes, and *Salmonella* in Commercial Beef Processing Plants. *Journal of Food Protection*, *66*(11), 1978–986.
- Barrette, W. C., Hannum, D. M., Wheeler, W. D., & Hurst, J. K. (1989). General mechanisms for the bacterial toxicity of hypochlorous acid: abolition of ATP production. *Biochemistry*, *28*(23), 9172–9178.
- Bauer Nanobubbles. (2016). Retrieved October 3, 2016, from <http://www.nanobubbles.com/>
- Bettelheim, K. A. (2007). The Non-O157 Shiga-Toxigenic (Verocytotoxigenic) *Escherichia coli* ; Under-Rated Pathogens. *Critical Reviews in Microbiology*, *33*(1), 67–87.
<https://doi.org/10.1080/10408410601172172>
- Bhunja, A. K. (2008). *Foodborne microbial pathogens: mechanisms and pathogenesis*. New York: Springer.

Block, S. S. (Ed.). (1991). *Disinfection, sterilization, and preservation* (4th ed). Philadelphia: Lea & Febiger.

Bosilevac, J. M., Guerini, M. N., Kalchayanand, N., & Koohmaraie, M. (2009). Prevalence and Characterization of *Salmonellae* in Commercial Ground Beef in the United States. *Applied and Environmental Microbiology*, 75(7), 1892–1900.

<https://doi.org/10.1128/AEM.02530-08>

Cabrera-Diaz, E., Moseley, T. M., Lucia, L. M., Dickson, J. S., Castillo, A., & Acuff, G. R. (2009). Fluorescent Protein-Marked *Escherichia coli* Biotype I Strains as Surrogates for Enteric Pathogens in Validation of Beef Carcass Interventions. *Journal of Food Protection*, 72(2), 295–303.

CDC. (2009, December). CDC - Disinfection & Sterilization Guideline: Disinfection - HICPAC.

Retrieved October 27, 2016, from

https://www.cdc.gov/hicpac/Disinfection_Sterilization/6_0disinfection.html

CDC. (2016, August). *Salmonella* Homepage | CDC. Retrieved October 30, 2016, from

<https://www.cdc.gov/Salmonella/>

Centers for Disease Control (CDC). (2007). Multistate Outbreak of *Salmonella* Serotype

Tennessee Infections Associated with Peanut Butter --- United States, 2006--2007.

MMWR, 56(21). Retrieved from

<https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5621a1.htm>

Centers for Disease Control (CDC). (2009). Investigation Information for Outbreak of *Salmonella*

Typhimurium Infections, 2008–2009 | *Salmonella* CDC. Retrieved September 30, 2016,

from <http://www.cdc.gov/Salmonella/2009/peanut-butter-2008-2009.html>

Centers for Disease Control (CDC). (2011a). CDC - *Salmonella* Agona Infections Linked to Whole, Fresh Imported Papayas. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/2011/papayas-8-29-2011.html>

Centers for Disease Control (CDC). (2011b). CDC - *Salmonella* Heidelberg Infections Linked to Ground Turkey. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/2011/ground-turkey-11-10-2011.html>

Centers for Disease Control (CDC). (2012). *National Shiga toxin-producing Escherichia coli (STEC) Surveillance Overview*. Atlanta, GA: US Department of Health and Human Services, CDC.

Centers for Disease Control (CDC). (2013a). CDC - *Salmonella* Typhimurium Infections Linked to Ground Beef - *Salmonella*. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/typhimurium-01-13/index.html>

Centers for Disease Control (CDC). (2013b). *Salmonella* | *Salmonella* Montevideo and *Salmonella* Mbandaka Infections Linked to Tahini Sesame Paste | May, 2013 | *Salmonella* | CDC. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/montevideo-tahini-05-13/index.html>

Centers for Disease Control (CDC). (2014a). CDC - Multistate Outbreak of Multidrug-Resistant *Salmonella* Heidelberg Infections Linked to Foster Farms Brand Chicken. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/heidelberg-10-13/index.html>

Centers for Disease Control (CDC). (2014b, January 23). Attribution of Foodborne Illness: Findings | Estimates of Foodborne Illness | CDC. Retrieved September 29, 2016, from <https://www.cdc.gov/foodborneburden/attribution/attribution-1998-2008.html>

Centers for Disease Control (CDC). (2015). Enteritidis Infections Linked to Bean Sprouts | November 2014 | *Salmonella* | CDC. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/enteritidis-11-14/index.html>

Centers for Disease Control (CDC). (2015). *Escherichia coli* O157:H7 Infections Linked to Costco Rotisserie Chicken Salad (Final Update) | November 2015 | *E. coli* | CDC. Retrieved September 30, 2016, from <http://www.cdc.gov/ecoli/2015/o157h7-11-15/index.html>

Centers for Disease Control (CDC). (2015). Multistate Outbreak of Multidrug-Resistant *Salmonella* | 4,[5],12:i:- and *Salmonella* Infantis Infections Linked to Pork (Final Update) | Multistate Outbreak of Multidrug-Resistant *Salmonella* | 4,[5],12:i:- or *Salmonella* Infantis Infections Linked to Pork (Final Update) | August 2015 | *Salmonella* | CDC. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/pork-08-15/index.html>

Centers for Disease Control (CDC). (2016). *Escherichia coli* O26 Infections Linked to Chipotle Mexican Grill Restaurants (Final Update) | November 2015 | *E. coli* | CDC. Retrieved September 30, 2016, from <http://www.cdc.gov/ecoli/2015/o26-11-15/>

Centers for Disease Control (CDC). (2016). Multistate Outbreak of *Salmonella* Poona Infections Linked to Imported Cucumbers (Final Update) | Multistate Outbreak of *Salmonella* Poona Infections Linked to Imported Cucumbers | September 2015 | *Salmonella* | CDC. Retrieved September 30, 2016, from <http://www.cdc.gov/Salmonella/poona-09-15/index.html>

- Centers for Disease Control (CDC). (2016). Multistate Outbreak of Shiga toxin-producing *Escherichia coli* O121 Infections Linked to Flour (Final Update). Retrieved September 30, 2016, from <http://www.cdc.gov/ecoli/2016/o121-06-16/>
- Cliver, D. O., & Riemann, H. (Eds.). (2002). *Foodborne diseases* (2nd ed). London ; San Diego, Calif: Academic Press.
- Donnenberg, M. S. (Ed.). (2013). *Escherichia coli: pathotypes and principles of pathogenesis* (2nd edition). Amsterdam: Academic Press.
- Dos Santos, M. S., Martendal, E., & Carasek, E. (2011). Determination of THMs in soft drink by solid-phase microextraction and gas chromatography. *Food Chemistry*, *127*(1), 290–295. <https://doi.org/10.1016/j.foodchem.2010.12.115>
- Fegan, N., Vanderlinde, P., Higgs, G., & Desmarchelier, P. (2004). The prevalence and concentration of *Escherichia coli* O157 in faeces of cattle from different production systems at slaughter. *Journal of Applied Microbiology*, *97*, 362–370.
- Food and Drug Administration (FDA). (2012). *Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins* (2nd ed.).
- Food Safety and Inspection Service. (1996). *Pathogen Reduction; Hazard Analysis Critical Control Point (HACCP) Systems: Final Rule. 9 CFR Part 304 et. al*, (No. Federal Register 61(144)) (pp. 38805–38989). United States Department of Agriculture.
- Food Safety and Inspection Service. (2011). *Northeastern Grocery Chain Recalls Ground Beef Products Due to Possible Salmonella Contamination* (Recall Release No. FSIS-RC-100-2011). Washington D.C. Retrieved from http://www.fsis.usda.gov/wps/portal/fsis/newsroom/!ut/p/a0/04_Sj9CPykssy0xPLMnM

z0vMAfGjzOINAg3MDC2dDbwMDIHQ08842MTDy8_YwNtMvyDbUREAzbjixQ!!/?1dmy&
current=true&urile=wcm%3Apath%3A%2Ffsis-archives-
content%2Finternet%2Fmain%2Ftopics%2Frecalls-and-public-health-alerts%2Frecall-
case-archive%2Farchives%2Fct_index205a

Food Safety and Inspection Service. (2013). *Salmonella and Campylobacter Verification Program for Raw Meat and Poultry Products* (No. FSIS Directive 10,250.1). Washington D.C.: United States Department of Agriculture. Retrieved from <http://www.fsis.usda.gov/wps/wcm/connect/ebf83112-4c3b-4650-8396-24cc8d38bf6c/10250.1.pdf?MOD=AJPERES>

Food Safety and Inspection Service. (2014). *Changes to Salmonella Verification Sampling Program: Analysis of Raw Beef for Shiga toxin-producing Escherichia coli and Salmonella* (Federal Register Docket No. FSIS-2012-0038). Washington D.C.: United States Department of Agriculture. Retrieved from <http://www.fsis.usda.gov/wps/wcm/connect/d3fdd61d-924a-4dd9-a363-a45b42b83163/2012-0038-A.pdf?MOD=AJPERES>

Food Safety and Inspection Service. (2015). Recall Summaries 2010. Retrieved September 27, 2016, from <http://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries/recall-summaries-2010>

Food Safety and Inspection Service. (2015). Recall Summaries 2011. Retrieved December 1, 2015, from <http://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries/recall-summaries-2011>

Food Safety and Inspection Service. (2015). Recall Summaries 2012. Retrieved December 1, 2015, from <http://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries/recall-summaries-2012>

Food Safety and Inspection Service. (2015). Recall Summaries 2013. Retrieved December 1, 2015, from <http://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries/recall-summaries-2013>

Food Safety and Inspection Service. (2015). Recall Summaries 2014. Retrieved December 1, 2015, from <http://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries/recall-summaries-2014>

Food Safety and Inspection Service. (2016). Recall Summaries 2015. Retrieved September 27, 2016, from <http://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries/recall-summaries-2015>

Gabbett, R. J. (2016, September 9). FSIS surprises industry with move toward testing beef primals. Retrieved September 28, 2016, from <http://www.meatingplace.com/ArticleRedirector/?code=2s68618&rType=4&allowguest=true>

Gragg, S. E., Loneragan, G. H., Nightingale, K. K., Brichta-Harhay, D. M., Ruiz, H., Elder, J. R., ... Brashears, M. M. (2013). Substantial within-Animal Diversity of *Salmonella* Isolates from Lymph Nodes, Feces, and Hides of Cattle at Slaughter. *Applied and Environmental Microbiology*, 79(15), 4744–4750. <https://doi.org/10.1128/AEM.01020-13>

Guentzel, J. L., Liang Lam, K., Callan, M. A., Emmons, S. A., & Dunham, V. L. (2008). Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral

- electrolyzed oxidizing water. *Food Microbiology*, 25(1), 36–41.
<https://doi.org/10.1016/j.fm.2007.08.003>
- Hricova, D., Stephan, R., & Zweifel, C. (2008). Electrolyzed water and its application in the food industry. *Journal of Food Protection*, 71(9), 1934–1947.
- Huang, A.-T., & Batterman, S. (2009). Formation of trihalomethanes in foods and beverages. *Food Additives & Contaminants: Part A*, 26(7), 947–957.
<https://doi.org/10.1080/02652030902897739>
- Huang, Y.-R., Hung, Y.-C., Hsu, S.-Y., Huang, Y.-W., & Hwang, D.-F. (2008). Application of electrolyzed water in the food industry. *Food Control*, 19(4), 329–345.
<https://doi.org/10.1016/j.foodcont.2007.08.012>
- Hussein, H. S., & Bollinger, L. M. (2005). Prevalence of Shiga toxin-producing *Escherichia coli* in beef. *Meat Science*, 71(4), 676–689. <https://doi.org/10.1016/j.meatsci.2005.05.012>
- Hussein, H. S., & Sakuma, T. (2005). Prevalence of Shiga Toxin-producing *Escherichia coli* in Dairy Cattle and Their Products. *Journal of Dairy Science*, 88(2), 450–465.
- Issa-Zacharia, A., Kamitani, Y., Miwa, N., Muhimbula, H., & Iwasaki, K. (2011). Application of slightly acidic electrolyzed water as a potential non-thermal food sanitizer for decontamination of fresh ready-to-eat vegetables and sprouts. *Food Control*, 22(3–4), 601–607. <https://doi.org/10.1016/j.foodcont.2010.10.011>
- Jadeja, R., & Hung, Y.-C. (2014). Efficacy of near neutral and alkaline pH electrolyzed oxidizing waters to control *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT 104 from beef hides. *Food Control*, 41, 17–20. <https://doi.org/10.1016/j.foodcont.2013.12.030>

