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Screening of Commercially Available Chlorine Based Sanitizers and their Efficacy in Reducing Microbial Load Levels of *E. coli* O157:H7 at High and Low Organic Load Environments

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**SCREENING OF COMMERCIALY AVAILABLE CHLORINE BASED SANITIZERS
AND THEIR EFFICACY IN REDUCING MICROBIAL COUNTS OF *E. coli* O157:H7
AT HIGH AND LOW ORGANIC LOAD ENVIRONMENTS**

A Thesis Presented

By

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**SCREENING OF COMMERCIALY AVAILABLE CHLORINE BASED
SANITIZERS AND THEIR EFFICACY IN REDUCING MICROBIAL
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ABSTRACT

SCREENING OF COMMERCIALY AVAILABLE CHLORINE BASED SANITIZERS AND
THEIR EFFICACY IN REDUCING MICROBIAL LOAD LEVELS OF *E. coli O157:H7* AT
HIGH AND LOW ORGANIC LOAD ENVIRONMENTS

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The presence of postharvest sanitizers has shown to be an effective approach to reducing microbial cross contamination in agricultural washing operations. However, choosing an appropriate sanitizer can be challenging due to produce commodity, processing conditions and interference with organic load. Current research shows a wide variety of methods to mimic the organic load of vegetable processing conditions, with paddle mixing and blender as the most commonly used. Controlling and understanding the physiochemical properties of wash water is key in maintaining sanitizer efficacy. The effects of simulated wash water preparation method on the physiochemical properties were tested at 0 and 50 COD (mg/L) and no significant difference was observed. However, at high levels of organic load results showed a significant difference between turbidity values at 1,500 COD. Free residual chlorine titration methods were compared,

using DPD-titrimetric and Iodometric method. Results showed a significant difference between titration methods in organic load heavy environments. Commercially available chlorine based sanitizers, Pure Bright™ Germicidal Bleach and Clorox® Germicidal Bleach, were compared to a concentrated solution of sodium hypochlorite. Pure Bright™ Germicidal Bleach showed to perform the best by reducing 7 log CFU/ml of *E. coli O157:H7* after 30 seconds in no organic load environments, whereas Clorox Germicidal bleach was able to reduce 7 log CFU/ml of *E. coli O157:H7* after 30 minutes. These studies aim to provide best management practices for small in medium growers in the implementation of antimicrobial solutions for the maintenance of water quality in postharvest washing solutions.

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LIST OF ABBREVIATIONS

CDC-Centers of Disease Control

TSB-Tryptone Soy Broth

TSA- Tryptone Soy Agar

NAL- Nalidixic Acid

FSMA-Food Safety Modernization Act

COD- Chemical Oxygen Demand

NTU- Nephelometric Turbidity Unit

ORP- Oxygen Reduction Potential

CFU-Colony Forming Units

FDA- Federal Drug Administration

EPA-Environmental Protection Agency

USDA-United States Department of Agriculture

PB- Pure Brigh™ Germicidal Bleach

SH: Sodium Hypochlorite

GB: Clorox® Germicidal Bleach

CHAPTER 1

INTRODUCTION

Fresh produce continues to be the leading food associated to foodborne outbreaks, where linked pathogens include *Escherichia coli* O157:H7, *Salmonella spp* and *Listeria monocytogenes* (Callejon et.al 2015). Foodborne illness associated with the consumption of fresh-cut produce in the United States have reached over 45 percent (Gombas et. al, 2017). Since fresh produce are a ready to eat product, the absence of a kill step increases the potential of pathogenic cross contamination, and thus the risk associated with their consumption could be minimized through good agricultural postharvest practices (Ghostlaw, Ramos, Kinchla, 2018). With the implementation of the Food Safety Modernization Act (FSMA) and the establishment of the “Produce Rule”, now requiring science-based minimum standard for the safe growing, harvesting, packing, holding and handing of fruits and vegetables for human consumption, has increased the need for sanitizer validation studies to ensure food safety. As stated by the Federal Drug Administration (FDA) in the “Produce Rule”, postharvest agricultural wash water is required to have no presence of generic *E. coli*. Not only is postharvest washing a method used for cooling produce after harvest, it is also a way of washing and removing soil and debris. However, it does not provide sufficient removal of microorganisms which increases the potential transfer of the pathogenic bacteria throughout the whole washing solution; Therefore, water maintenance is key in reducing microbial load for potential pathogenic cross-contamination (Joshi, Mhendran, Alagusundaram, Norotnm Tiwari, 2013).

While sanitizer has proven to be a good tool in reducing the potential of cross contamination in postharvest wash water (Luo, Nou, Millner, Zhou, Shen, Yang, and Shelton, 2012), the wide range of processing methods used to generate simulated wash water makes it

challenging to make comparison between sanitizer validation studies. Besides the preparation method, produce commodity used in the studies can also play a role in the changes of the physicochemical characteristics of the solutions, potentially providing large scatter in the results for sanitizer concentration and their efficacy in presence of organic matter (Holvoet et al., 2012; Callejon et al., 2015; Gil et al., 2015; Sharma and Reynnells 2016). Our goal with this study is to be able to provide guidance on the implementation of sanitizers for small and medium leafy green processors, specifically on the use of chlorine based sanitizers for produce washing and food contact surfaces. Before testing the sanitizers, we must first understand how the preparation methods can affect the physicochemical properties of the simulated wash water, and how the detection method used for testing free residual chlorine levels in solution can affect the overall results of a study when looking at the depletion behavior of chlorine in a high organic load environment.

1.1 Objectives

1. To compare preparation methods and test their effects on the physicochemical properties of simulated wash water solutions.
2. To investigate the impact of organic load and bacteria on free residual chlorine detection methods in simulated wash water.
3. To study the effects of organic load and time on the efficacy of commercial chlorine based sanitizers on *E. coli* O157:H7 inactivation in simulated wash water conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Recent outbreaks associated with fresh fruits and vegetables have resulted in an increased interest in improved on-farm food safety practices. In light of the implementation of “The Food Safety Modernization Act” (FSMA) and the Produce Safety Rule, agricultural wash water is now required to have no detectable generic *E. coli*, which can indicate the potential presence of fecal contamination (Food & Drug Administration [FDA], 2017). While postharvest washing helps to remove field heat, soil and debris from produce this process can be a source of cross-contamination if water quality is not adequately maintained with the potential of becoming a vector for the spread of pathogens (FDA, 2008). The addition of an antimicrobial solution to agricultural wash water is a known practice that can reduce cross contamination of pathogens, such as *E. coli* O157:H7, *Listeria monogytogenes* and *Salmonella* spp. (Luo, Nou, Millner, Zhou, Shen, Yang, & Shelton, 2012).

The use of sanitizing agents in wash water has proven to be a good means to ensure and control water quality. However, choosing an appropriate sanitizer for a vegetable processing operation can be challenging due to the open nature of the farm processing operations, as well of the produce itself (Holvoet et al., 2012; Callejon et al., 2015; Gil et al., 2015; Sharma and Reynnells 2016). A survey by the Mid-Atlantic region stated that 47% of growers wash their produce first with just water and another 22.4% wash their produce with some sort of disinfectant (Marine, Martin, Adalja, Mathew & Everts, 2016). One of the problems stemming from the implementation of the Produce Rule is the lack of guidance provided for small and medium growers as to how to use and

implement sanitizers that best fit their processing operations. Therefore, it is critical to first understand what properties of processing water have the strongest effects on sanitizers' efficacy in preventing cross contamination (Gil, Selma, López-Galvez & Allende, 2009). However, the wide range of processing methods being used to simulate wash water in a laboratory setting make it challenging to make comparisons between sanitizer validation studies. Not only are there a variety of processing methods, there is also a wide range of produce commodities being used to produce desired organic load levels. High organic load levels present in wash water can cause an increase in potential pathogens transfer to uncontaminated plants (Gombas et al., 2017; Allende, Selma, López-Gálvez, Villaescusa & Gil, 2008), due to the accumulation of organic load causing the sanitizer quenching capacity to decrease and thus affect its sanitizing capacity (Beuchat et al., 2001). Due to the scatter approach, the capacity to compare the efficacy of different postharvest sanitizer studies is limited. The lack of a standard model for laboratory replication of simulated wash water, making it difficult to compare previous work on sanitizer efficacy, in presence of high and low organic load as well as microbial counts, and does not provide a clear guide for growers to implement such practices in their processing operations.

There is a wide range of research conducted on chlorine and chlorine based sanitizers. This work focuses on the efficacy of commercially available products in presence of organic matter, and their ability of reducing pathogenic cross-contamination in wash water. The majority of previous published work has been done using a concentrated solution of sodium hypochlorite as a model for chlorine sanitizers. Most commercial chlorine based sanitizer use sodium hypochlorite as an active disinfectant ingredient, however this only makes up a small percentage of the solution and the rest is just labeled "other ingredients". Products like Pure Bright™ and Clorox® Germicidal bleach where sodium hypochlorite only makes up 6.00% and the other 94% is "other

ingredients”. The lack of validation works available on commercial chlorine based system makes it challenging to compare and recommend best management practices on the implementation of sanitizer in postharvest wash water for small and medium growers.

2.2 Physiochemical properties of wash water

Previous and current work have utilized a myriad of different measurements of water quality in attempts of quantifying the effects of organic matter on sanitizers. Measurements such as Turbidity (NTU), Chemical Oxygen Demand (COD), Oxidation Reduction Potential (ORP), pH, Biochemical Oxygen Demand (BOD) and most recently UV254 have been proposed and previously used (Barrera, Blenkinsop, & Warriner, 2012; Luo et al., 2011; Selma, Allende, Lopez-Galvez, Conesa & Gil, 2008; Suslow, 2004; Chen & Hung, 2016). While these have provided useful measurements of water quality, they all have their limitations and will not be equally effective under different processing conditions. For our work we mainly focused on four characteristics, these being COD, turbidity, ORP and pH; where we used both COD and turbidity as our methods for quantifying organic matter in our solutions.

Chemical Oxygen Demand and turbidity are two of the most common methods used in research as indicators of organic load in simulated wash water. Turbidity is a measure of the particulate present in water, which can be composed of organic and inorganic particles and also plant material and it is reported in Nephelometric Turbidity Unit (NTU) (World Health Organization, 2006). COD is a measurement of the amount of oxygen required to oxidize soluble organic matter in solution (Luo, 2007). In laboratory use, a COD test involves the the introduction of a strong oxidizer in excess into the test sample to oxidize the organic matter in solution to carbon dioxide and water under acidic conditions. This allows for the quantification of organic matter

degradation by measuring the organic material in solutions that has the capacity of being oxidized (Rice, Bridgewater, & American Public Health Association 2012).

Oxidation Reduction Potential (ORP) is a measure of the the relative intensity of the electron activity in solution (Rice, Bridgewater, & American Public Health Association, 2012). This means is that ORP can be used as a measure for water quality, which allows for the monitoring of antimicrobials solutions levels in a postharvest wash water system (Suslow, 2004). However, a limitation with the use of ORP is that it is only feasible for a system that use a chlorine based sanitizer because of its ability to be a strong oxidizer. While ORP measurements are a rapid and single value assessment tool for the disinfection potential of an antimicrobial solution (Suslow, 2004), readings can be affected by the pH and temperature of the washing system, as well as the presence of organic matter (Rice, Bridgewater, & American Public Health Association 2012).

pH is as quantitative measure of the acidity or basicity of a solutions. Understanding the pH of a washing system can help determine the optimum conditions for antimicrobial solutions to be added. For example, the optimum pH for a chlorine as a produce sanitizer is in the 6.5-7.5 range to achieve the greatest antimicrobial effectiveness (Gombas et. al, 2017). The introduction of organic matter to solution can disrupt the pH of the water, causing the efficacy of the sanitizer to be affected.

2.3 Preparation methods for simulated wash water

To effectively test the efficacy of produce washing sanitizers we must conduct studies simulating on farm conditions of wash water with adequate and realistic loads of organic material. The amount and type of organic load plays an important role in sanitizer depletion (Gombas et. al, 2017), and thus the most critical attribute to mimic in order to effectively test sanitizer disinfecting

capacity. Organic matter degrades overtime through biochemical reactions, where in large surface areas the presence of high organic load levels is less likely (Chaulk and Sheppard, 2011). However, in produce washing systems where there is a much smaller surface area, there can be an influx of organic matter constantly being introduced to the water causing a more rapid increasing of organic load levels (Ghostlaw, Ramos, Kinchla, 2018). Upon comparing research studies, a wide range of preparation methods for simulated wash water was observed with the most common methods being a paddle mixer and a blender. **Table 1** showcases examples of the range of preparation methods used for generating simulated wash water using leafy greens. In order to develop a standard model, we must first understand how the preparation method used to replicate organic load for simulated wash. Mechanical methods for breaking down the vegetative material can affect the physicochemical properties of the simulated wash water. Blenders have the ability to completely homogenize and breakdown the sample which allows for the inner cellular components of the produce to be in solution. On the other hand, a paddle mixer only has the ability to breakdown the material partially which when compared side by side with a blender, can cause differences in the organic load characteristics (Ghostlaw, Ramos, Kinchla, 2018). Besides the nature of the processing method, the preparation of the produce prior to creating simulated wash water can also affect the physicochemical characteristics of the water. For example, the removal of outer layers of produce, like lettuce and cabbage, removes any residual dirt which may be present, which is an essential step for decreasing any possible environmental contamination being introduced into solution. Chemical coatings and waxes can also affect the organic load values and potentially give incorrect COD for example (Baur, Klaiber, Hammes, Carle, 2004; Harris, Beauchat, Kajs, Ward, Taylor, 2011). In commodities such as spinach, that do not have outer layers to remove, any sanitizer residue present in the surface of the leaves will be introduced into water model. In order

to create a standard preparation model, we need to first understand the physicochemical properties of the wash water and how these can vary by produce which can be seen in **Table 2**. Differences between COD and turbidity values can be observed between different commodities.