- Laster, B. A., Harris, K. B., Lucia, L. M., Castillo, A., & Savell, J. W. (2012). Efficacy of trimming chilled beef during fabrication to control *Escherichia coli* O157:H7 surrogates on subsequent subprimals. *Meat Science*, *90*(2), 420–425.
<https://doi.org/10.1016/j.meatsci.2011.08.011>
- Laufer, A. S., Grass, J., Holt, K., Whichard, J. M., Griffin, P. M., & Gould, L. H. (2015). Outbreaks of *Salmonella* infections attributed to beef – United States, 1973–2011. *Epidemiology and Infection*, *143*(9), 2003–2013. <https://doi.org/10.1017/S0950268814003112>
- Li, M., Malladi, S., Hurd, H. S., Goldsmith, T. J., Brichta-Harhay, D. M., & Loneragan, G. H. (2015). *Salmonella* spp. in lymph nodes of fed and cull cattle: Relative assessment of risk to ground beef. *Food Control*, *50*, 423–434.
<https://doi.org/10.1016/j.foodcont.2014.09.011>
- Liao, L. B., Chen, W. M., & Xiao, X. M. (2007). The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. *Journal of Food Engineering*, *78*(4), 1326–1332. <https://doi.org/10.1016/j.jfoodeng.2006.01.004>
- Mani-López, E., García, H. S., & López-Malo, A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International*, *45*(2), 713–721.
<https://doi.org/10.1016/j.foodres.2011.04.043>
- McEvoy, J. M., Doherty, A. M., Sheridan, J. J., Blair, I. S., & McDowell, D. A. (2003). The prevalence of *Salmonella* spp. in bovine faecal, rumen and carcass samples at a commercial abattoir. *Journal of Applied Microbiology*, *94*(4), 693–700.
<https://doi.org/10.1046/j.1365-2672.2003.01898.x>

- Najjar, M. B., & Meng, J. (2009). *Risk Assessment of Disinfection Byproducts in Poultry Chilled in Chlorinated Water*. Joint Institute for Food Safety and Nutrition and Department of Nutrition and Food Science.
- Niebuhr, S. E., Laury, A., Acuff, G. R., & Dickson, J. S. (2008). Evaluation of nonpathogenic surrogate bacteria as process validation indicators for *Salmonella enterica* for selected antimicrobial treatments, cold storage, and fermentation in meat. *Journal of Food Protection*, 71(4), 714–718.
- Park, H., Hung, Y.-C., & Chung, D. (2004). Effects of chlorine and pH on efficacy of electrolyzed water for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *International Journal of Food Microbiology*, 91(1), 13–18.
[https://doi.org/10.1016/S0168-1605\(03\)00334-9](https://doi.org/10.1016/S0168-1605(03)00334-9)
- Particle Sciences. (2012). *Zeta Potential (ZP): An Overview - Particle Sciences, Drug Development* (Technical Brief No. Volume 2). Bethlehem, PA. Retrieved from <http://www.particlesciences.com/news/technical-briefs/2012/overview-of-zeta-potential.html>
- Patel, P. D. (Ed.). (1995). *Rapid Analysis Techniques in Food Microbiology*. Boston, MA: Springer US. Retrieved from <http://link.springer.com/10.1007/978-1-4615-2662-9>
- Rao, M. V. (2007). *Acidified Sodium Chlorite (ASC) Chemical and Technical Assessment* (pp. 1–12). Food and Agriculture Organization. Retrieved from http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/68/Acidified_Sodium_Chlorite.pdf

- Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., ... Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases*, 17(1), 7–15. <https://doi.org/10.3201/eid1701.P11101>
- Schwan, C. (2015). *Characterizing differences in shiga toxin-producing Escherichia coli (STEC) to pre-rigor and chilled beef carcass surfaces*. Kansas State University, Manhattan, KS.
- Sofos, J. N. (2008). Challenges to meat safety in the 21st century. *Meat Science*, 78(1–2), 3–13. <https://doi.org/10.1016/j.meatsci.2007.07.027>
- Sohaib, M., Anjum, F. M., Arshad, M. S., & Rahman, U. U. (2016). Postharvest intervention technologies for safety enhancement of meat and meat based products; a critical review. *Journal of Food Science and Technology*, 53(1), 19–30. <https://doi.org/10.1007/s13197-015-1985-y>
- Soli, K. W., Yoshizumi, A., Motomatsu, A., Yamakawa, M., Yamasaki, M., Mishima, T., ... Miyamoto, T. (2010). Decontamination of fresh produce by the use of slightly acidic hypochlorous water following pretreatment with sucrose fatty acid ester under microbubble generation. *Food Control*, 21(9), 1240–1244. <https://doi.org/10.1016/j.foodcont.2010.02.009>
- Sperandio, V., & Hovde, C. J. (Eds.). (2015). *Enterohemorrhagic Escherichia coli and other shiga toxin-producing E. coli*. Washington, DC: ASM Press.
- Stephens, T. P., Loneragan, G. H., Thompson, T. W., Sridhara, A., Branham, L. A., Pitchiah, S., & Brashears, M. M. (2007). Distribution of *Escherichia coli* O157 and *Salmonella* on hide surfaces, the oral cavity, and in feces of feedlot cattle. *Journal of Food Protection*, 70(6), 1316–1526.

- Stopforth, J. D., Mai, T., Kottapalli, B., & Samadpour, M. (2008). Effect of Acidified Sodium Chlorite, Chlorine, and Acidic Electrolyzed water on *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* Inoculated on Leafy Greens. *Journal of Food Protection*, 71(3), 625–628.
- Tsuge, H. (Ed.). (2014). *Micro- and nanobubbles: fundamentals and applications*. Singapore: Pan Stanford Publishing.
- United States Department of Agriculture Food Safety and Inspection Service. (2011). *Shiga Toxin-producing Escherichia coli in certain raw beef products* (No. Docket No. FSIS-2010-0023). Retrieved from <https://www.gpo.gov/fdsys/pkg/FR-2011-09-20/pdf/2011-24043.pdf>
- US Environmental Protection Agency. (2001). *Stage 1 Disinfectants and Disinfection Byproducts Rule* (No. EPA 816-01-014).
- USDA ERS - Cost Estimates of Foodborne Illnesses. (n.d.). Retrieved November 3, 2015, from <http://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx#.VDW27r4mUfy>
- USDA Food Safety and Inspection Service. (2015). *FSIS Compliance Guideline HACCP Systems Validation*. Retrieved from http://www.fsis.usda.gov/wps/wcm/connect/a70bb780-e1ff-4a35-9a9a-3fb40c8fe584/HACCP_Systems_Validation.pdf?MOD=AJPERES
- USDA Food Safety and Inspection Service. (2015). *Sampling verification activities for shiga toxin-producing Escherichia coli (STEC) in raw beef products* (No. FSIS Directive 10,010.1 Rev. 4). Washington D.C.

- USDA Food Safety and Inspection Service. (2015). *Use of non-pathogenic Escherichia coli (E. coli) cultures as surrogate indicator organisms in validation studies*. Retrieved from http://askfsis.custhelp.com/app/answers/detail/a_id/1392/kw/Can%20non-pathogenic%20surrogate%20bacteria%20be%20used%20in%20a%20commercial%20processing%20plant%20to%20validate%20process%20control%3F/session/L3RpbWUvMTM2MDc5NDY2NC9zaWQvQUc1YmxOaWw%3D
- USDA Food Safety and Inspection Service. (2016). *Safe and suitable ingredients used in the production of meat, poultry, and egg products* (No. FSIS Directive 7120.1 Rev. 37). Washington D.C.
- Vizzier-Thaxton, Y., Ewing, M. L., & Bonner, C. M. (2010). Generation and detection of trihalomethanes in chicken tissue from chlorinated chill water. *The Journal of Applied Poultry Research*, 19(2), 169–173. <https://doi.org/10.3382/japr.2009-00129>
- Waters, B. W., & Hung, Y.-C. (2014). The effect of organic loads on stability of various chlorine-based sanitisers. *International Journal of Food Science & Technology*, 49(3), 867–875. <https://doi.org/10.1111/ijfs.12379>
- Yang, Y., Luo, Y., Millner, P., Shelton, D., & Nou, X. (2012). Enhanced Chlorine Efficacy against Bacterial Pathogens in Wash Solution with High Organic Loads: Enhanced Chlorine Efficacy against Pathogens. *Journal of Food Processing and Preservation*, 36(6), 560–566. <https://doi.org/10.1111/jfpp.12000>
- Zhou, B., Luo, Y., Nou, X., Lyu, S., & Wang, Q. (2015). Inactivation dynamics of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in wash water during

simulated chlorine depletion and replenishment processes. *Food Microbiology*, 50, 88–96. <https://doi.org/10.1016/j.fm.2015.03.004>

Appendix A - Temperature Charts from In-Plant Validation Study

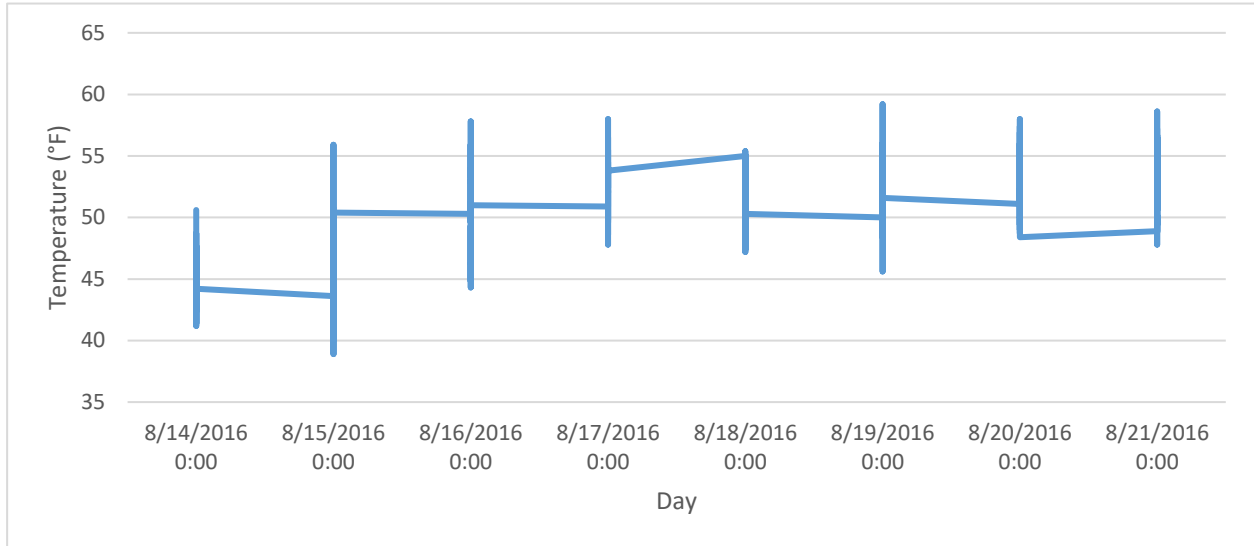


Figure 5.7 Temperature of Processing Room during Preliminary In-Plant Study

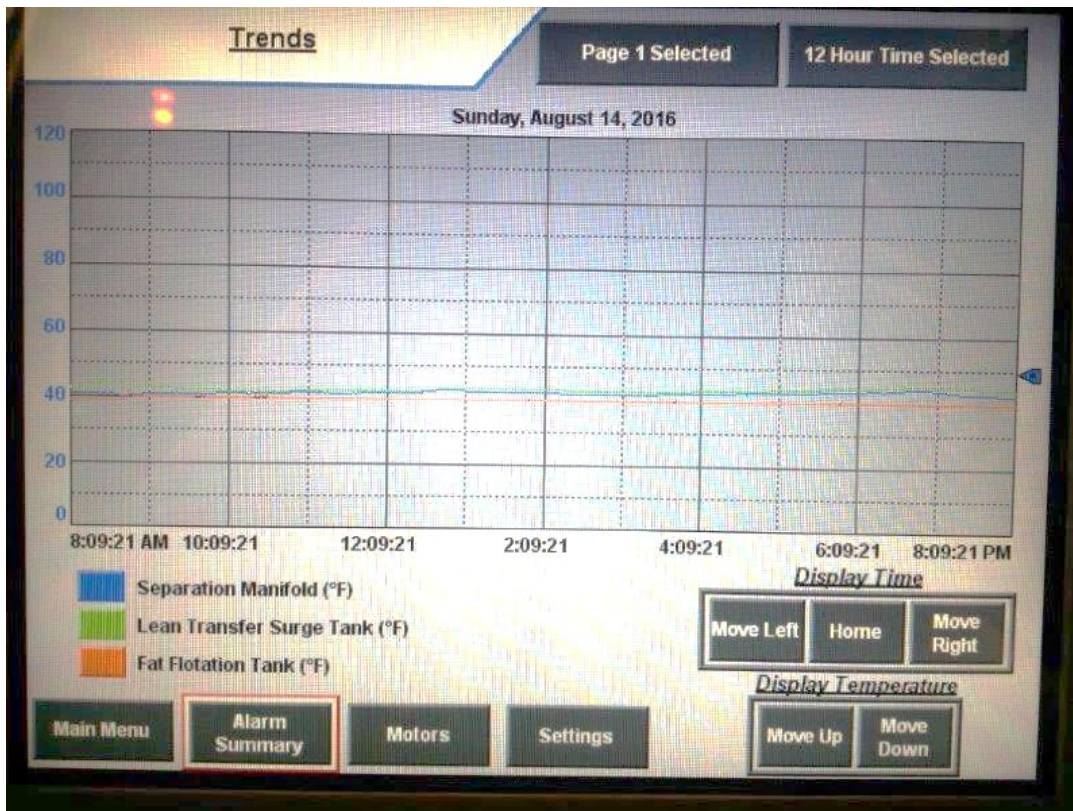


Figure 5.8 Temperature of water in recirculating solution during preliminary water experiment