Table 1: Comparison of simulated wash water preparation methods from previous work

Purpose	Organic load method	Produce Model	Source
Evaluate minimum free residual chlorine levels required to inactivate <i>E. coli</i> O157:H7 and potential for THM generation in spinach, simulating dynamic washing conditions.	Stomacher	Spinach	Gómez-López et. al., (2014)
Testing efficacy of chlorine dioxide and sodium hypochlorite in <i>E. coli</i> inactivation in process water after cross-contamination in pre-washing tank	Knife	Lettuce	López-Gálvez, Gil, Truchado, Selma & Allende (2010)
Evaluate the efficacy of electrolyzed water in combination with salt on <i>E. coli</i> O157:H7 inactivation in vegetable washing systems	Stomacher	Iceberg Lettuce	Gómez-López et al.(2015).
Inactivation of <i>Salmonella</i> , <i>E. coli</i> O157:H7, and non-O157 STEC in chlorinated solutions in varying concentrations of free chlorine	Juicer to create extract	Lettuce and Tomatoes	Shen, Luo, Nou, Wang, & Millner (2013)
Investigate the of <i>E. coli</i> O157:H7 from inoculated lettuce leaves to inoculated pieces during washing and the efficacy of PAA and chlorine sanitizers in reducing the transfer of <i>E. coli</i> O157:H7	High Speed Blender	Lettuce	Zhang, Ma, Phelan & Doyle (2009)
Testing the efficacy of chlorine treatments against <i>E. coli</i> O157:H7 during pilot-plant scale processing of iceberg lettuce and assessing the relationship between the physiochemical parameters of wash water and <i>E. coli</i> O157:H7 inactivation	Blender	Iceberg Lettuce	Davidson, Kaminski, & Ryser (2014)
The use of chlorine in water disinfection strategies in maintain	Stomacher	Butter head lettuce	Van Haute, Sampers, Holvoet & Uyttendaele, M. (2013)

microbial wash water quality without targeting the fresh-cut lettuce			
Investigate the effect of reusing wash water on the changes of water quality and the effect of water quality and microbial growth of packaged romaine lettuce	Knife	Romaine Lettuce	Luo, Y. (2007)

Table showcases the range of methods used to prepare the simulated wash water solutions, as well as the range in produce used.

It is critical to test sanitizers in presence of varying organic load levels. This to best understand how sanitizers would perform in a farm processing operation, and also accounting for the sanitizers quenching capacity (Gonzalez, Luo, Ruiz-Cruz & Cevoy, 2004). There are a variety of factors that make scaling up of laboratory research into industry application challenging, which could be eased with the implementation of a standardized organic load replication method (Beauchat et al 2001; Gil et al., 2009; Gombas et al., 2017). Standardizing a preparation method to replicate organic load seen in industry and on farm wash water will help to provide a controlled an appropriate environment for sanitizer validation research.

Table 2: Physiochemical properties of simulated wash water for varying produce commodities

Produce	Time of measurement	COD (mg/L)	pH	Turbidity (NTU)	ORP (mV)	Source
Lettuce	2hr	218.6	7.2	87.4		Selma, et al., (2008)
Escarole	2hr	173.6	7.3	95.7		
Chicory	2hr	33	7.8	42.4		
Carrot	2hr	18	7.6	0.6		
Onion	2hr	747.3	7.1	5040.4		
Spinach	2hr	68	7.5	88.9		
Sugar snap peas	Approximately 1hr	30± 5	8.0 ± 0.1	5.2 ± 1.1		Van Haute, Uyttendaele, Sampers (2013)
Iceberg Lettuce	2-3 hr	119				Baur, Klaiber, Hammes & Carle (2004)
Lettuce	3 hr	2550	5.6		868	Davidson et al. 2014
Iceberg lettuce (Company 1)	2hr	465 ± 2	7.34 ± 0.01	13.8 ± 0.9		Van Haute, Sampers, Holvoet & Uyttendaele, (2013)a
Iceberg lettuce (Company 2)	2hr	1,405 ± 57	7.2 ± 0.1	72.6 ± 6.6		
Spinach (Facility A)	5-8hr		7.33±2.19	0.058±0.053	N/A	Barrera et al., 2012
Spinach (Facility B)	4-8hr		7.53±0.11	0.036±0.036	383±127	
Spinach (Facility C)	30hr		7.47 ±0.26	0.123 ± 0.27	598 ±152	
Tomato (Facility A Primary Tank)	4hr	390	7.0–7.5	38	950	Zhou et al., 2014
Tomato (Facility B Primary Tank)	8hr	732	5.5-6.5	74.90	1100	
Tomato (Facility C Primary Tank)	4hr	519.5	6.5–7.0	107.0	870	

Table sourced from: Ghostlaw, Ramos, Kinchla (2018). This tables gives a brief overview of the methods that are used to measure the physiochemical properties of water that are used to establish water quality and the limitations to these tests.

2.4 Chlorine kinetics and detection methods

Chlorine is a disinfectant most commonly used for water and wastewater treatments. When added to water, hydrochloric and hypochlorous acids are formed, where the hypochlorous acid (HOCl) is the “bleaching” or disinfectant capacity of the reaction seen in the **Figure 1**.

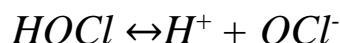
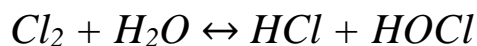


Figure 1: Sodium hypochlorite reaction with water

The two chemical species formed by chlorine in water are hypochlorous acid, HOCl, and hypochlorite ion, OCl⁻, and are defined as free available or free residual chlorine (Gombas et. al, 2017). These two compounds have a disinfection ability, and are key for controlling microbial loads in both wash water and food contact surfaces when using chlorine based systems. In solutions with a pH ranging between 6.5 and 8.5, both species will be present with HOCl is the more germicidal of the two (Harp, 1995).

Studies have shown that HOCl is the most effective form of chlorine when it comes to inactivating pathogens (Luo et al., 2012). However, maintaining adequate levels of free available chlorine can be challenging in produce washing operations. The deterioration of water quality can be seen due to the accumulation of soil, debris and plant particles during processing, which causes an increase in both turbidity and COD, and thus a decrease in sanitizer efficacy (Luo et al., 2012). The longer the organic matter sits in the wash water, free available chlorine levels will continue to deplete to the point of no chlorine available for disinfection. Any pathogenic bacteria present will be able to survive and spread all throughout the wash water causing potential cross contamination to uncontaminated produce. In large fresh produce processing facilities, using chlorine as a

sanitizer, periodic monitoring and replenishment of chlorine is a common practice. However the continuous addition of chlorine into high organic load solution can generate noxious chlorine by-products and chlorine off-gassing (Cornell 1996; Suslow, 2001). It is critical for a sanitizer, like chlorine, to be tested in presence of different levels of organic load, even with small-scale studies, to better understand the efficacy and availability of free residual chlorine over time. This will help render more comparable results to on-farm conditions and will account for the sanitizer quenching capacity in processing water (Gonzalez et. al., 2004).

The use of chlorine as a produce sanitizer has been widely studied, due to its widespread use in industry and the availability of ORP probes or systems to monitor chlorine in large wash tanks (Shen et. al, 2013). However, due to the nature of the wash tank, the constant addition of produce and the constant movement of water, reports have shown that ORP readings do not fully reflected the free residual chlorine levels within the wash tank (Devkota et al., 2000; Kim & Hensley, 1997; Zhou et al., 2014). These discrepancies are due to displacement of the chlorine and

water reaction.

There are a variety of analytical methods used to measure chlorine levels in washing systems, both free residual and total. Free residual or free available chlorine represents the amount of chlorine available that has the oxidizing capacity. Whereas total chlorine is the sum of all forms of chlorine in solution.

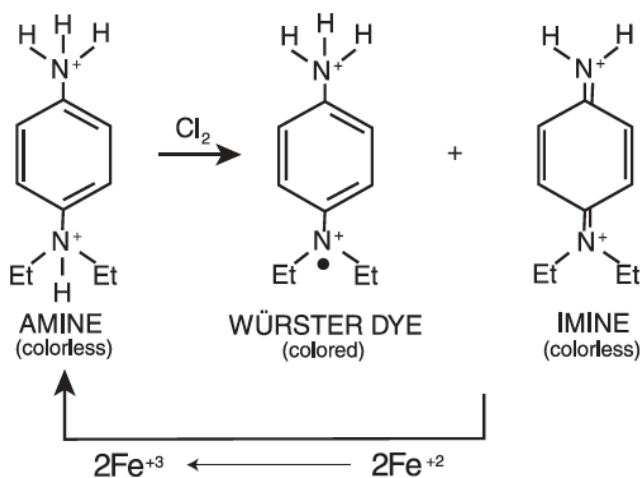


Figure 2: DPD reaction with chlorine (Harp 1995)

N,N-diethyl-p-phenylenediamine (DPD)

methods are the one of the most common methods seen throughout academic research for

quantifying the levels of free residual chlorine in water quality studies. The DPD titration method is based on the chemical reaction where DPD is oxidized by chlorine to create a bright magenta-colored compound, where this compound will then be titrated with a ferrous reducing agent to a colorless endpoint (Harp, 1995) reaction seen in **Figure 2**. Another standard method is an iodometric titration, which is one of the oldest methods for determining chlorine. The reaction is based on the interaction with a sodium thiosulfate solution, where chlorine reacts with potassium iodide and a starch indicator is added to form a starch-iodide complex that is titrated to the endpoint where the blue colored starch-iodide complex disappears (Harp 1995). The Iodometric reaction can be seen in **Figure 3**.

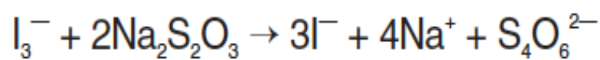
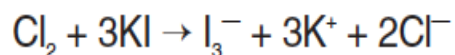


Figure 3: Iodometric Titration Equation (Harp 1995)

Limitation are associated to both methods. For example, detection range poses an issue for the DPD titration and interferences with organic material in solutions causing a disruption in the formation of the Würster dye, which can make the visual detection of the endpoint challenging. Limitations for both methods must be taken into consideration when choosing a method for measuring chlorine specifically when conducting studies for chlorine depletion in wash water and its ability to reduce microbial loads of pathogenic bacteria, since chlorine efficacy can be affected by the organic load present in wash water, pH and contact with metals, such as iron (Ghostlaw, Ramos, Kinchla, 2018).

With such a variety of detection methods for free residual chlorine, it is important to keep in mind which test will work best in high organic load environments. Studies should look at the impact different produce have on detection methods capability and inferences with varying organic load levels for free residual chlorine detection.

2.5 Chlorine Sanitizer Applications

Chlorine has been one of the most heavily studied sanitizers, due to its low cost and efficacy in reducing pathogen cross-contamination. Besides price and efficacy, it can be utilized for both produce washing and food contact surfaces. Commercial brands like Pure Bright™ Germicidal bleach and Clorox® Germicidal bleach are readily accessible at a low cost, but chlorine concentrations in solution may not exceed 25ppm as stated by the Environmental Protection Agency (Environmental Protection Agency [EPA], 2007; EPA 2010). However, even with the availability of commercial available chlorine base antimicrobial solutions it can be seen in previous published work that the use of a concentrated sodium hypochlorite solution as a model sanitizer is common for validation studies. Thus, making it challenging to compare and recommend best management practices on the implementation of sanitizer in postharvest wash water for small and medium growers because if the commercial brands of chlorine sanitizer will perform the same in farm processing conditions as seen in **Table 3**. More studies should be done using commercially available chlorine based sanitizer in efforts to identify the real performance of commercial antimicrobial solutions in reducing pathogenic cross-contamination in high organic load environments.