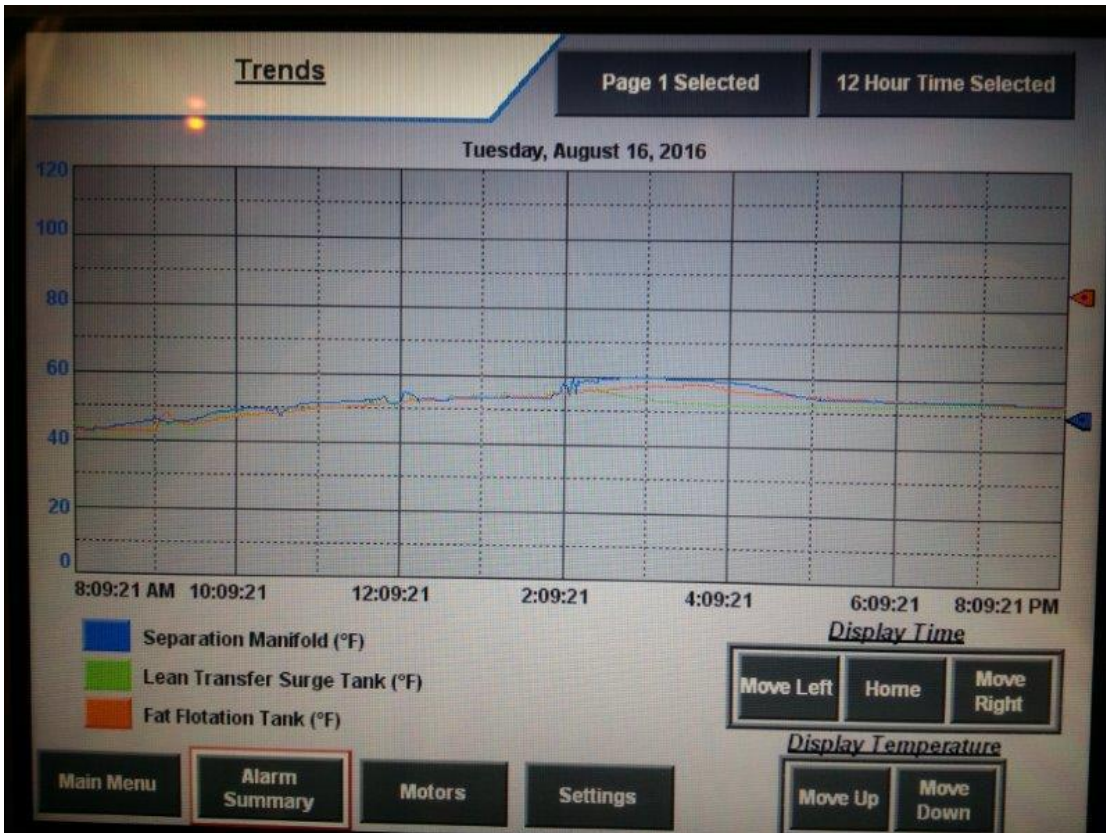


Figure 5.10 Temperature of water in recirculating water, Day 1 Preliminary Study

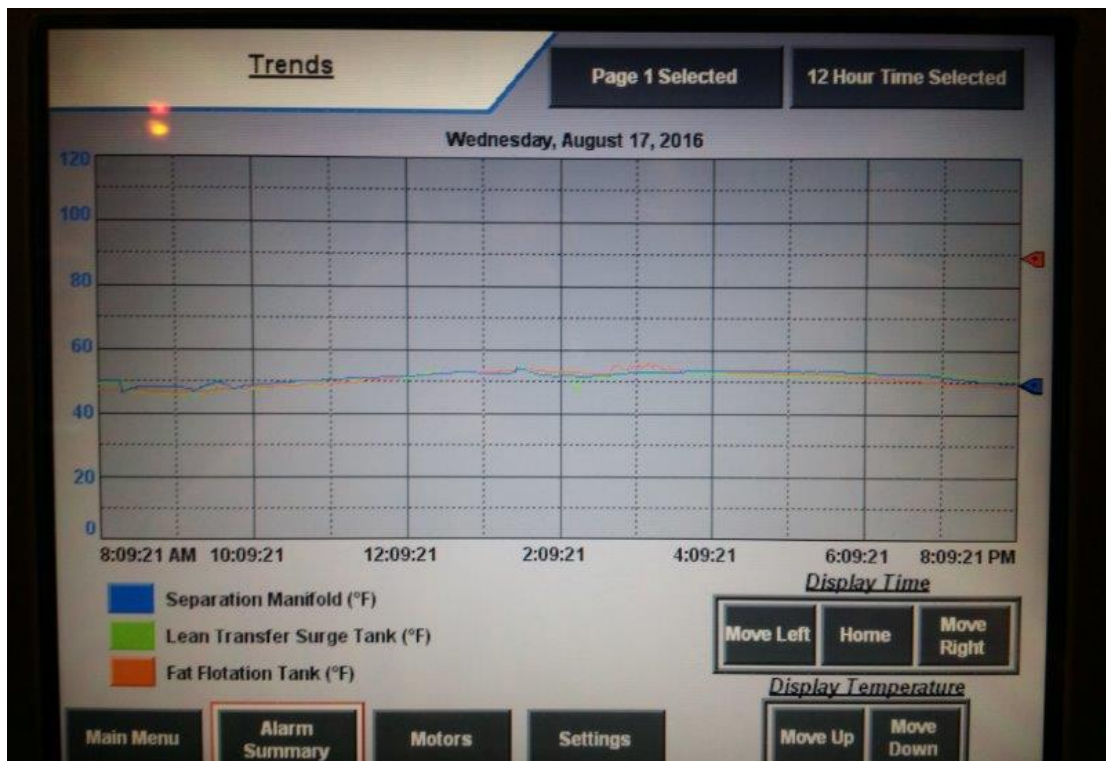


Figure 5.9 Temperature of water in recirculating water, Day 2 Preliminary Study

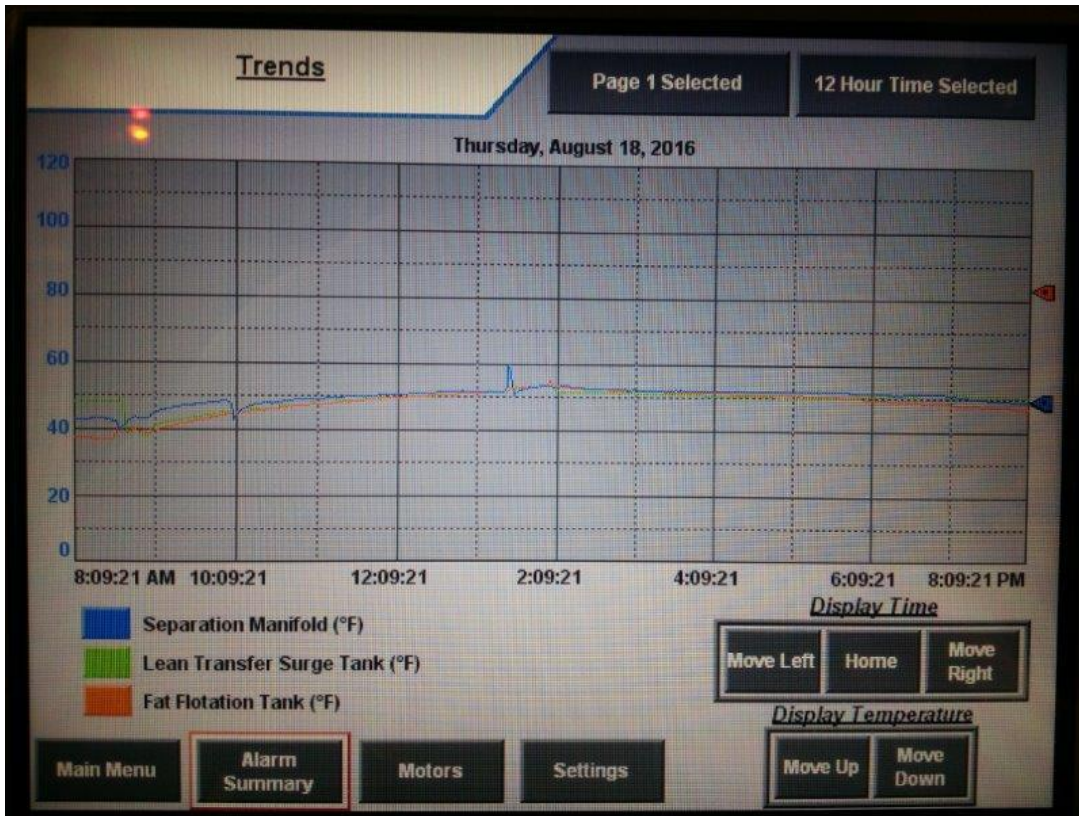


Figure 5.12 Temperature of recirculating water, Day 3 Preliminary Study

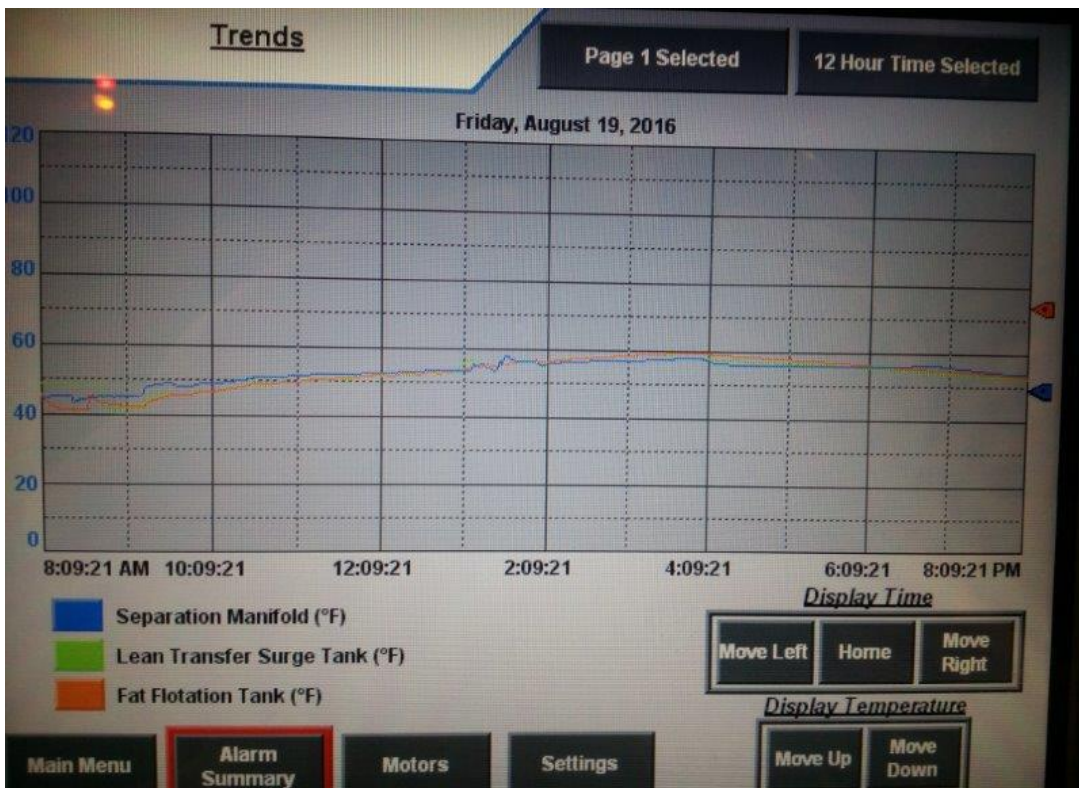


Figure 5.11 Temperature of recirculating water, Day 4 Preliminary Study

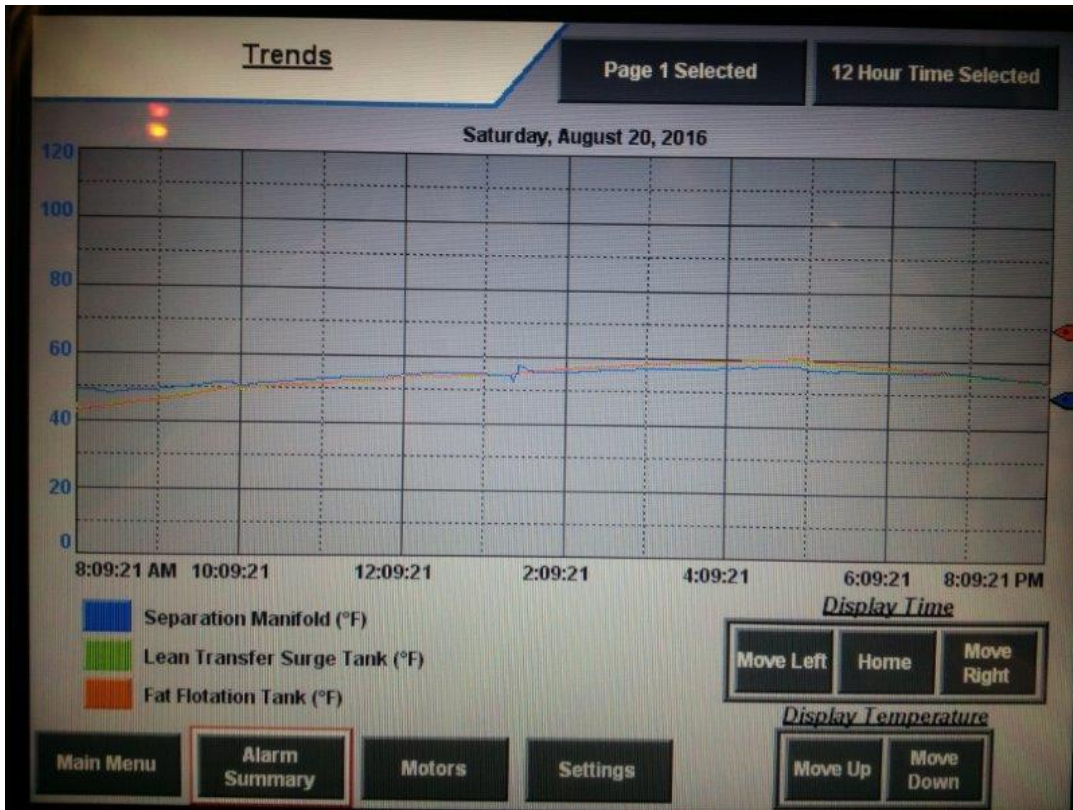


Figure 5.14 Temperature of recirculating water, Day 5 Preliminary Study

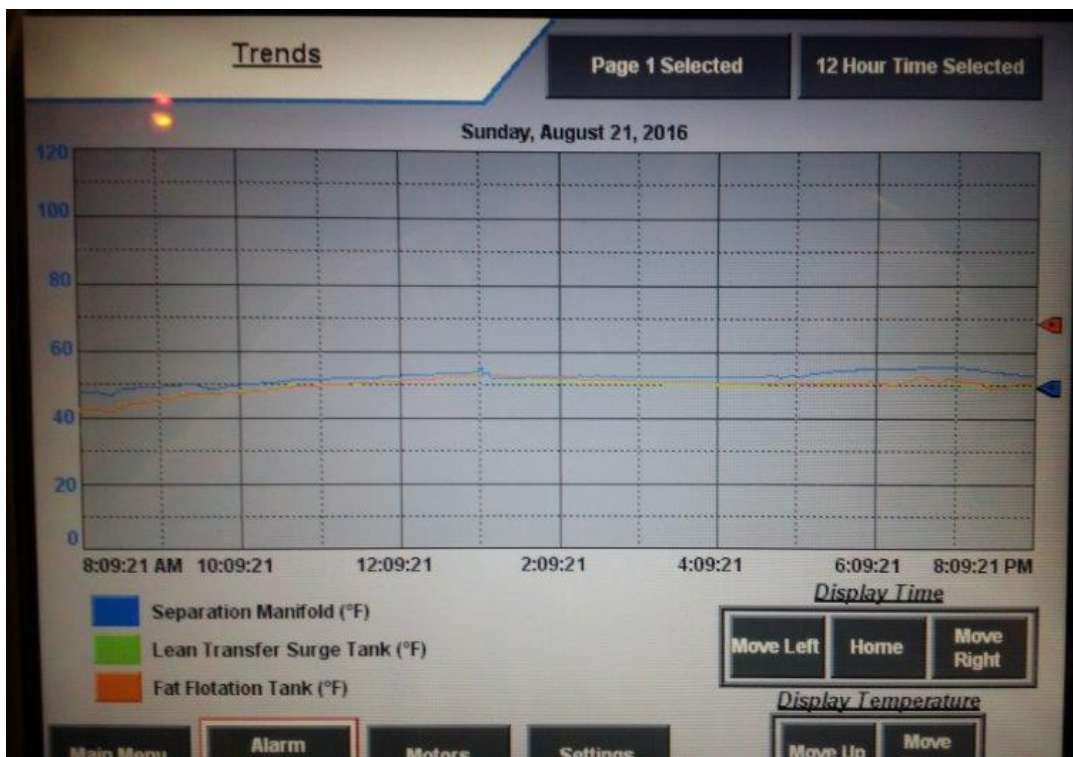


Figure 5.13 Temperature of recirculating water, Day 6 Preliminary Study

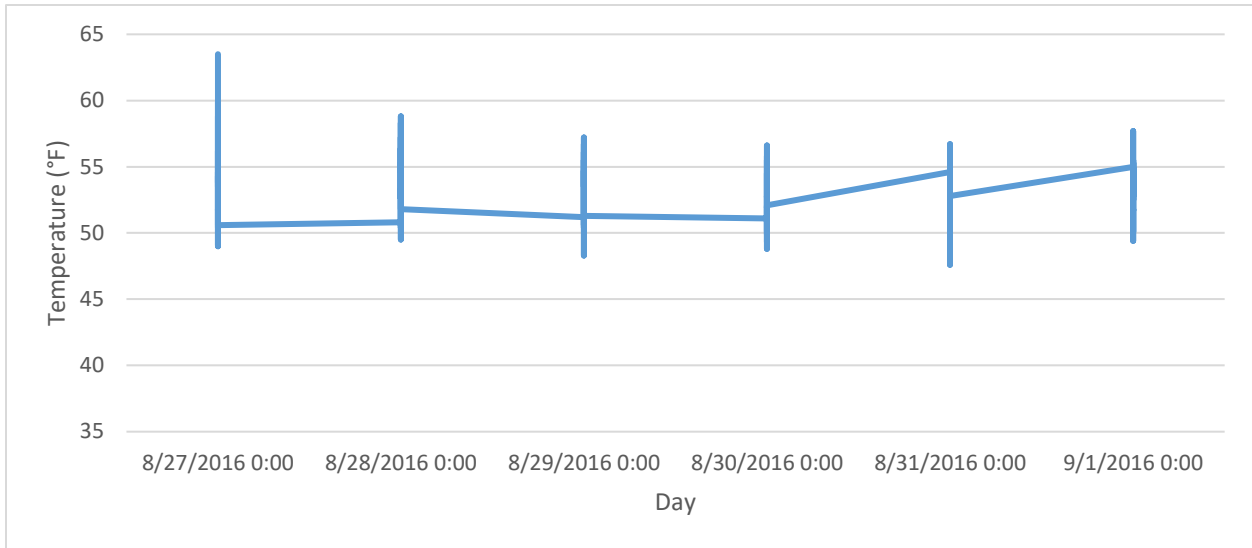


Figure 5.16 Temperature of Processing Room during Optimized Validation Study



Figure 5.15 Temperature of recirculating water, Preliminary water and Day 1 of Optimized Study