Table 3: Summary of free residual chlorine detection methods and the sanitizers used in wash water quality studies

Purpose	Chlorine Sanitizer Used	Free residual chlorine detection method	Produce	Reference
Compare efficacy of antimicrobial solutions at various concentrations on cut cilantro.	Sodium hypochlorite, acidified sodium chlorite citric acid sodium chlorite	Chlorine Photometer	Cilantro	Allende, McEvoy, Tao, & Luo (2009)

Evaluate the effects of sanitizer pH and initial chlorine concentration of NaOCl on chlorine demand of different fresh produce wash waters at different organic load.	Sodium Hypochlorite	DPD-FEAS Titration	Romaine lettuce	Chen & Hung (2017)
Investigate the dynamic changes in organic load, pH and free concentration and the relationship between bacterial survival and the real time free chlorine concentration.	Sodium Hypochlorite and phosphoric acid-based acidulant	Automated Analytical Platform	Romaine lettuce Iceberg lettuce Cabbage	Luo, Zhou, Van Haute, Nou, Zhang, Teng & Millner (2018)
Study provided a feasible method for studying sanitizer/bacteria interaction and their effect on free chlorine levels.	Sodium Hypochlorite	DPD-FEAS Photometric	Romaine lettuce	Teng, Luo, Alborzi, Zhou, Chen, Zhang, & Wang (2018)
Pilot-scale evaluation of a new process aid and its impact on enhancing the antimicrobial efficacy of chlorinated water against pathogen survival and cross-contamination.	Sodium Hypochlorite	DPD method using Chlorine Photometer	Iceberg lettuce Spinach	Lou et. al (2012)
Study performed sampling visits within a commercial lettuce processing facility to determine the changes in free chlorine concentration during typical processing activities.	Chlorine- Not specified	Not specified, but monitored with ORP probe	Iceberg lettuce	Murray, Aldossari, Wu & Warriner (2018)
Efficacy of sanitizers to inactivate Escherichia coli O157: H7 on fresh-cut carrot shreds under simulated process water conditions.	Acidified sodium chlorite citric acid-based sanitizer Tsunami 100	Not specified	Carrots	Gonzalez et al. (2004)
The efficacy of chlorine dioxide and sodium hypochlorite was evaluated by assessing <i>E. coli</i> inactivation in process water and fresh-cut iceberg lettuce after cross-	Sodium Hypochlorite	DPD-FEAS Titration	Iceberg lettuce	Lopez-Galvez et al. (2010)

contamination in pre-washing tank.				
Inactivation of <i>Salmonella</i> , <i>E. coli</i> O157:H7, and non-O157 STEC in chlorinated solutions in varying concentrations of free chlorine	Sodium Hypochlorite	Chlorine Photometer	Iceberg lettuce Tomatoes	Shen et al. (2013)

2.6 Conclusion

The need for a standardized preparation method for simulated wash water was seen after reviewing the research work available in agricultural wash water. More comparisons studies are needed, not only between preparation methods, but also between produce used for simulated wash water. More work is needed to best determine the depletion rates of commercially available chlorine sanitizer over long period of time in high organic load environments and conditions mimicking those seen in processing operations, to compare depletion rates observed in research when using concentrated sodium hypochlorite solutions.

CHAPTER 3

ASSESSMENT OF PREPARATION METHODS TO PRODUCE A POSTHARVEST WASH WATER MODEL FOR FOOD SAFETY VALIDATION STUDIES

3.1 Introduction

With the implementation of “The Food Safety Modernization Act” there has been an increase in food safety research, specifically produce safety, in efforts to better understand the needs of produce processing operations in compliance with FSMA’s rule regarding the presence of generic *E. coli* in postharvest agricultural wash water. Sanitizers have been proven to be an effective tool in maintaining water quality, however choosing an appropriate sanitizer for vegetable processing operations can be challenging. Mimicking farm wash water conditions is key to assessing sanitizer behavior at high organic load concentrations. Current research shows a wide range of different preparation methods to model organic load levels to mimic on-farm conditions. However, different produce can potentially affect the physicochemical properties of the wash water making it challenging to make comparisons on sanitizer effectivity from published work. Currently there is no standard for simulated wash water model. In order to develop such model, we must first understand how preparation methods can affect the physicochemical properties of the wash water, and thus the efficacy of sanitizers for future validation studies. Our work will focus mainly on leafy green processing operations and conditions using baby spinach as our commodity model due to the increase in outbreaks related to leafy greens in recent years like the *E. coli* O157:H7 outbreak with contaminated baby spinach in 2007. During the 2015 agricultural season a farm survey was conducted to asses wash water conditions of 10 farms in Western Massachusetts,

USA. Each sample survey was test for NTD (Turbidity), ORP (Oxidation Reduction Potential), COD (Chemical Oxygen Demand), and pH. To determine the physicochemical characteristics of post-harvest wash water in this region. Based on our findings, we modeled the organic load levels for bench top trials on the average values observed on-farm, choosing COD and NTU levels as our organic load indicators. Laboratory trials were performed to determine the effects of organic load generated using paddle mixer and a on the physiochemical properties of the wash water. This study aims to identify a suitable preparation method to best represent leafy green processing conditions on farm, for future commercial sanitizer screening studies in efforts to provide best management practices for produce wash water quality.

3.2: Materials and methods

3.2.1 Farm Survey in Pioneer Valley

Three wash water samples were obtained from each farm engaged in produce washing and cooling processes. Seven out of ten farms were leafy green processing operations using dunk tanks. The other three operated carrots, squash and melons; and were also included in the ten farms surveyed in this study. Water samples were taken and transported in Whirl pack bags (Nasco, Fort Atkins, WI) to the lab for analysis. Physicochemical properties analyzed included: Turbidity (NTU) using HACH 2100Q portable Turbidimeter (HACH Company, Loveland CO 80539), pH using the Thermo Scientific Orion Star A221 pH meter (Thermo Scientific, Waltham MA 02451), ORP using ORP/ATC electrode, 967961 attachment using Thermo Scientific Orion Star A221 (Thermo Scientific, Waltham MA 02451) and Chemical Oxygen Demand (COD) using a HACH DRB200 Digital Reactor Block (HACH Company, Loveland CO 80539).

3.2.2 Organic load wash water preparation

Baby spinach (*Spinacia oleracea*) was purchased from a local grocery store and stored at 4°C for no than 48 hours. For homogenized leafy matter solutions (blender), 40g of baby spinach was prepared with 200ml of distilled water and mixed using a high speed blender (Coolife Professional Kitchen Blender, Guangdong, China). For paddle mixing (stomacher) solutions, 40g of baby spinach was prepared with 200ml of distilled water and mixed using a Stomacher (Bagmixer 400 CC, Interscience Laboratories Inc., Woburn, MA). Organic load solutions prepared with both a blender and stomacher were filtered through cheesecloth and diluted to a final volume of 1,500ml with chemical oxygen demand concentrations of 50, 100, 400, 700, 1,000, 1,500 mg/l. Solutions were refrigerated for 24 hours at 4°C before analysis.

3.2.3 Analysis of physicochemical properties of simulated wash water

The physicochemical properties used for the analysis of simulated wash water were: Turbidity (NTU) measured using the HACH 2100Q portable Turbidimeter (HACH Company, Loveland CO 80539), Chemical Oxygen Demand (COD) and using the HACH DRB200 Digital Reactor Block (HACH Company, Loveland CO 80539), Oxygen Reduction Potential (ORP) and pH were measured using HANNA instruments HI901C1-01 with both ORP and pH probe attachments (HANNA Instruments Inc., Woonsocket RI).

3.2.4 Statistical Analysis

Three samples were taken for each treatment and all experiments were performed in triplicate. The data was partitioned and assessed by an F-test and Analysis of variance (ANOVA) and Duncan

Multiple Range Test performed using SAS where statistical significance was set at $p < 0.05$ (SAS Institute Inc., Cary, NC, USA).

3.3: Results and Discussion

3.3.1 Farm Survey and analysis of physiochemical properties

To better understand the properties of postharvest agricultural wash water of the Western Massachusetts region, a farm survey was conducted during the 2015 agricultural season where seven out of the ten farms were leafy green processors, as seen in **Table 4**.

Table 4: Processing characteristics of farms surveyed in Western Massachusetts, USA during 2015

Farm	Produce Type	Processing Method
1	Leafy Greens	Dunk Tank
2	Leafy Greens	Dunk Tank
3	Leafy Greens	Dunk Tank
4	Leafy Greens	Dunk Tank
5	Leafy Greens	Dunk Tank
6	Leafy Greens	Dunk Tank
7	Leafy Greens	Hydro Cooler
8	Carrots	Tumble Washer
9	Squash	Spray Washer
10	Melons	Brush Washer

Based on the results from the farm survey and previous work, we chose COD and turbidity as indicators of organic load and their link to water quality (Barrera, Blenkinsop, & Warriner, 2012; Luo et al., 2011; Selma, Allende, Lopez-Galvez, Conesa & Gil, 2008; Suslow, 2004; Chen & Hung, 2016). Target values were chosen as were 50mg/L and 100 mg/L COD and 100 NTU respectively. Bench top laboratory trials were conducted to make comparisons between common

preparation methods, a paddle mixer (stomacher) and a blender and their effects on the physicochemical properties of wash water.

3.3.2 Comparison of organic load preparation methods

Simulated wash water samples were prepared with baby spinach using two different methods, a stomacher and a blender. For each treatment we analyzed the physicochemical characteristics mentioned in section 3.2.3. Our goal was to evaluate if different processing methods had a significant effect on the physicochemical properties of the simulated wash water.

To better understand the properties of postharvest agricultural wash water of the Western Massachusetts region, a farm survey was conducted during the 2015 agricultural season where seven out of the ten farms were leafy green processors. Based on the results from the farm survey and published work, we chose COD and turbidity as our parameters of organic load quantification due to their relationship to water quality (Barrera, Blenkinsop, & Warriner, 2012; Luo et al., 2011; Selma, Allende, Lopez-Galvez, Conesa & Gil, 2008; Suslow, 2004; Chen & Hung, 2016). Target COD values were 50mg/L and 100 mg/L respectively, then values of 100 NTU for controlled turbidity studies. Values were chosen based on averages from wash water farm survey.

Bench top laboratory trials were conducted to make comparisons between common preparation methods, a paddle mixer (stomacher) and a homogenized leafy matter (blender) at observed on farm organic load levels for future sanitizer validation studies. Simulated wash water samples were prepared with baby spinach using two different methods, a stomacher and a blender due to their common use in research work. Our goal was to evaluate if different processing methods had a significant effect on the physicochemical properties of the simulated wash water, and could in turn affect sanitizer efficacy in solution. The nature of the preparation method could cause

changes in the physicochemical properties of the wash water where a blender, which which causes a complete breakdown of baby spinach leaves, compared a stomacher which renders only a partial breakdown on the spinach leaves. These small differences can affect the properties of the simulated wash water, thus making it challenging to compare results to a wide range of commodities (Ghostlaw, Martinez, Kinchla, 2018)

COD (mg/L) was used as measure of organic load for our simulated wash water, and then analyzed the physiochemical properties in simulated wash water. Results showed no statistical differences between preparation methods at 50 and 100 mg/L among the physiochemical properties analyzed in this study as seen in **Table 5**.

Table 5: Physicochemical Properties of simulated wash water at 50 and 100 mg/L

Physicochemical properties	0 COD		50 COD		100 COD	
	Blender	Stomacher	Blender	Stomacher	Blender	Stomacher
ORP (mv)	333 a	333 a	378 a	375 a	346 a	352 a
pH	5.9 a	5.9 a	6.0 a	6.1 a	6.2 a	6.2 a
Turbidity (NTU)	0.11 a	0.1a	8.6 a	8.3 a	15.7 a	15.5 a

^a Preparation methods were compared at 0, 50 and 100 COD for each wash water property Mean values in the same row that are not followed by the same letter are significantly different (Duncan’s Multiple Range Test, P=0.05)

After evaluating the effects of simulated wash water preparation methods using COD as a measure of organic load, we repeated the experiment this time changing the method of organic load quantification. We used turbidity, as it has also been used in previous work as an indirect method of organic load quantification (Gombas et. al, 2017). The turbidity target value was chosen based on average turbidity values observed in a farm survey conducted in leafy green processing operations of the Western Massachusetts region. The physicochemical properties of the simulated

wash water are presented in **Table 6**. Results showed no significant difference between preparation methods when looking at their effects on the physicochemical properties of the solution at 100 NTU.

Table 6: Physicochemical properties of simulated wash water at 100 NTU

Physicochemical properties	0 NTU		100 NTU	
	Blender	Stomacher	Blender	Stomacher
ORP (mv)	274 a	274 a	242 a	213 a
pH	5.8 a	5.8 a	6.3 a	6.5 a
Turbidity (NTU)	0 a	0 a	589 a	558 a

Preparation methods were compared at 0 and 100 NTU for each wash water property. Mean values in the same row that are not followed by the same letter are significantly different (Duncan Multiple Range Test P=0.05)

Organic load quantification methods were looked at separately in each experiment. The goal of the study was to evaluate the effects of the preparation method using baby spinach as a model to generate the organic load levels seen on-farm on the physicochemical properties of the wash water. Organic load measure methods were kept separate to assess each condition independently and later assess the relationship between COD and NTU when used for generating simulated wash water at increasing levels of organic load. Overall no significant difference was observed between preparation methods and the physicochemical properties of the simulated wash water ($p > 0.05$).