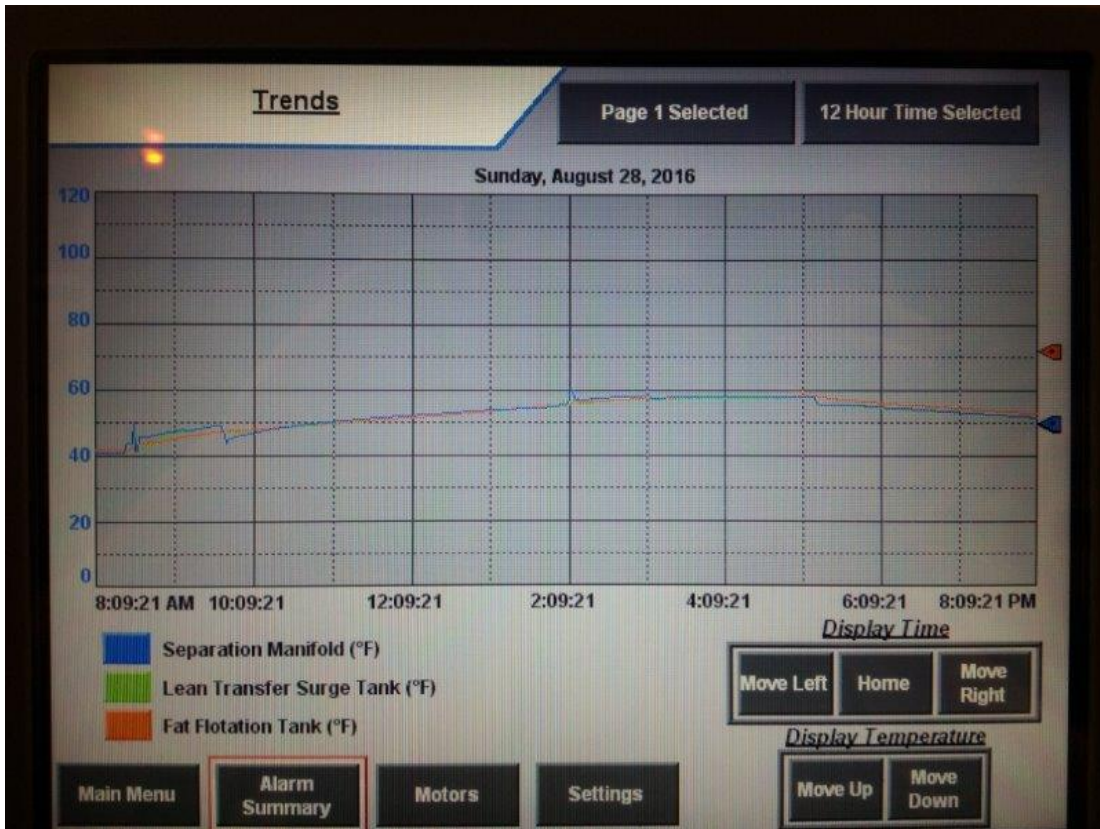


Figure 5.18 Temperature of recirculating water, Day 2 Optimized Study

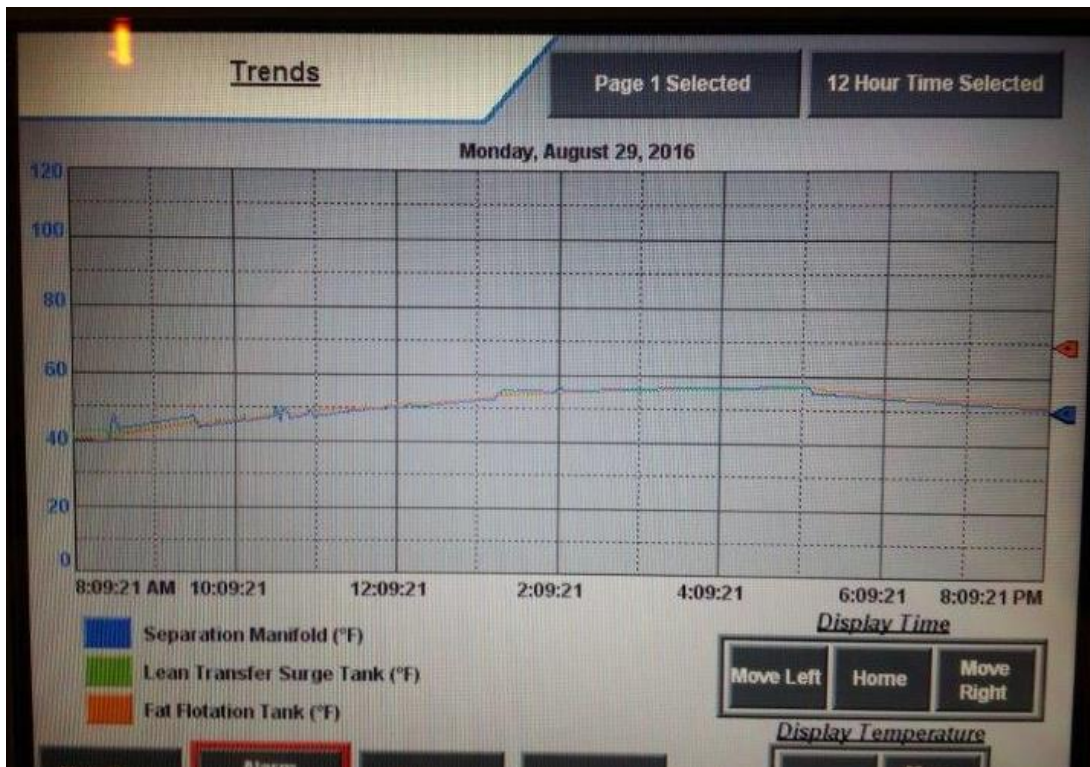


Figure 5.17 Temperature of recirculating water, Day 3 Optimized Study



Figure 5.20 Temperature of recirculating water, Day 4 Optimized Study

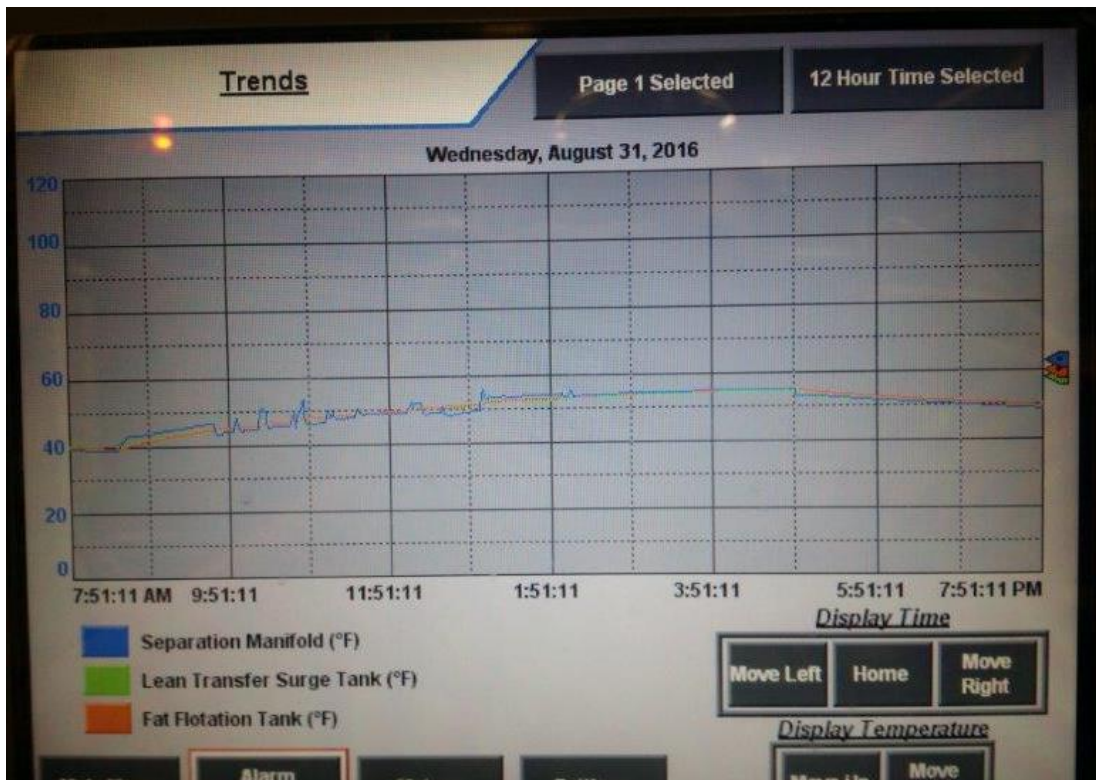


Figure 5.19 Temperature of recirculating water, Day 5 Optimized Study

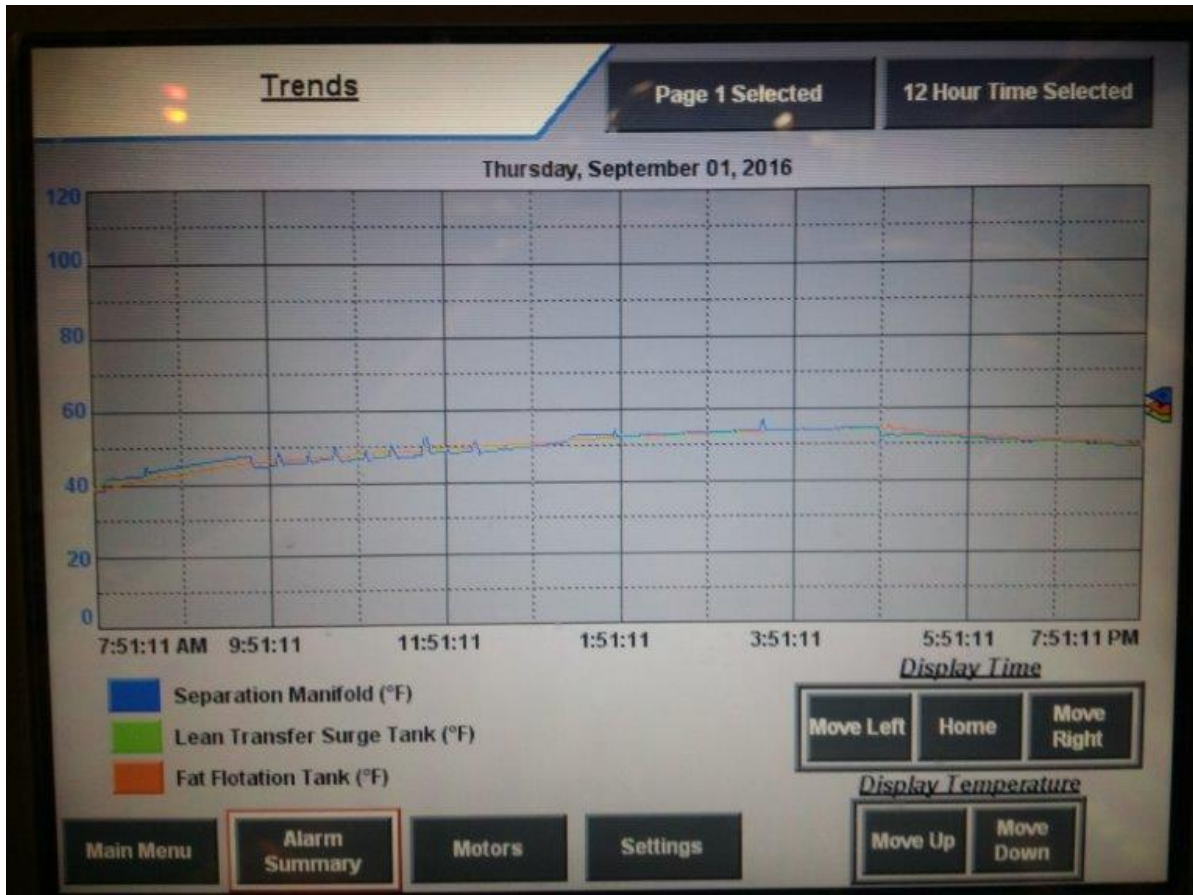


Figure 5.21 Temperature of recirculating water, Day 6 Optimized Study

Appendix B - Cesco-Bauer Chemical Readings during In-Plant Studies

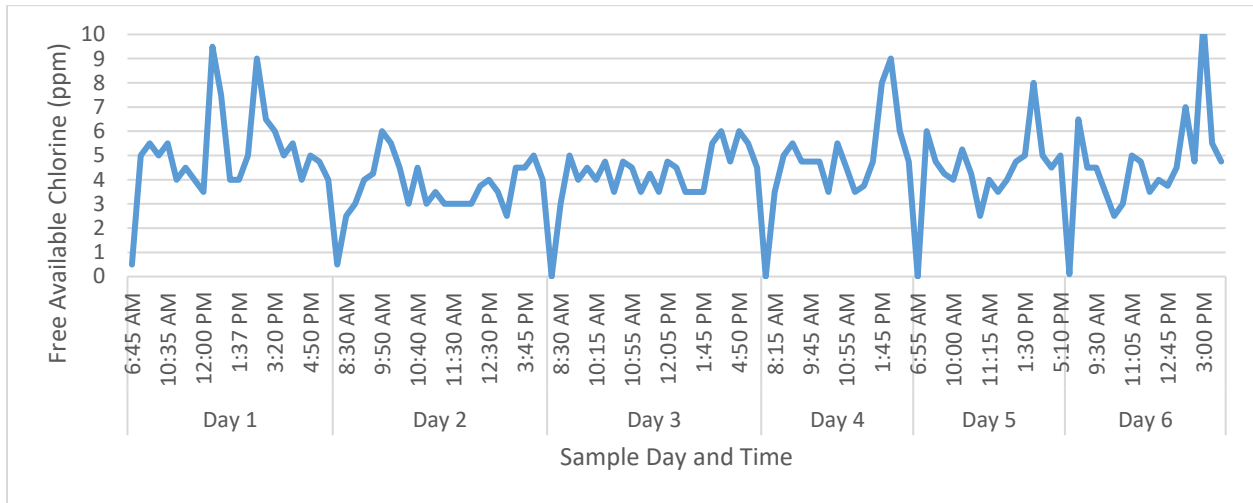


Figure 5.22 Free Available Chlorine at point of meat contact (Vortex) in Recirculating Water over 6-day Preliminary Study

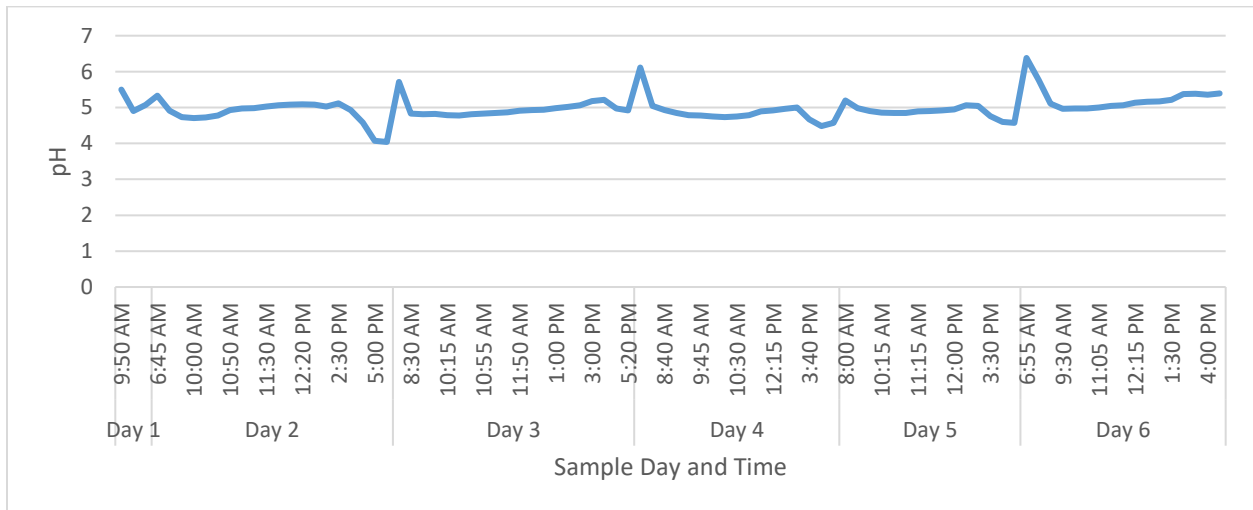


Figure 5.23 pH of Recirculating Water over 6-day Preliminary Study, recorded at flotation tank