3.3.3 Comparison between paddle mixer and homogenized leafy matter at increasing COD (mg/L) concentrations

In the previous experiment preparation methods were compared at known organic load values using both COD and turbidity as indicators of the organic material, due to their use in the produce industry, and data showed no significant difference between preparation methods at

known COD and NTU values when looking at the physicochemical properties of simulated wash water.

Besides looking at the differences between preparation methods at values seen on farm leafy green processors in the Western Massachusetts region, experiments also looked to test if at high levels of organic load, the preparation method would affect the physicochemical properties of the simulated wash water. The next study focused at testing the turbidity values of the simulated wash water when using COD as a measurement of organic load in the system. More focus was put on testing and understanding the use of COD and turbidity in generating simulated wash water, due to their use in the produce industry as organic load indicators, where the presence of organic load is known to impact the efficacy and quenching capacity of chlorine based system for the monitoring of water quality in produce washing operations.

Simulated wash water was generated using baby spinach and processed using a stomacher and a blender to achieve COD levels of 0, 400, 700, 1,000 and 1,500 mg/L, which were chosen to showcase worst case scenarios in leafy green processing like those seen in previous work like Luo, Zhoum Van Haute, Nou, Zhang, Teng & Miller, 2018. Comparison between blender and stomacher turbidity values at increasing levels of organic load can be seen in **Figure 4**. At COD levels of 0, 100, 400, 700 and 1,000 no significant difference was observed between preparation methods. However, at 1,500 COD there is a significant difference in turbidity values between stomacher and the blender method, where the blender yielded a higher turbidity than the stomacher both at 1,500 COD.

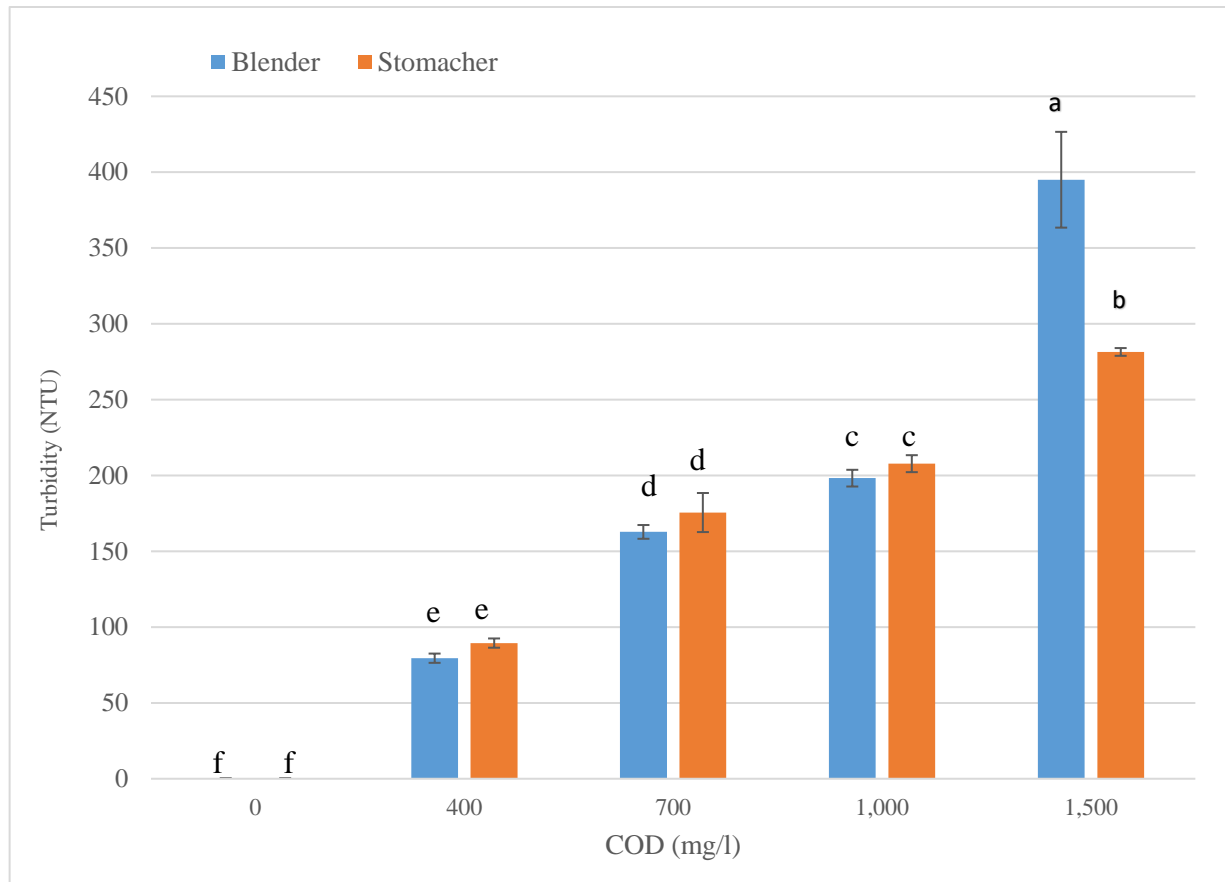


Figure 4: Comparison between Blender & Stomacher turbidity values at increasing organic load levels
 Turbidity values of simulated wash water preparation methods were compared at increasing levels of organic load of 0, 400, 700, 1,000 and 1,500 COD. Same letter grouping represents no statistical differences. Significant difference between Blender & Stomacher at 1,500 mg/L (Duncan’s Multiple Range Test, P=0.05).

Results showed, that while 1,500 COD was not representative of organic load levels observed in survey conducted on leafy green processing water in the Western Massachusetts, these levels have been reported in simulated wash water research studies (Luo et. al, 2018; Weng, Luo, Li,Zhou, Jacangelo and Schwab, 2016; Chen and Hung, 2016; Van Haute, Sampers, Holvoet and Uyttendaele, 2013). Further studies continued to use the blender as the preferred preparation method, because it proved to be more time efficient for experiments and required less amounts of

produce to achieve both low and high levels of organic load, in comparison to a paddle mixing method like the Stomacher, to be used for sanitizer validation work.

The physicochemical properties of the wash water need to be understood and maintained to ensure the efficacy of the antimicrobial solution added to the washing system in efforts to properly conduct validation studies on their use in wash water. Being that organic load is an essential parameter that should be monitored in washing systems (Gombas et. al, 2017), a study was conducted to evaluate the use of COD and turbidity measurements for monitoring organic load in wash water solutions.

The use of turbidity measurements has been occasionally used in the produce industry in reference to the amount of organic load (Gombas et. al, 2017). However, results may be impacted based on the amount of soil and debris in the washing system, as well as any color developed in water. COD on the other hand, is a direct measurement of organic load and chlorine demand, depletion rate of free available chlorine, in a system. One of the main differences between methods is the cost and time of each. The COD assay uses heat and a strong oxidizer to oxidize the organic material present and thus measuring the amount of oxidizing agent consumed in the reaction. The reaction takes approximately two hours and one of the main components of the assay is mercury. The implementation of this method is not adequate for a small or medium scale farmer due to its high cost (example: HACH DRB200 Digital Reactor Block \$1,670.08) and use of toxic and corrosive chemical which require separate disposal protocols. While the use of a turbidity meter is fairly low in cost when compared to a COD measuring device (example: SPER Scientific Direct Turbidity Meter-860040 \$350.00) it is not a direct measurement of organic load, and thus the produce being washed will affect the clarity of the water and thus the values reported. In studies like Selma, Allende, Lopez-Galvez, Conesa and Gil (2008) reported NTU values varied

significantly when depending on the produce washed, where lettuce had a reported 87.4 NTU versus Onions reported 5040.4 NTU.

Using the blender method, we processed baby 50g of baby spinach and 200ml of dH₂O, filtered through cheesecloth and diluted to achieve COD values of 50, 100, 400, 700, 800, 1,000 and 2,000 mg/L. Study evaluated the NTU values of each COD concentration and results are shown in **Figure 5**. Results showed that an increase in organic load showed an increase in NTU values which corresponded with a linear relationship between turbidity and COD.

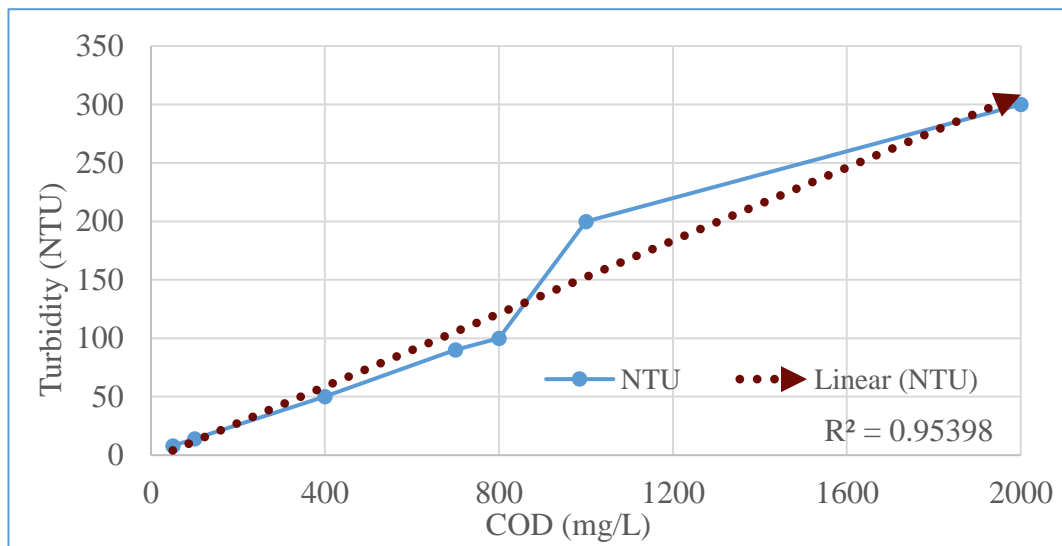


Figure 5: Relationship between COD and turbidity at increasing levels of organic load

At increasing levels of organic load data showed a linear relationship, when increasing COD levels as a measure of organic load.

Previous studies such as that done by Luo et. al (2012) showed that when processing leafy greens like spinach and lettuce there was a linear increase in COD and turbidity in the wash water in relation to the amount of produce that was being washed. Future studies continued to use COD as the organic load monitor, instead of turbidity measurements due to its precision and accuracy in directly measuring organic load and chlorine demand.

3.4 Conclusion

This study illustrated the need for a standard preparation method to produce simulated wash water solutions for laboratory trials. While the on farm observed concentrations of organic matter, established from the data obtained in the farm survey, of 50 & 100 mg/l and 100 NTU, showed no statistical difference between the stomacher and blender methods ($p>0.05$), high levels of organic matter in solution reflected a statistical difference between methods and their effects on the physiochemical properties of the simulated wash water. Ultimately the Blender (homogenized leafy matter) method worked best for our future sanitizer validation studies. The blender proved to be more time efficient, as well as providing a better control at mimicking on farm organic load levels at low, high and very high levels when compared to the stomacher (paddle mixer).

CHAPTER 4

ASSESSMENT OF ANALYTICAL METHODS TO DETECT FREE RESIDUAL CHLORINE IN AGRICULTURAL WASH WATER FOR SANITIZER VALIDATION

4.1 Introduction

Previous work done on chlorine based sanitizers in produce washing operations indicates that the DPD-titrimetric is the method preferred when using a titration to detect free residual chlorine levels in wash water solutions. Another common method is the use of test kits, for example the HACH free chlorine test kit, which mimics the DPD reaction seen in **Figure 2** where the intensity of color due to the formation of the Würster dye correlates with the amount of free chlorine present in the sample. In this study, besides comparing DPD methods for free residual chlorine detection, studies also evaluated the efficacy of an IOD-titration which is also categorized as a standard method for free residual chlorine detection in wash water solutions, specifically for sodium hypochlorite based chlorine sanitizers. The goal of the studies was to compare commonly used free chlorine detection to assess the best fit method for detection at high levels of organic load. Upon comparing all three detection methods, further studies were conducted to evaluate the differences between DPD and IOD titrations methods, which led to subsequent studies where the interaction between organic load, sanitizer and *E. coli* O157:H7 was tested and used to evaluate their effects on both DPD and IOD titrations. The main goal of the studies was to establish the best fit analytical method for free residual chlorine detection in samples with heavy organic load solutions.