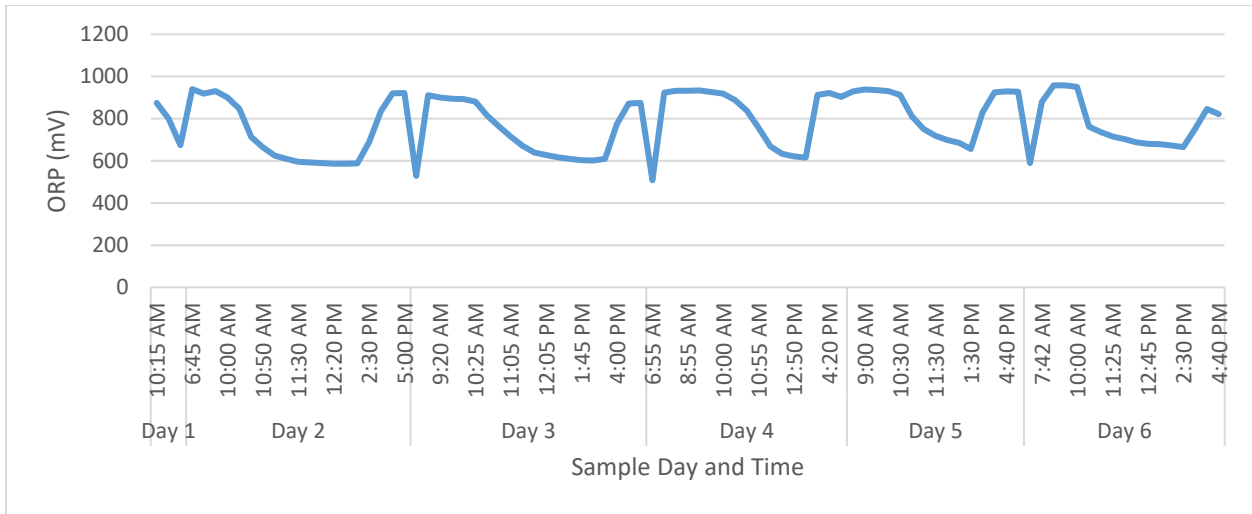


Figure 5.24 ORP of Recirculating Water over 6-day Preliminary Study, recorded at flotation tank

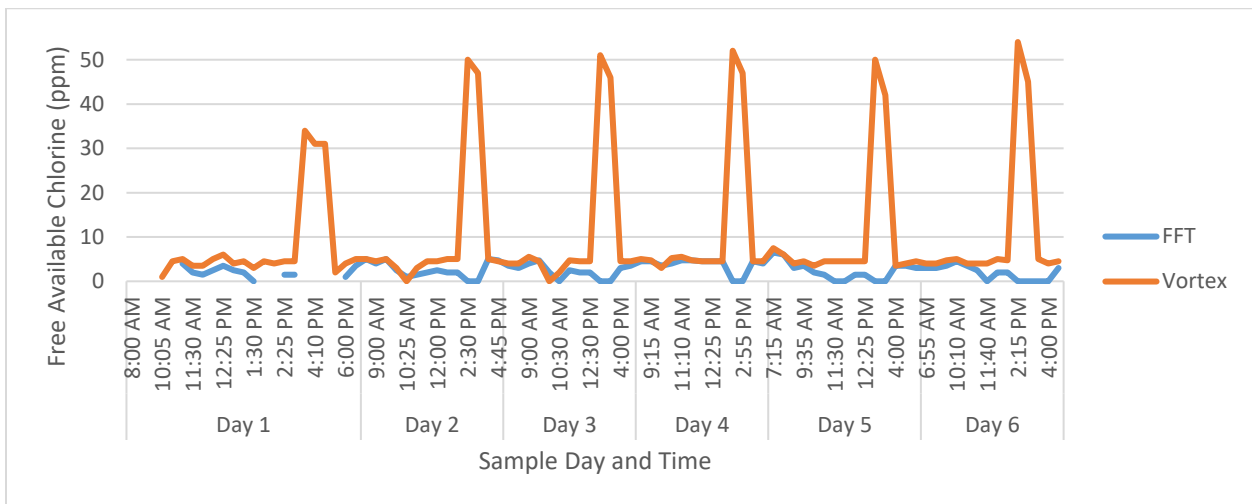


Figure 5.25 Free Available Chlorine in Recirculating Water over 6-day Optimized Validation Study

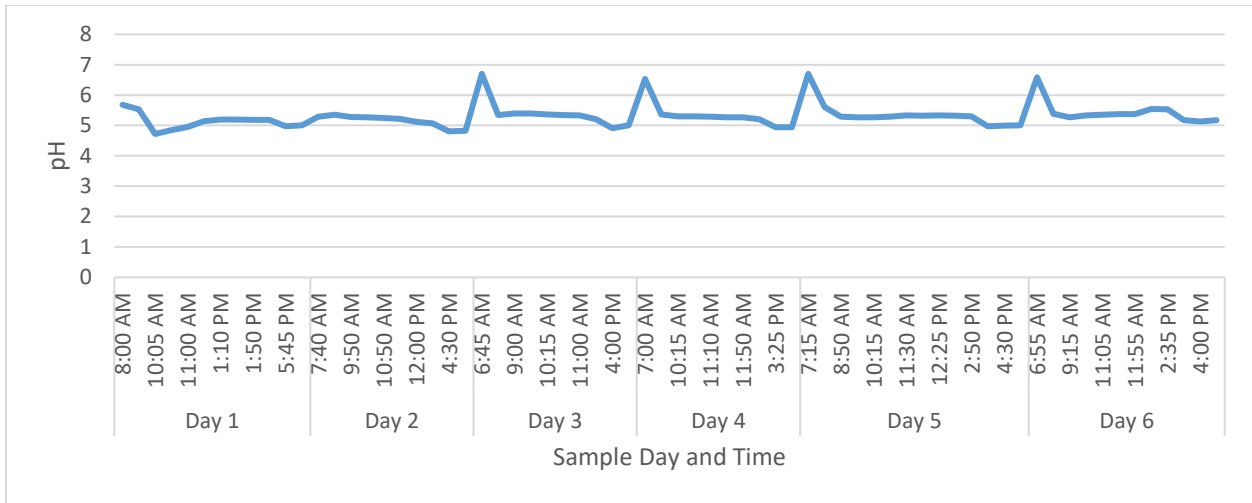


Figure 5.26 pH of Recirculating Water over 6-day Optimized Validation Study, recorded at flotation tank

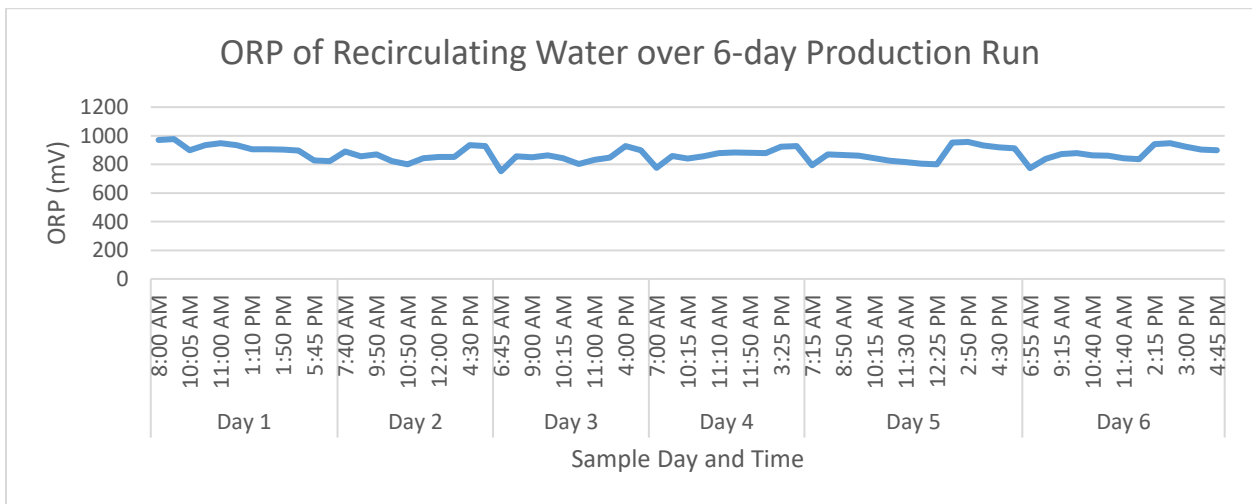


Figure 5.27 ORP of Recirculating Water over 6-day Optimized Validation Study, recorded at flotation tank

Appendix C - Extra Graphs from In-Plant Study

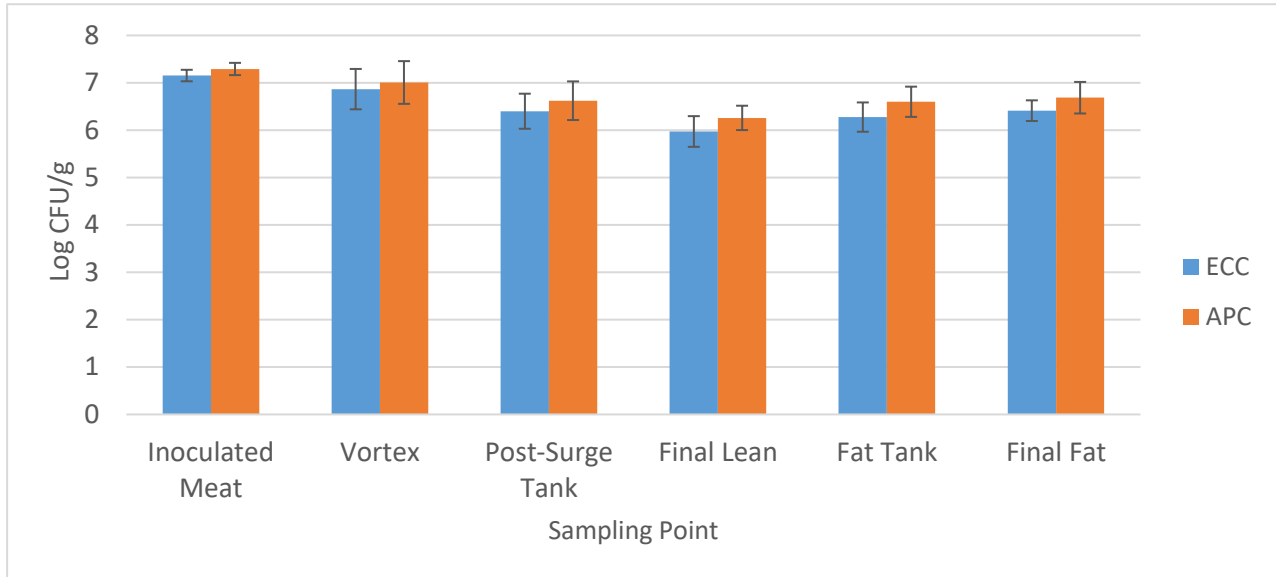


Figure 5.28 Average Recovery of Organisms on Inoculated Meat during Preliminary Study