4.2 Materials and Methods

4.2.1 Organic load wash water preparation

Baby spinach (*Spinacia oleracea*) was purchased from a local grocery store and stored at 4°C for less than 48hrs. Organic load solutions were prepared using baby spinach with distilled water using a high speed blender (Coolife Professional Kitchen Blender, Guangdong, China). Organic load solutions were then filtered through cheesecloth and diluted to desired COD levels of 50, 100, 400, 500 and 700 mg/l based on levels seen in previous work (HACH DRB200 Digital Reactor Block, Ames, IA). Samples were diluted for a total volume of 1,500ml for each sample and kept in the refrigerator for 24hrs at 4°C before analysis.

4.2.2 Preparation of Sodium Hypochlorite (NaOCl) solutions

25ppm of Sodium Hypochlorite (NaOCl) solutions were prepared by diluting at a ratio of 1:10 (Clorox® germicidal bleach: dH₂O). Free residual chlorine concentration was measured using two different titration methods, namely an Iodometric Titration (ASTM D2022-89, 2016) and DPD-titrimetric titration (Rice & Bridgewater, 2012) and one test kit being the HACH Kit for Free Chlorine testing (Free chlorine Color Disc Test it Model CN-66F, HACH, Ames, IA). Titrations were performed using a HANNA Instruments HI901C1-01 (HANNA Instruments Inc., Woonsocket RI).

4.2.3 Comparison of Free Residual chlorine testing method comparison study

Free residual chlorine levels were tested using IOD and DPD titrations in addition to the HACH free chlorine test kit, to compare their ability to accurately detect chlorine levels in presence of organic material. Organic load levels chosen were based on our farm survey where 50mg/l was

the average value of COD levels observed in postharvest agricultural wash water on farm. Organic load samples were prepared at 0 and 50mg/L for a total volume of 1,500ml and stored in the refrigerator for 24hrs 4°C before analysis. Free chlorine levels were tested after adding 25ppm of a ratio of 1:10 (Clorox® germicidal bleach: dH₂O) to solution and mixing for 30 seconds. Samples were taken from the same solution and tested at the same time for all three detection methods.

4.2.4 *E. coli* O157:H7 strain preparation

E. coli O157:H7 strain was obtained from ATCC (ATCC 43894 Manassas, VA) and grown to 100µg/ml nalidixic acid resistance. A single colony of the strain was grown in Tryptone soy broth (abbreviated TSB, Thermo Scientific™, Waltham, MA). Strain was stored in glycerol and TSB at -80°C. Strain was regrown in TSB for 18-24hrs for use and plated on Tryptone soy agar (abbreviated TSA, Thermo Scientific™, Waltham, MA) treated with 100µg/ml nalidixic acid. The samples were inoculated to obtain 10⁷ CFU/ml of *E. coli* O157:H7 concentrations in samples.

4.2.5 Comparison study of free residual chlorine titration methods at increasing organic load levels

A comparison study was done in two separate experiments, both of which to assess the efficacy and sensitivity of IOD and DPD titration methods. In the first experiment free residual chlorine levels were tested at 0,100, 400 and 500mg/L (HACH DRB200 Digital Reactor Block, Loveland, CO) after adding sanitizer to solution and mixing for 30s, running each titrations side by side at the same time. For the second experiment we tested free residual chlorine levels this time at 0,100,700mg/L (HACH DRB200 Digital Reactor Block, Loveland, CO) inoculating samples to obtain 10⁷ CFU/ml of *E. coli* O157:H7.

4.2.6 Statistical Analysis of Free Residual Chlorine detection studies

Three samples were taken for each treatment and all experiments were performed in triplicate. Analysis of variance (ANOVA), and data was partitioned and assessed using an F-test, test were performed using SAS where statistical significance was set at $P=0.05$ (SAS Institute Inc., Cary, NC, USA).

4.3 Results & Discussion

4.3.1 Comparison study between IOD and DPD titrations and a HACH kit for free residual chlorine detection at two low organic load levels (0 and 50mg/l)

Free residual chlorine detection levels were compared between three different detection methods, two of which were titrations and one being a rapid testing kit. **Figure 6** illustrates detection method and organic load combinations, which were all treated with 25ppm of a 1:10 ration of Clorox® Germicidal bleach and dH₂O at organic load levels of 0 and 50 mg/l, which are representative of observed on farm conditions from wash water farm survey

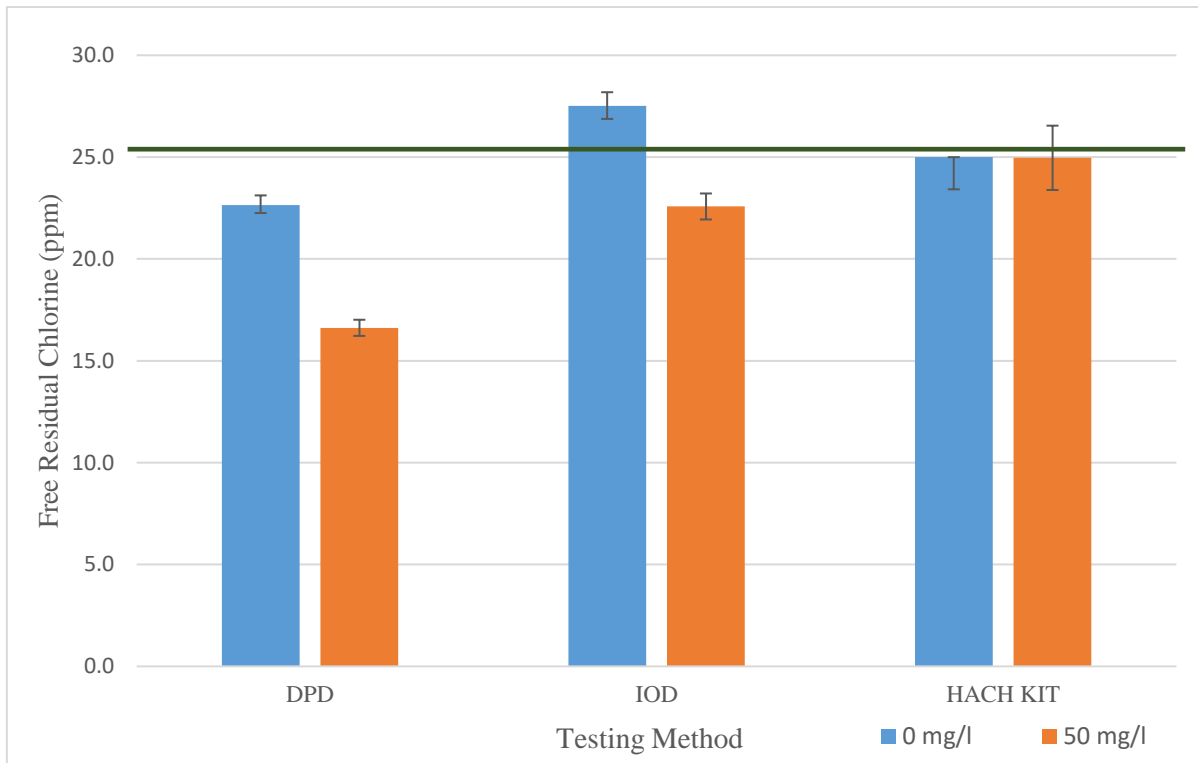


Figure 6: Free residual chlorine concentrations detected using different analytical methods at two COD levels

*Indicates statistical significance among free residual chlorine detection methods at 50 mg/L using Two-way anova for variance analysis followed by F-test, P=0.05).

Line represents sanitizer concentration added initially (25ppm of Clorox® Germicidal bleach)

No significant difference was observed between free residual chlorine detection methods with no presence of organic load in solution. However, the presence of organic load of 50 COD (mg/L) had a significant effect on the detected sanitizer concentration for both DPD and IOD titration ($P > 0.05$) when compared to the HACH kit. When looking at **Figure 6**, the results shows that the HACH kit seems to be the best fit method when comparing sanitizer levels in presence and absence of organic material, where Figure 4 shows that presence of organic load had no

significant effect on detected sanitizer concentration when compared to the control which had no organic load in solution (0 COD).

However, while conducting the experiment, we observed that due to the green color of our 50 mg/L organic load stock created using baby spinach, made it challenging to effectively detect the free residual chlorine level change when using the HACH kit. The kit uses a color wheel, as seen in **Figure 7**, which correlates the level of free residual chlorine in solution to the intensity of the Würster dye (bright magenta in color) formed and compared to the color wheel seen in **Figure 7**. The higher the levels of free residual chlorine in solutions, the more intense the magenta color will be. The kit has a maximum value of detection is 10ppm and the color wheel reports free residual chlorine levels in increments. In colored solutions such as the 50mg/L organic load stock, it was challenging to effectively identify the color formed from the reaction to the color wheel

from the kit. Both the HACH test kit and the DPD titration follow the same reaction, in which a DPD (N, N-diethyl-p-phenylenediamine) reagent reacts with chlorine to produce a bright magenta color known as the Würster dye. The more chlorine present in solution, the brighter the color. For the DPD titration, the titrant Ferrous Ammonium Sulfate (FAS) will be dispensed till the



Figure 7: HACH free chlorine test kit

solution becomes colorless; whereas the HACH test kit compares the intensity of the Würster dye formed to a color wheel to identify the ppm of free residual chlorine in solution. The downfall with both methods, is that they both have a small test range for free residual chlorine detection. The HACH kit has a range of 001-10ppm whereas the DPD titration has a range of 0.01-5ppm of chlorine. In our case a solution of 25ppm must be further diluted prior to analysis which can cause

increase variability between test, and since chlorine is known to dissociate into its two main components (HOCl and OCl⁻) in water, diluting the solution further can cause interferences with the formation of the HOCl which is the germicidal component of the reaction (Gombas et. al 2017)

Overall, results showed that while the HACH kit seemed to work well in both no presence and absence of organic load in solution, the identification of the proper free residual chlorine values can be biased based on the person performing the analysis and in turn introduce variability between samples and would not be precise enough for our future experiments involving sanitizer screenings at increasing organic load levels. DPD, IOD and HACH kit comparison results showed a significant difference in detected sanitizer concentration at 50 mg/L ($P < 0.05$). The HACH kit results showed no significant difference between organic load levels of 0 and 50 mg/L. Further studies were conducted to explore the dynamics of organic load and sanitizer and their effects the detection capabilities of both DPD and IOD titrations, as a preferred method of free residual chlorine detection for laboratory studies.

4.3.2 Comparison between analytical method (IOD and DPD titrations) for free residual chlorine detection at increasing organic load levels

Upon concluding that the rapid test kit from HACH would not fit our future experiments, we looked closer at the interaction between sanitizer and organic load using two different titration methods for the detection of free residual chlorine in solution for future sanitizer screening studies. DPD titration and the IOD titration were compared at at 0,100, 400 and 500 mg/L organic load levels all treated with 25ppm of Clorox® germicidal bleach in. Results can be seen in **Figure 8**. Results showed that there was a significant difference between DPD and IOD free residual chlorine concentrations detected at 100, 400 and 500 mg/L in which a significant interaction between

sanitizer concentration and organic load at 100, 400 and 500 mg/l was observed. From **Figure 8** we can see a decrease in free residual chlorine concentration in solution, with the addition of organic load in both titration results. For the IOD titration we see that at 0mg/L we have 25ppm and once organic load is introduced we have an average of a 5ppm decrease overall organic load is introduced we have an average of 5ppm decrease overall organic load levels tested.

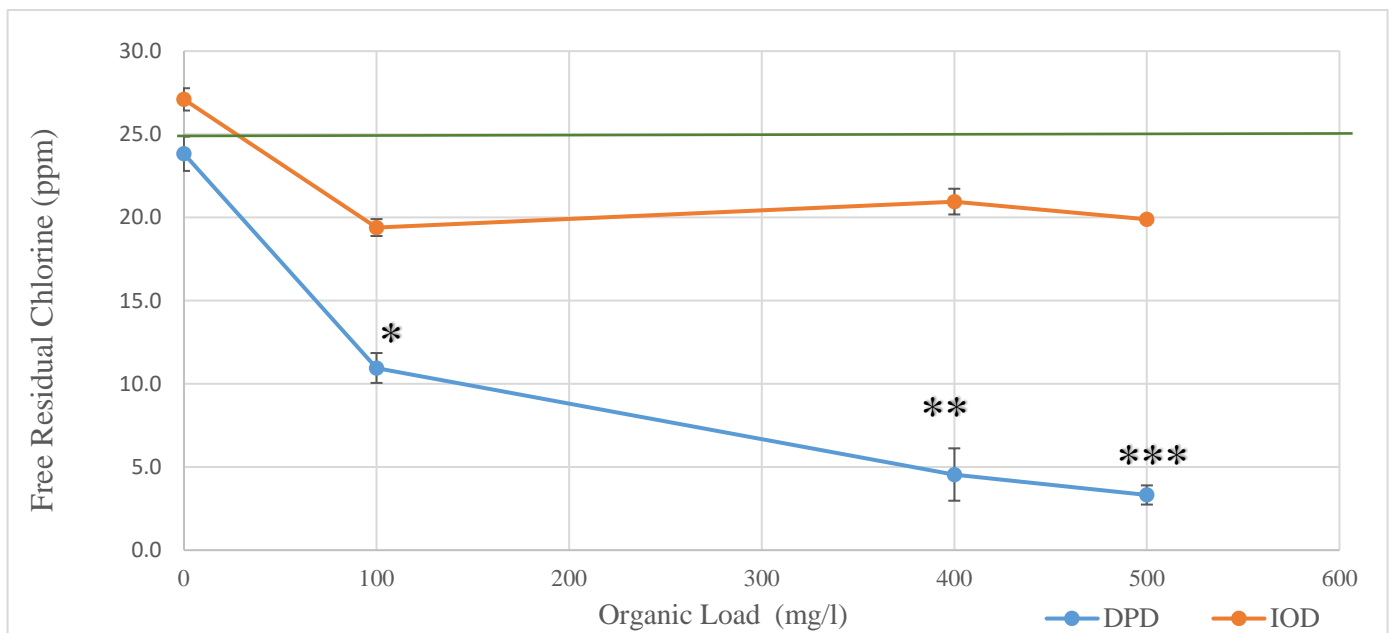


Figure 8: Free residual chlorine detection of different levels of COD using 2 titration methods (IOD and DPD)

*Represents significant difference between titration methods at 100 mg/L (F-test $P < 0.05$)

**Represents significant difference between titration methods at 100 mg/L (F-test $P < 0.05$)

***Represents significant difference between titration methods at 100 mg/L (F-test $P < 0.05$)

Line represents initial sanitizer concentration added- 25ppm of Clorox® Germicidal Bleach

However, we can see that the results obtained from the DPD titration show a significant decrease in concentration over all levels of organic load when compared to the results seen for the IOD titrations going from around 25ppm initially at 0mg/L to 11ppm at 100 mg/L and 3ppm at 500. The interaction between titration method and sanitizer concentration was also highly

significant where DPD and IOD results showed to be statistically different. Titration graphs can be seen in **Figure 9** where it can be seen that with increasing organic material the DPD titration had problems finding the endpoint of the reaction.

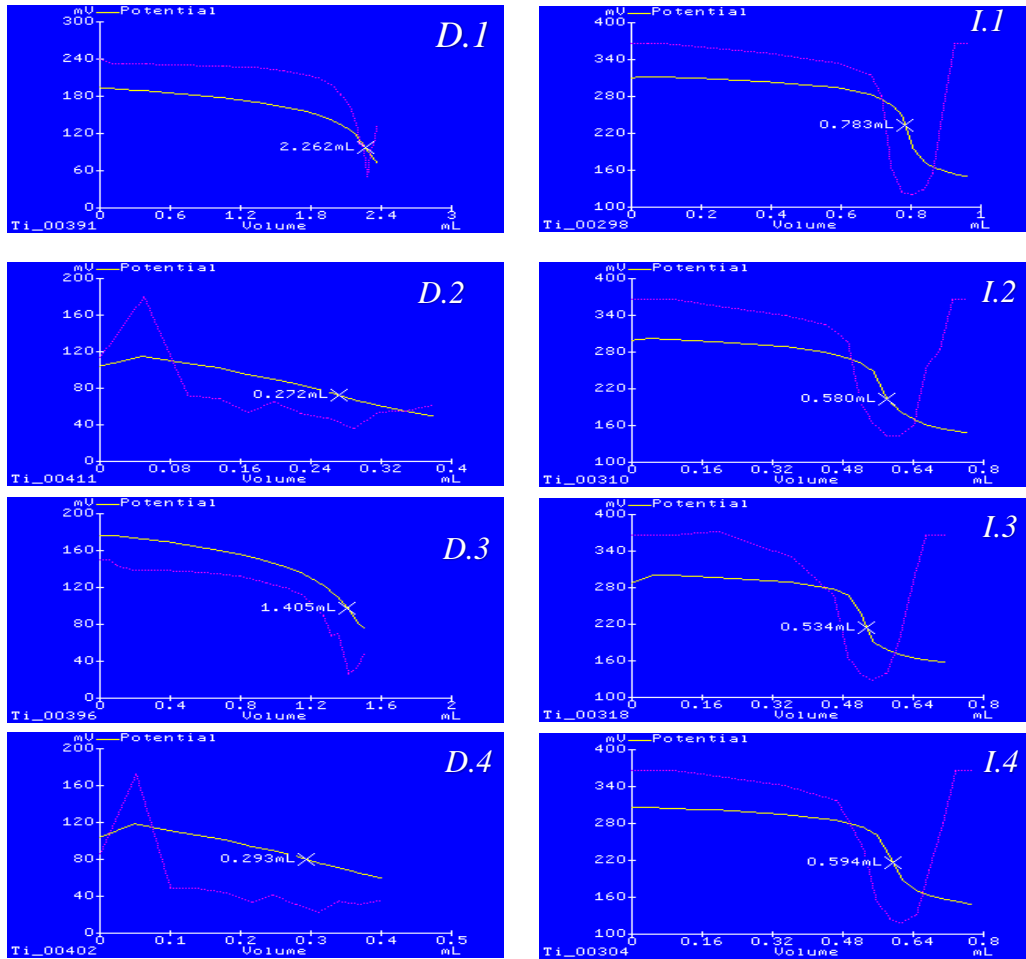


Figure 9: DPD and IOD titration screenshots in increasing organic load solutions treated with 25ppm of germicidal bleach

^D Represents titration values for DPD titration and ^I Represents titration values for IOD titration

¹ 0 COD, ² 100 COD, ³ 400 COD and ⁴ 500 COD

Previous work has shown that the introduction of organic matter to a wash solution causes free residual chlorine concentrations to decline (Gombas et. al 2017), however the large

discrepancy in results seen from both titrations in this experiment, when titration samples were taken from the same solution and ran at the same time was alarming. The DPD titration method is a standard method for free residual chlorine analysis and commonly used in research, however when tested in presence of varying organic load levels it proved to not be as effective as we expected. This titration is very color and pH dependent, where the solution will turn a bright magenta color (Würster dye) in presence of chlorine and titrate to a colorless solution. Due to the nature of our simulated wash water solutions, created using baby spinach and deionized water, yielding varying shades of green it is possible that this color interference would cause the equipment to not find the endpoint. One critical problem when using a DPD method for wastewater or in this case simulated wash water is the interference one from turbidity and color (Harp 1995). An increase in organic load results in a linear increase in turbidity (Ghostlaw, Ramos, Kinchla, 2018), which can explain the issues in the discrepancies when using a DPD titration for simulated wash water systems with high levels of organic load which was discussed previously in **Figure 4**.

While DPD methods are one of the most commonly used in previous published work as previously seen in **Table 3** our results showed that in high organic load solutions, interferences with turbidity and color, in this case shades of green due to the use of baby spinach as our produce model for simulated wash water, can cause this method to be ineffective and render inaccurate readings by missing to find the correct end point of the titration reaction.

4.3.3 Comparison study of IOD and DPD titrations for free residual chlorine detection at 0,100 and 700 mg/l inoculated with E. coli O157:H7

Before choosing one titration method over another, we tested both titrations not only in presence of increasing organic load levels, but also in presence of microbial counts by inoculating

samples with 10^7 CFU/ml of *E. coli* O157:H7 in stationary phase (refer to **Table 9** in appendix for *E. coli* O157:H7 ATCC 43894 growth curve) and tested the interaction between organic load, sanitizer and bacteria and their effects on free residual chlorine concentration readings. The focus for this study was to see how the interaction between organic and microbial loads would affect the titrations ability to detect free residual chlorine levels due to the results seen in previous work where organic load has a significant effect on the DPD titration. Samples were tested at three levels of organic load- 0, 100 and 700 mg/l- at four different treatments, these being: treatment 1- sanitizer, treatment 2-no sanitizer, treatment 3- sanitizer + bacteria and treatment 4- control (no sanitizer or bacteria).

Results showed no significant difference between organic load and treatments on free residual chlorine levels except for treatment 1 and 3 at 700 mg/L ($p < 0.001$) as seen in **Table 9**. Results show that the presence of high organic load levels will have a significant impact on the free chlorine levels detected, regardless of presence or absence of bacteria. A significant interaction was also observed between method and treatment ($p < 0.05$), in other words the presence of increasing organic load had a significant effect on the free residual chlorine detection capacity of the DPD titration methods as seen in our previous study.

Table 7: Titration method comparison within bacteria, organic load and sanitizer treatments

COD (mg/L)	Titration Method	Sanitizer	No Sanitizer + Bacteria	Sanitizer + Bacteria	Control
		Treatment 1 (ppm)	Treatment 2 (ppm)	Treatment 3 (ppm)	Treatment 4 (ppm)
0	DPD	16.76	0.50	13.74	0.50
	IOD	21.63	1.78	19.19	1.78
100	DPD	4.09	0.50	3.15	0.50
	IOD	15.33	1.78	15.57	1.78
700	DPD	0 *	0.50	2.69**	0.50
	IOD	16.9*	1.78	23.7**	1.78

Values of 0.50 represent 0ppm for the DPD titration and 1.78 represents 0ppm for the IOD titrations.

*Highly significant difference seen between titration methods in treatment 1 at 700mg/L ($p < 0.001$).

** Highly significant difference seen between titration methods in treatment 3 at 700mg/L ($p < 0.001$).

In comparison to the previous study, the responses observed for the DPD titration, with increasing organic load showing a significant decrease in free chlorine levels in solution, were consistent. High organic load levels present in solution caused an interference with the equilibrium of the DPD titration, as previously mentioned, by not allowing for the formation of Würster dye and thus the completion of the reaction. This leading to the inability of finding an endpoint to the titration, making it challenging for us to use this method in further studies. Results showed that the IOD titration method would be the best alternative for free residual chlorine detection in high organic load scenarios for our future sanitizer validation studies.

4.4 Conclusion

Free residual chlorine levels were directly affected by the presence of organic matter in solution. While in a wash water environment of 50 mg/L treated with 25ppm of sanitizer the HACH kit seemed to be the best fit, we observed that any interference with the formation of the Würster dye would make interpreting free residual chlorine levels challenging. This pattern was also observed upon testing the DPD titration at increasing organic load levels. Both the HACH kit and the DPD titration failed to detect free residual chlorine levels effectively in organic load ridden environments, which suggest that for studies requiring high organic load levels the IOD titration would be the best fit under the experimental conditions. We also observed a significant interaction between bacteria and free residual chlorine levels in organic load solutions, which will discussed in the next chapter.

CHAPTER 5

COMMERCIAL CHLORINE BASED SANITIZER SCREENING

5.1 Introduction

The presence of sanitizers in postharvest agricultural wash water have shown to be an effective approach to reducing microbial cross contamination in agricultural washing operations (Luo, Nou, Millner, Zhou, Shen, Yang, and Shelton, 2012). However, choosing an appropriate sanitizer for a processing operation can be challenging due to processing conditions and produce commodity, which can lead to the degradation of sanitizer levels due to the interference with organic load present in solution. Cross contamination with *E. coli* O157:H7 in agricultural wash water has been widely studied, yet little research has focused on investigating the efficacy of commercially available chlorine based systems as sanitizer sources for postharvest applications. The goal of this study was to evaluate and compare two commercially available chlorine based sanitizers, whose active ingredient is sodium hypochlorite, being Clorox® Germicidal bleach and Pure Bright™ Ultra Bleach to a sodium hypochlorite concentrated solution commonly used in research, to test their efficacy in reducing *E. coli* O157:H7 counts in high and low organic load solutions. Experiments tested the depletion of chlorine overtime at high and low organic load levels before conducting the final experiment where all three sanitizing solutions were compared at high and low organic load levels. This study aims to help better understand the performance of commercially available chlorine based antimicrobial solutions in reducing microbial loads in a wash water system in comparison to a sodium hypochlorite solution commonly used in research, in efforts to provide guidance on sanitizer implementation for small and medium leafy green processing operations.

5.2 Materials and Methods

5.2.1 Organic load wash water preparation

Baby spinach (*Spinacia oleracea*) was purchased from local grocery store and stored at 4°C for no more than 48hrs after purchase. . Organic load solutions were prepared using 40 g of baby spinach with 200 ml distilled water using a high speed blender (Coolife Professional Kitchen Blender, Guangdong, China). Organic load solutions were then filtered through cheesecloth and diluted to desired COD levels of 100 and 700 mg/L (HACH DRB200 Digital Reactor Block, Ames, IA) with a total volume of 1,500ml for each sample and kept refrigerated for 24hrs at 4°C before analysis.