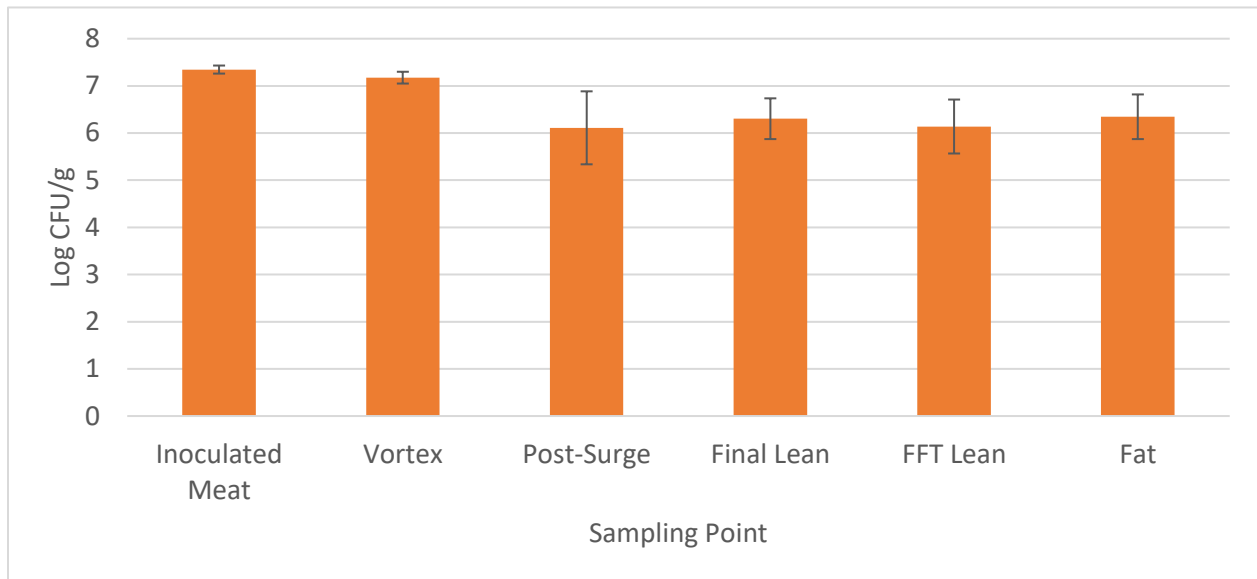


Figure 5.29 Average Recovery of Total Aerobic Plate Counts on Inoculated Meat during Optimized Study

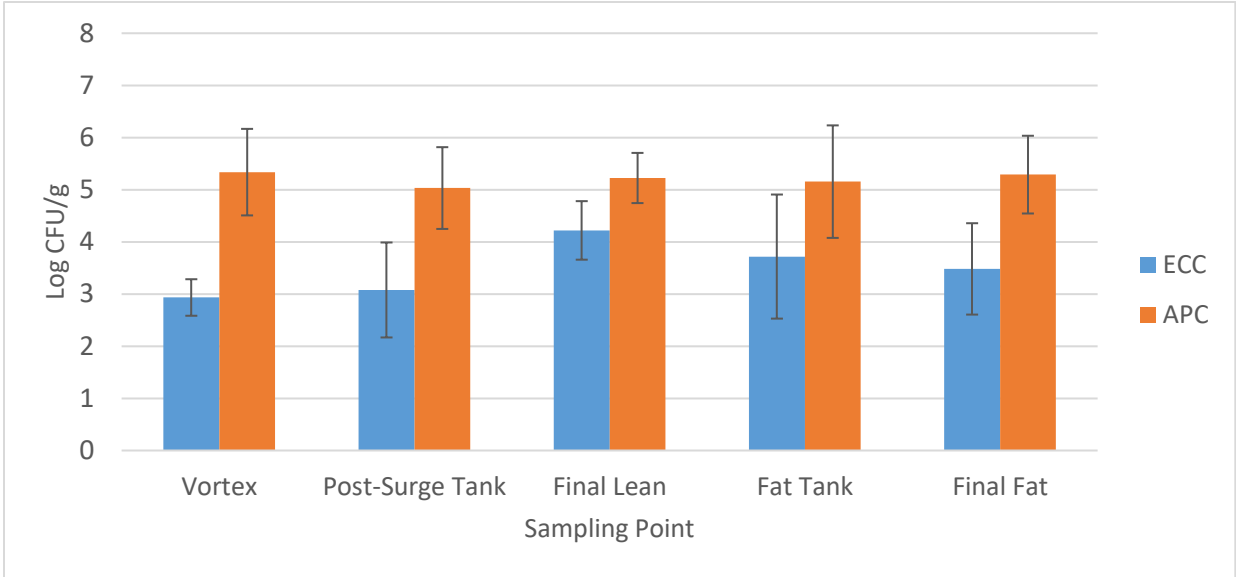


Figure 5.30 Average Recovery of Organisms on Pick-Up Meat during Preliminary Study

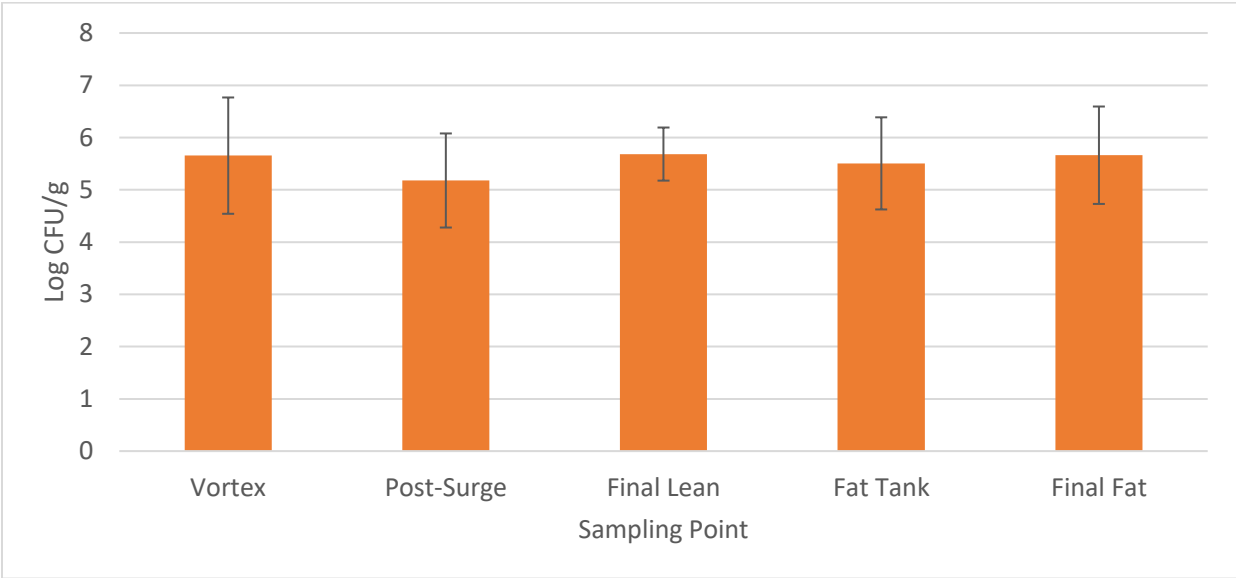


Figure 5.31 Average Recovery of Total Aerobic Plate Counts on Pick-Up Meat during Optimized Study

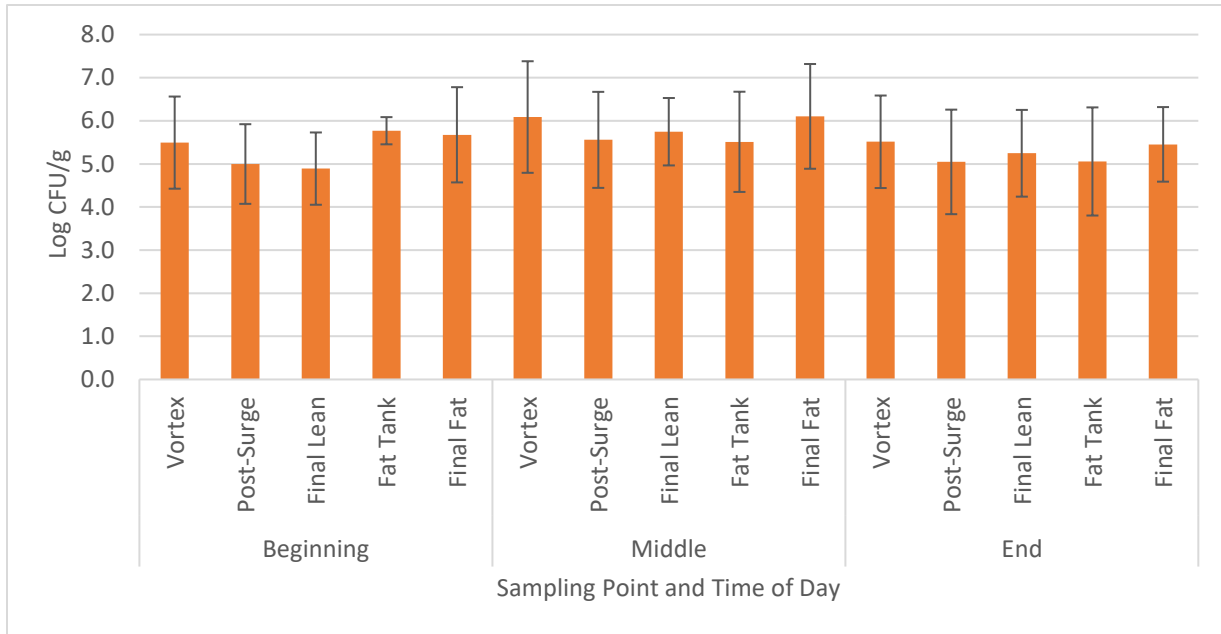


Figure 5.32 Average Recovery of Total Aerobic Plate Counts on 24-hour Pick-Up Meat during Optimized Study

Appendix D - SAS Code

Pure Culture Benchtop Data (Chapter 4.1) Statistical Analysis

```
proc sort data=pc out=pc;  
  by Day Chlorine Organism Temperature pH nanobubbles time;  
run;
```

```
data pc_pre; set pc;  
  if time="pre";  
  pre_selective=selective;  
  pre_injury=injury;  
  drop selective injury time;  
run;
```

```
data pc_post; set pc;  
  if time="post";  
  drop time;  
run;
```

```
data pc2; merge pc_pre pc_post;  
  by Day Chlorine Organism Temperature pH nanobubbles;  
  reduc_s=pre_selective-selective;  
  reduc_i=pre_injury-injury;  
run;
```

```
data pc3; set pc2;  
  drop phnano pre_selective pre_injury selective injury;  
  rename reduc_s= sel  
         reduc_i= inj;  
run;
```

```
proc transpose data=pc3 out=pc4(rename=(Col1=reduc_Name_=Media));  
  by Day Chlorine Organism Temperature pH nanobubbles;  
  var sel inj;  
run;
```

```
proc sort data=pc4;  
  by Day Chlorine Organism Temperature pH nanobubbles media;  
run;
```

```
data pc4; set pc4;
```

```

rename Chlorine = Cl
      Temperature = Temp
      Organism = org
      nanobubbles = nb;
run;

*ods rtf file="C:\Users\Chris\Documents\KSU Consulting\Amanda
Wilder\output_pure_culture.rtf";

title 'Log Reductions (Pre-Post) for Selective Media';
proc mixed data=pc2 plots=none;
  class Day Chlorine Organism Temperature ph nanobubbles;
  model reduc_s=Chlorine|Organism|Temperature|ph|nanobubbles/ddfm=kr;
  random Day Day*Chlorine Temperature*Day(Chlorine);
  lsmeans Chlorine|Organism|Temperature|ph|nanobubbles/pdiff=all;
  ods output lsmeans=lsm_select diffs=diff_select tests3=test_select;
run;

title 'Log Reductions (Pre-Post) for Injury Media';
proc mixed data=pc2 plots=none;
  class Day Chlorine Organism Temperature ph nanobubbles;
  model reduc_i=Chlorine|Organism|Temperature|ph|nanobubbles/ddfm=kr;
  random Day Day*Chlorine Temperature*Day(Chlorine);
  lsmeans Chlorine|Organism|Temperature|ph|nanobubbles/pdiff=all;
  ods output lsmeans=lsm_injury diffs=diff_injury tests3=test_injury;
run;

title 'Log Reductions (Pre-Post) for Both Media';
proc mixed data=pc4 plots=none;
  class Day Cl org temp ph nb media;
  model reduc=Cl|Org|Temp|ph|nb|media/ddfm=kr;
  random Day Day*Cl Temp*Day(Cl) Org*pH*nb*Day(Cl*Temp);
  lsmeans Cl|Org|Temp|ph|nb|media/pdiff=all;
  ods output lsmeans=lsm_media diffs=diff_media tests3=test_media;
run;
quit;

*ods rtf close;

proc export
  data=work.lsm_select
  dbms=xlsx
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\select.xlsx"

```



```
replace;  
sheet="LSMeans Select";  
run;
```

```
proc export  
  data=work.diff_select  
  dbms=xlsx  
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\select.xlsx"  
  replace;  
  sheet="Diffs Select";  
run;
```

```
proc export  
  data=work.test_select  
  dbms=xlsx  
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\select.xlsx"  
  replace;  
  sheet="Tests Select";  
run;
```

```
proc export  
  data=work.lsm_injury  
  dbms=xlsx  
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\injury.xlsx"  
  replace;  
  sheet="LSMeans Injury";  
run;
```

```
proc export  
  data=work.diff_injury  
  dbms=xlsx  
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\injury.xlsx"  
  replace;  
  sheet="Diffs Injury";  
run;
```

```
proc export  
  data=work.test_injury  
  dbms=xlsx  
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\injury.xlsx"  
  replace;  
  sheet="Tests Injury";  
run;
```

```
proc export
  data=work.lsm_media
  dbms=xlsx
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\media.xlsx"
  replace;
  sheet="LSMeans Media";
run;
```

```
proc export
  data=work.diff_media
  dbms=xlsx
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\media.xlsx"
  replace;
  sheet="Diffs Media";
run;
```

```
proc export
  data=work.test_media
  dbms=xlsx
  outfile="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\media.xlsx"
  replace;
  sheet="Tests Media";
run;
```

In-Plant (Optimized Study) Statistical Analysis (Chapter 5.4)

```
PROC IMPORT OUT= WORK.innoc
  DATAFILE= "C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\A. Wilder In-
  Plant Data Vahl.xlsx"
  DBMS=xlsx REPLACE;
  sheet="Freeze";
RUN;
```

```
PROC IMPORT OUT= WORK.vortex
  DATAFILE= "C:\Users\Chris\Documents\KSU Consulting\Amanda
  Wilder\InPlantData.xlsx"
  DBMS=xlsx REPLACE;
  sheet="PickUp";
RUN;
```

```
proc sort data=innoc out=innoc;
  by rep trt;
run;
```

```
proc sort data=vortex out=vortex;
  by rep trt ;
run;
```

```
ods rtf file="C:\Users\Chris\Documents\KSU Consulting\Amanda Wilder\output_inplant.doc"
style=journal;
```

```
proc mixed data=innoc plots=none;
  class rep trt sample;
  model ecc = trt/ddfm=KR;
  random rep;
  repeated/group=sample;
  lsmeans trt/pdiff adjust=Tukey;
run;
```

```
proc mixed data=innoc plots=none;
  class rep trt sample;
  model apc = trt/ddfm=KR;
  random rep;
  repeated/group=trt;
  lsmeans trt/pdiff adjust=Tukey;
run;
```

```
proc mixed data=vortex plots=none;
  class trt rep sample;
  model ecc=trt | sample/ddfm=kr;
  random rep;
  repeated/subject=rep*trt type=csh;
  lsmeans trt | sample/pdiff adjust=tukey;
  lsmeans trt*sample/slice=trt;
  lsmeans trt*sample/slice=sample;
run;
```

```
proc mixed data=vortex plots=none;
  class trt rep sample;
  model apc=trt | sample/ddfm=kr;
  random rep;
  repeated/subject=rep*trt type=cs;
  lsmeans sample/pdiff adjust=tukey;
run;
```

```
ods rtf close;
```

Appendix E - Aquaox 5000™ SDS



Safety Data Sheet AQUAOX DISINFECTANT 5000

SECTION I – IDENTIFICATION

Product Name: Aquaox Disinfectant 5000 | Product Number: AX5000
Product Description: Mixed Oxidant Water, 0.4 – 0.5% solution, generated Electro-
Chemically from Diluted Brine

Container Size: 275 Gallons 30 Gallons
5 Gallons 1 Gallon

CAS Number: None (Mixture)

Recommended Use: This product is an NSF60 certified liquid oxidant for use in all water systems including drinking water systems to restore chlorine residuals and remove reaction by-products. The maximum use limit for water treatment is 1510ppm (0.15%).

Restricted Use: This product is not for human or animal use.

Manufacturer: Aquaox LLC
Address: 220 S. Second Street
Dillsburg, PA
17019

Number: (800) 790-7520
Chemtrec Emergency Number:(800)-424-9300

SECTION II – HAZARDS IDENTIFICATION

The following values are obtained using the guidelines prepared by the National Fire Protection Association (NFPA) and the American Coatings Association.

- HMIS Rating:
- Health = 1
 - Flammability = 0
 - Physical = 0

- Reactivity = 0

NFPA/HMIS Definitions

0 = Minimal Hazard

1 = Slight Hazard

2 = Moderate Hazard

3 = Serious Hazard

4 = Severe Hazard

Personal Protection Index: B (Eye Protection and Gloves)

Hazard Information Disclosures:

TSCA: All chemicals in this product are listed on the EPATSCA inventory list.

CERCLA / SARA: This product does not fall under any hazardous categories under SARA Sections 311 and 312.

OSHA: This product is not a hazardous chemical as defined by the OSHA Hazard Communication Standard, 29 CFR § 1910.1200.

Product Label on Hazard Information:



**WARNING Harmful if Ingested Irritant (Skin and Eye)
Cause Irritation to Mucous Membranes and Respiratory
Tract if Prolonged or Repeated Inhalation**

**Handle with Gloves and Safety Glasses Wash Hands
after Handling Product Keep out of Reach of Children**

**SECTION III –
COMPOSITION AND INFORMATION ON INGREDIENTS**

<u>Component(s)</u>	<u>CAS #</u>
Water	7732-18-5
Hypochlorous Acid	7790-92-3
Sodium Hypochlorite	7681-52-9
Sodium Chloride	7647-14-5

The product contains approximately 5000ppm free available chlorine (FAC).

**SECTION IV –
FIRST-AID MEASURES**

Skin Contact: In case of contact, flush with plenty of water. Cold water may be used. Wash clothing before reuse. Seek medical attention if skin irritation occurs.

Eye Contact: Check for and remove any contact lenses. Flush eyes with running water for at least 15 minutes with eyelids open. Cold water may be used. Seek medical attention if eye irritation occurs.

Inhalation: If inhaled, remove to fresh air. Seek medical attention if not breathing or breathing is difficult.

Ingestion: If swallowed, rinse mouth with water and drink plenty of fluids. Seek medical attention if discomfort occurs.

**SECTION V –
FIRE-FIGHTING MEASURES**

Not Applicable, this product is Non-Flammable and Non-Explosive. No extinguishing techniques or equipment are required.

**SECTION VI –
ACCIDENTAL RELEASE MEASURES**

In case of spill or leakages, dike spill with inert absorbent materials (e.g. sand, “oil-dry” or other commercially spill absorbents) to contain and soak spilled liquid. Place wastes into an appropriate waste disposal container.

**SECTION VII –
HANDLING AND STORAGE**

Handling: No special handling requirements; follow use instructions on product label. Open air or good room ventilation and appropriate PPE are adequate for the safe use of this product.

Storage: Keep container tightly closed in a dry and well-ventilated place at room temperature. Avoid light exposure, freezing and heat.

**SECTION VIII –
EXPOSURE CONTROLS AND PERSONAL PROTECTION**

OSHA PEL: Unknown.

Cal/OSHA PEL: Unknown.

NIOSH REL: Unknown.

ACGIH TLV: Unknown.

Engineering Control: None Required. Open air or good room ventilation is adequate for the safe use of this product.

Personal Protective Equipment (PPE):

Protective Clothing: Wear proper personal protective equipment and clothing to care for spill situation. Product can bleach clothing, equipment and furniture.

Hand Protection: Handle with gloves; use rubber, neoprene, or other chemically impervious gloves when handling this product.

Eye Protection: Wear safety glasses when handling this product.

**SECTION IX –
PHYSICAL AND CHEMICAL PROPERTIES**

Physical State: Liquid
Color: Clear
Odor: Slight Chlorine Odor pH: 8.0 – 9.5
Specific Gravity (H₂O = 1 at 20°C): 1.06 – 1.08
Viscosity: Comparable to Water Boiling Point:
Comparable to Water Melting Point / Range: NA
Evaporation Rate: Comparable to Water
Solubility: Complete in Water
Flash Point: NA
Flammability: Non-Flammable
Explosive Limits: Non-Explosive
Vapor Pressure (mmHg @ 20°C): NA
Vapor Density: NA

**SECTION X –
STABILITY AND REACTIVITY**

Reactivity: Not Reactive under recommended handling and storage conditions.
Chemical Stability: Stable under recommended handling and storage conditions.
Hazardous Reactions: Product is Not Hazardous.
Conditions to Avoid: Direct light exposure, freezing and heat.
Materials to Avoid Strong oxidizing agents, strong acids and organic materials.
Hazardous Decomposition Products: May form under fire conditions; nature of decomposition products is unknown.
Hazardous Polymerization: Will not occur.

**SECTION XI –
TOXICOLOGICAL INFORMATION**

Route of Entry / Exposure: Skin Contact

Eye Contact

Inhalation

Ingestion

Potential Acute Health Effects:

Skin Contact: Depending on the degree of unprotected contact and individual sensitivity with the product, skin contact may cause irritation, redness, rash and itchiness.

Eye Contact: Depending on the duration and individual sensitivity with the product, unprotected eye contact may cause moderate eye irritation, pain, redness, watering and itchiness.

Inhalation: Excessive, prolonged or repeated inhalation may cause irritation to mucous membranes and upper respiratory tract.

Ingestion: This product may be harmful if ingested. Depending on the amount swallowed, ingestion may cause irritation to mouth, throat, stomach and gastrointestinal tract.

Potential Chronic Health Effects:

Carcinogenic Effects: Not Applicable.

Mutagenic Effects: Not Applicable.

Teratogenic Effects: Not Applicable.

Developmental: Not Applicable.

Numerical Measures of Toxicity: Unknown.

**SECTION XII –
ECOLOGICAL INFORMATION**

Product presents no hazards to the environment. Product is bio-degradable and eco-friendly.

**SECTION XIII –
DISPOSAL CONSIDERATIONS**

Dispose of in accordance with all applicable Federal, State and Local regulations. Always contact a licensed disposal company to assure compliance.

**SECTION XIV –
TRANSPORT INFORMATION**

DOT: Not DOT regulated. No DOT label required.

IATA: Not dangerous good.

IMDG: Not dangerous good

OSHA: No label required.

**SECTION XV –
REGULATORY INFORMATION**

See “Hazard Information Disclosures” under Section II.

**SECTION XVI –
OTHER INFORMATION**

Preparation Date of Latest Revision: May 15, 2015

Disclaimer:

This Safety Data Sheet (SDS) was prepared in accordance with the provisions and requirements of 29 CFR § 1910.1200(g) and discloses the physical and health hazards of all hazardous chemicals contained in the product described in this SDS. Unless otherwise noted, this SDS does not describe or disclose all of the chemicals/components in the product, some of which may be Trade Secrets.

The information included in this SDS is based on data developed or compiled by Aquaiox from open literature, independent laboratory studies, and other available scientific evidence, and is believed to be accurate and complete to the best of our knowledge. However, Aquaiox makes no warranty with respect thereto. Anyone intending to use the product described in this SDS should satisfy herself that the Product (1) is suitable for their particular purposes and intended uses, and (2) meets any safety and health standards applicable thereto. It is the obligation of each user of the product described in this SDS to determine and comply with all statutes, local, state and federal requirements, which are applicable to its use, storage and disposal.

Abbreviations:

ACGIH: American Conference of Industrial Hygienists
CAL/OSHA: California Division of Occupational Safety and Health
CAS Number: Chemical Abstracts Service Register Number
CERCLA: Comprehensive Environmental Response Compensation and Liability Act
DOT: Department of Transportation
EPA: Environmental Protection Agency
GRAS: Generally Recognized as Safe
HMIS: Hazardous Materials Identification System
IATA: International Air Transport Association
IMDG: International Maritime Dangerous Goods
NA: Not Applicable
NIOSH: National Institute for Occupational Safety and Health
OSHA: Occupational Safety and Health Administration
PEL: Permissible Exposure Limits
REL: Recommended Exposure Limits
SARA: Superfund Amendment and Reauthorization Act of 1986
TLV: Threshold Limit Values
TSCA: Toxic Substances Control Act of 1976