5.2.2 E. coli O157:H7 strain preparation

E. coli O157:H7 strain was obtained from ATCC (ATCC 43894 Manassas, VA) and grown to 100µg/ml nalidixic acid resistance. A single colony of the strain was grown in Tryptone soy broth (abbreviated TSB, Thermo Scientific™, Waltham, MA). Strain was stored in glycerol and TSB at -80°C. Strain was regrown in TSB for 18-24hrs for use and plated on Tryptone soy agar (abbreviated TSA, Thermo Scientific™, Waltham, MA) treated with 100µg/ml nalidixic acid. The samples were inoculated to obtain 10⁷ CFU/ml of *E. coli* O157:H7 concentrations in the samples.

5.2.3 Preparation of Sodium Hypochlorite (NaOCl) solutions for chlorine depletion studies

25ppm of Sodium Hypochlorite (NaOCl) solutions were prepared by diluting at a ratio of 1:10 (Clorox germicidal bleach: dH₂O). Free residual chlorine concentration was measured using two different titration methods, namely an Iodometric Titration (ASTM D2022-89, 2016). Titrations

were performed using a HANNA Instruments HI901C1-01 automatic titrator (HANNA Instruments Inc., Woonsocket RI).

5.2.4 Chlorine depletion studies

Chlorine depletion studies were performed in three separate experiments. Simulated wash water solutions were prepared at organic load levels of 0, 100, 400, 500 and 700 mg/L (HACH DRB200 Digital Reactor Block, Ames, IA). Samples were treated with 25ppm of germicidal bleach following EPA regulation 5813-1(Germicidal Bleach, Clorox Company, Oakland CA) and mixed for 30s before free residual chlorine levels were measured using IOD titration (ASTM International Standard Methods of Sampling and Chemical Analysis in Sodium Hypochlorite Solutions method, West Conshohocken, PA). The first experiment tested free residual chlorine levels at 0, 100, 400 and 500mg/L organic load levels, right after adding the sanitizer. For the second experiment chlorine levels were tested in samples with 0, 100 and 700 mg/L at three separate time points after the addition of the sanitizer of 30 seconds, 1hour and 2 hour intervals. For the final study, the selected time intervals were tested and added 10^7 CFU/ml *E. coli* O157:H7 to our organic load samples and tested the free chlorine levels at the three time intervals previously mentioned. Microbiological analysis was performed for samples at all three time points, plated on TSA treated with 100 μ g/ml of nalidixic acid and incubated for 24hrs at 37°C.

5.2.5 Preparation of Sodium Hypochlorite (NaOCl) solutions for commercial sanitizer screenings

Sanitizing solutions of 25ppm were prepared using a 1:10 ratio of sanitizer and deionized water. Clorox® Germicidal bleach with 5.75% available chlorine (Germicidal Bleach, Clorox

Company, Oakland CA) and Pure Bright™ Germicidal bleach with 5.75% available chlorine (Pure Bright Ultra Bleach, KIK International Inc., Ontario, CA) were used as our model commercial chlorine sanitizers, and a sodium hypochlorite solution with 5% available chlorine as our control solution, based on its use in previous published work (Ricca Chemical Company LLC, Arlington, TX).

5.2.6 Screening of commercial chlorine based systems sanitizers in high and low organic load solutions

Two commercial brands of chlorine sanitizers, being Clorox® Germicidal Bleach and Pure Bright™ both of which yielding 5.75% of available chlorine, were compared to concentrated solution of sodium hypochlorite with 5% available chlorine. This in efforts to evaluate the antimicrobial capabilities of a commercial product when compared to an antimicrobial solution commonly used in previous published work. Sanitizers where tested in organic load solutions of 0 and 700 mg/L inoculated with 7 log CFU/ml of E. coli O157:H7 by adding 25ppm of the sanitizer. After the addition of the sanitizer, samples were taken at 30 seconds and 30 minutes and tested the free residual chlorine concentration and the microbial load in solution. Microbiological analysis was performed for samples at all three time points, plated on TSA treated with 100µg/ml of nalidixic acid and incubated for 24 hours at 37°C.

5.2.7 Statistical Analysis

Three samples were taken for each treatment and all experiments were performed in triplicate. Data was partitioned and assesses by an F-test and Analysis of variance (ANOVA), Duncan's

Multiple Range Test were performed using SAS where statistical significance was set at $p < 0.05$ (SAS Institute Inc., Cary, NC, USA).

5.3 Results & Discussion

5.3.1 Chlorine depletion at increasing organic load levels

Free residual chlorine levels were tested at organic load levels of 0, 100, 400 and 500 mg/L treated with 25 ppm of a Clorox® Germicidal Bleach. Samples were prepared using baby spinach and deionized water filtered through cheesecloth to generate a homogenous solution with dissolved solids. Varying concentrations of organic load were chosen to evaluate the effects the amount of organic load present in solution to the depletion of antimicrobial chemicals. Analysis was done on each sample after adding and mixing sanitizer solution for 30 seconds.

Results showed that free residual chlorine levels significantly depleted once organic load was present in solution, as seen in **Figure 10**. Data showed a significant interaction between the presence of organic matter and the amount of free residual chlorine present in solution ($p < 0.05$). Sanitizer depletion behavior observed across all levels of organic load of 100, 400 and 500 COD (mg/L) showed a significant difference when compared to solutions not containing organic load (0 COD), showing that an increase in organic load would cause a significant decrease in initial free residual chlorine levels in solution. Upon the introduction of organic load into solution free residual chlorine levels showed a significant reduction in initial sanitizer levels from 25 ppm added to 19, 20, 21 ppm in 100, 400 and 500 COD samples respectively. However, while there was an observed initial depletion, results showed that 100, 400 and 500 COD were statistically similar, meaning there was no significant difference in initial sanitizer concentration at increasing levels of organic load.

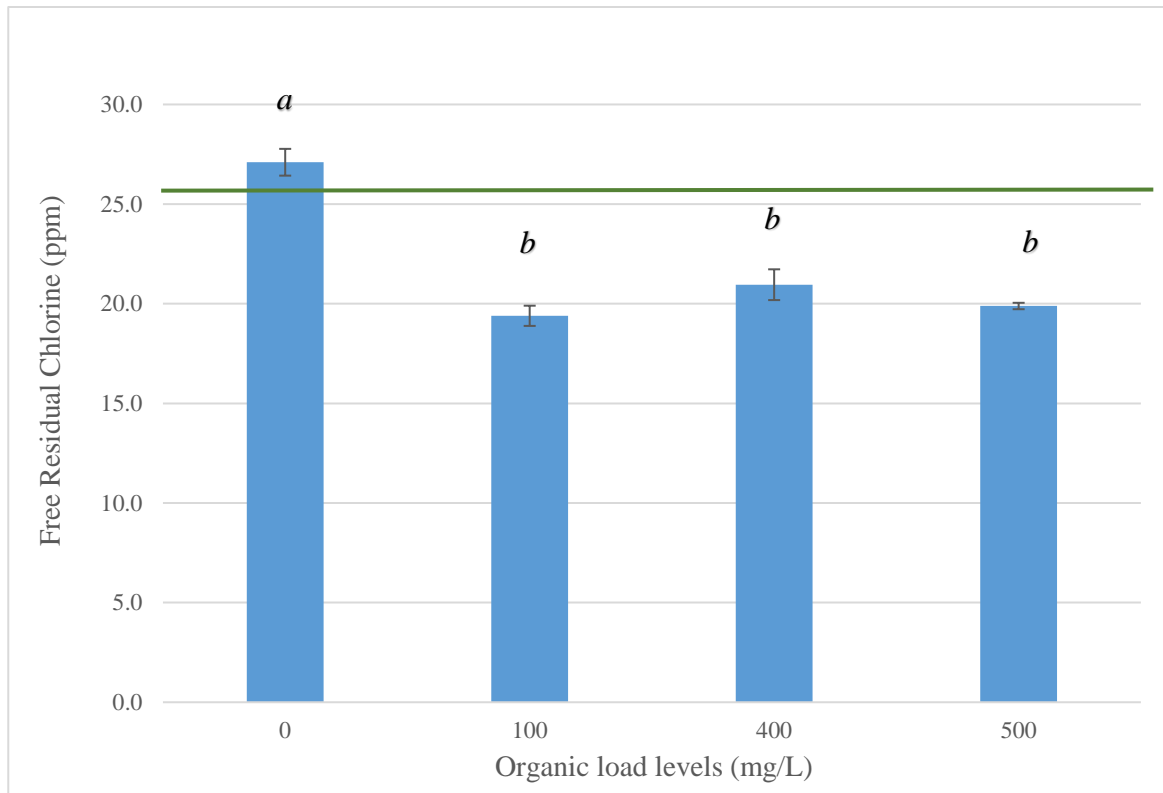


Figure 10: Free residual chlorine levels at increasing organic load after mixing for 30 seconds

Different letter grouping shows statistical differences among organic load sanitizer combinations using Duncan Multiple Range test (P=0.05). Experiment conducted in triplicate. Line represents amount of sanitizer initially added (25ppm Clorox® Germicidal bleach).

Upon introduction of organic matter into a wash water solution, free available chlorine concentrations are known to rapidly decline (Gombas et. al 2017). Data shown in **Figure 10** shows how the introduction of organic load can rapidly impact the concentration of residual chlorine in solution in which the rapid reaction between organic load and chlorine can cause the discrepancy in sanitizer concentration added and the concentration detected (Gomez-Lopez, Lannoo, Gil and Allende, 2014; Shen, Luo, Nou, Wang and Millner, 2013; Zhou, Luo, Nuo, Lyu, and Wang 2015; Zhou, Luo, Nou and Millner; 2014).

5.3.2 Chlorine concentration overtime at high and low organic load levels with and without *E. coli* O157:H7

In previous studies, results showed that the presence of organic matter in solution had a significant effect on the depletion of sanitizer concentration, as also seen in previous published work (Gomez-Lopez et. al, 2014; Luo, 2007; Luo, Nou, Millner, Zhou, Shen, Yang, Wu, Wang, Feng, and Shelton, 2012; Zhou et. al, 2015). This study focused on the interaction between organic load and time on sanitizer concentration, since contact time and sanitizer concentration are two main factors in pathogen inactivation in produce washing systems (Gombas et. al 2017). Samples were tested at 30 seconds, one hour and two hours (Time 0, 1 and 2 respectively) after the addition of 25ppm of Clorox® Germicidal bleach based on EPA Reg. No. 5813-1 (EPA, 2007). The interaction between organic load and time was highly significant ($p < 0.001$) showing that the presence of organic matter had a significant effect on free residual chlorine depletion at 100 and 700mg/L for overtime as seen in **Figure 11.A**. Free residual chlorine depletion pattern overtime for both organic load levels (100 and 700 mg/L) was consistent with that observed in previous experiments where the presence organic material, showed a significant effect on the depletion of free residual chlorine sanitizer like that seen in **Figure 11.A**. Significance was observed at time 2 and time 3 (1 hour and 2 hours after mixing, respectively). While the free residual chlorine levels continued to deplete overtime, our results showed that after 30 minutes Clorox® Germicidal bleach was able o inactivate the bacteria present (data not shown).

For the second study, all combinations of organic load and sanitizer, samples were inoculated to obtain 10^7 CFU/ml of *E. coli* O157:H7 in stationary phase (refer to **appendix** for *E. coli*

O157:H7 growth curve). We analyzed the interaction between organic load, time and inoculum on sanitizer concentration over a period of two hours.

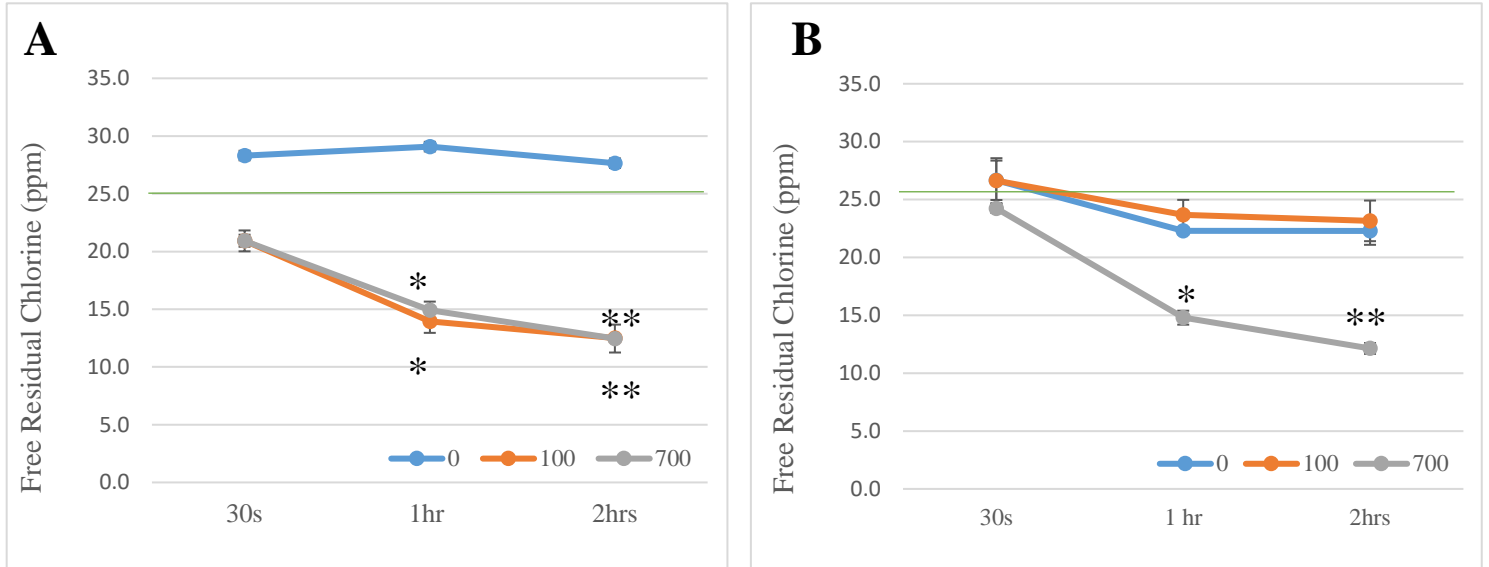


Figure 11: Sanitizer depletion overtime

Sanitizer levels were tested at three time points- 30s, 1 hr. and 2hrs - after adding 25ppm

^A Shows the free residual chlorine depletion overtime without bacteria

^B Shows the depletion of free residual chlorine overtime with *E. coli* O157:H7 at 10^7 CFU/ml

*Represents highly statistical difference between 0, 100 and 700 COD at 1 hour ($P < 0.001$)

** Represents highly statistical difference between 0, 100 and 700 COD at 2 hours ($P < 0.001$)

Line represents initial sanitizer concentration added (25 ppm Clorox® Germicidal bleach)

The interaction between organic load and time showed to be highly significant ($P < 0.001$), showing that high organic load solutions cause a higher depletion of sanitizer concentration overtime. Highly significant differences in sanitizer concentration were observed at time 2 and time 3 (1 hour and 2 hours after mixing, respectively) in 700 COD samples as seen in **Figure 11.B**. The presence of bacteria showed no significant interaction with the sanitizer depletion over time. At high organic load (700mg/L), the sanitizer depletion pattern was consistent with our previous studies, regardless of the presence of bacteria in solution. However, in low organic load solutions (100mg/L) the presence of bacteria had a significant effect on sanitizer concentration, where in

solutions inoculated with 7 log CFU/ml of *E. coli* O157:H7 sanitizer concentration showed to deplete less overtime when compared to non-inoculated samples. Results shown in **Figure 11. A & B.**

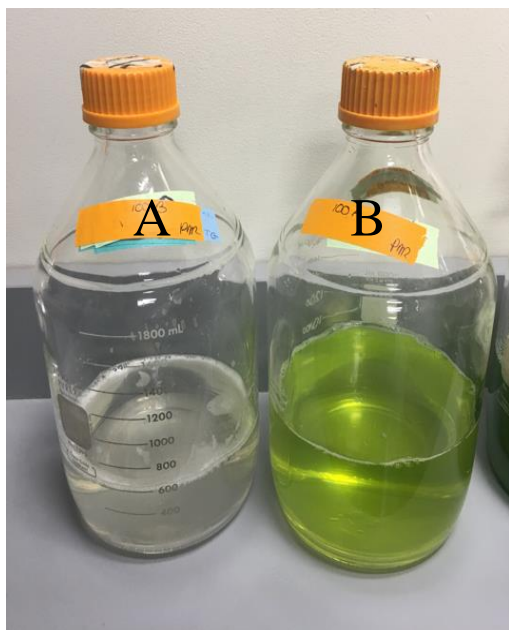


Figure 12: 100 mg/l solutions treated with 25ppm of sanitizer

- A. Without Bacteria
- B. With Bacteria

COD solutions of 100 mg/L treated with 25ppm of sanitizer can be seen in **Figure 12.** Sample A (to the left) was not inoculated with *E. coli* O157:H7, while sample B (to the right) was. Upon looking at both solutions we can see that the inoculated solution maintains its vibrant green color, whereas the non-inoculated solution is colorless. This phenomenon was observed during our study after adding sanitizer to the solution, where inoculated samples at 100 COD (mg/L) showed less sanitizer depletion overtime when compared to non-inoculated samples (results shown in **Figure 11. A & B.**) The vibrant green color began to fade approximately at 10 minutes after adding the sanitizing

solutions, until it became colorless and remained this way during the course of the two-hour study.

Virto, Manas, Alvarez, Condon, & Raso (2005) showed that Gram-negative microorganisms can have an increased resistance to free residual concentrations than Gram-positive microorganisms. This study also showed that the presence of TSB in solution, in this case our simulated inoculum in wash water, can have an increase microbial resistance to chlorine (Virto, et al, 2005). Previous studies have also shown that that organic matter can have a protective effect against chlorine, in which this effect would result in a higher chlorine demand of organic compounds which in turn

would cause a rapid decline in available free chlorine (Kotula, Kotula, Rose, Pierson, and Camp, 1997; Lyndon, and Gordon, 1998; Nikaido, 1996).

Overall, results show that both organic load and time are two main factors affecting sanitizer depletion. In the case of low organic load samples, like 100 COD (mg/L), when conducting bench top trials without presence of bacteria, the properties of the solutions can be affected with the use of a chlorine based antimicrobial solution. The use of both a chlorine based sanitizer and low levels of organic load used for simulated wash water, like 100mg/L solutions, should be taken into consideration we concluding laboratory trials for sanitizer efficacy studies.

5.3.3 Screening of commercially available chlorine based sanitizer and their efficacy in reducing E. coli O157:H7 loads in high and low organic load solutions.

For our final study we compared two commercially available chlorine based sanitizers- Pure Bright™ Germicidal bleach and Clorox® Germicidal Bleach, with 5.75% of sodium hypochlorite active ingredient, against a common sanitizing agent used in research work which concentrated sodium hypochlorite is yielding 5% free residual chlorine. The reagents are shown in **Figure 13**. Our goal with this study was to evaluate the microbial inactivation capability of commercially available products to a concentrated sodium hypochlorite solution. The study also, aimed to assess the efficacy of chlorine based sanitizers and their depletion overtime; in efforts to

provide best practice recommendations for postharvest wash water quality controls for small and medium leafy green processors.

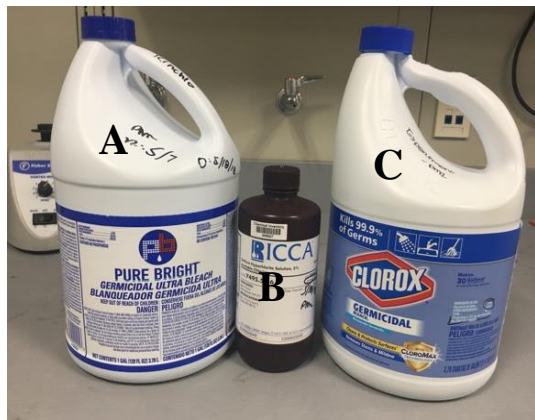


Figure 13: Chlorine solutions for sanitizer screening study

- A. Pure Bright™ Germicidal bleach
- B. Sodium Hypochlorite 5% residual chlorine
- C. Clorox® Germicidal bleach

All three sanitizers were tested in both no and high organic load environments inoculated to obtain 10^7 CFU/ml of *E. coli* O157:H7. We tested free residual chlorine levels at two separate time points, 30 seconds and 30 minutes (Time 0 and 1 respectively) based on the results obtain in our previous studies were Clorox®

Germicidal bleach completely inactivated the microbial load present in solution after 1 hour. We also determined the microbial load present in solution at

three time points, before the addition of sanitizer and after adding sanitizer at 30 seconds and 30 minutes (Time 0, 1 and 2 respectively). All solutions were maintained at room temperature and optimal pH for chlorine disinfecting performance, between 6.5-7.5 (Gombas et. al 2017).

Free residual chlorine levels were tested for all three sanitizers at 0 and 700 mg/L, in presence and absence of *E. coli* O157:H7, during a period of 30 minutes chosen based on results obtained from previous experiment looking and the sanitizer depletion overtime. Results showed that the interaction between organic load, bacteria and time was significant. All three sanitizers- Pure Bright™ Germicidal bleach, Clorox® Germicidal Bleach and sodium hypochlorite concentrated solution- showed a significant reduction in free residual chlorine levels after 30 seconds and 30 minutes after being added to high organic load environments of 700mg/L. The interaction between organic load and time was significant for all three sanitizing solutions at 30 seconds after the addition of the sanitizing solution. However, 30 minutes after adding sanitizer

only Pure Bright™ Germicidal bleach and Clorox® Germicidal Bleach sanitizer showed a significant depletion at 700 mg/L, as seen in **Figure 14**.

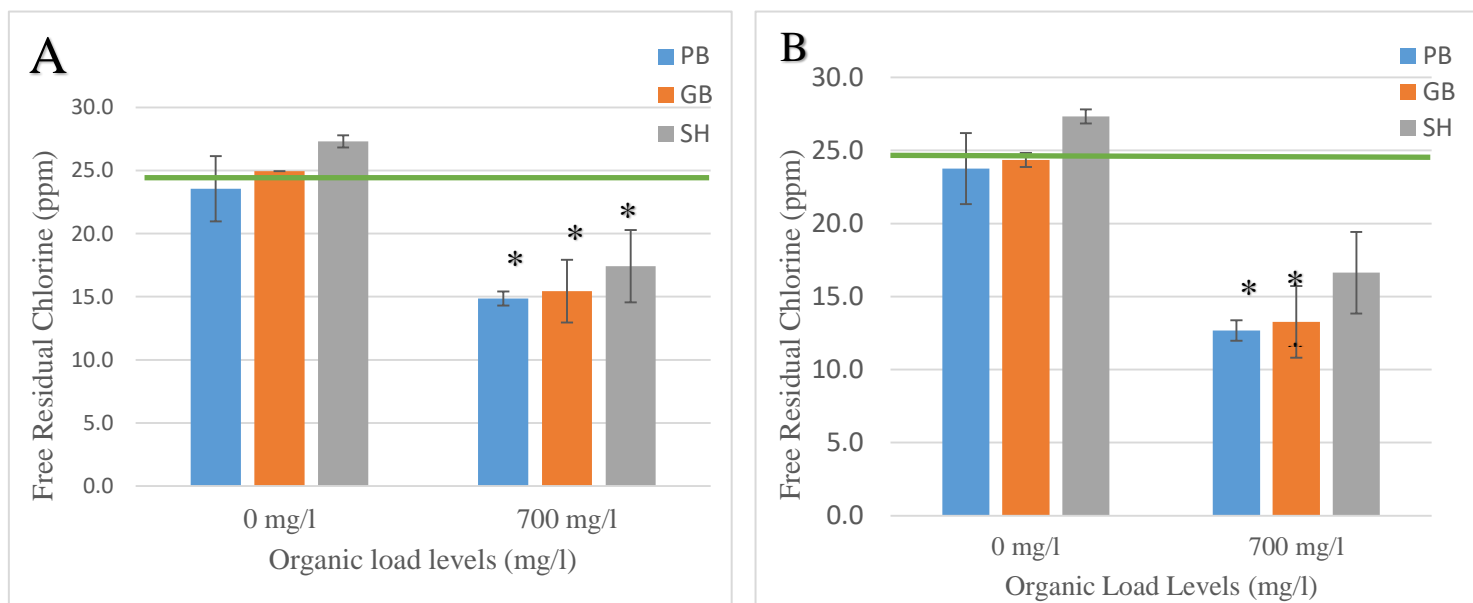


Figure 14: Comparison between sanitizers and their depletion rates in low and high organic load levels

^A Sanitizer Depletion after 30 seconds

^B Sanitizer Depletion after 30 minutes

*Represents highly significant difference in free residual chlorine levels between 0 COD and 700 COD ($p < 0.0001$).

PB: Pure Bright™ Germicidal Bleach, **GB:** Clorox® Germicidal Bleach, **SH:** Sodium Hypochlorite

For sodium hypochlorite sanitizer there was an initial reduction immediately after adding sanitizer in high organic load solutions of 700 mg/L, and same was observed for Clorox® Germicidal Bleach and Pure Bright™ Germicidal Bleach. However, after 30 minutes the free residual chlorine levels did not change, in comparison to Pure Bright™ Germicidal Bleach and Clorox® Germicidal Bleach where the free residual chlorine levels continued to deplete. After 30 seconds of the addition of the sanitizers, all three sanitizers showed no significant difference amongst each other at 700 mg/L and all showed a significant depletion in comparison to the concentrations seen at no organic load solutions (Control- 0mg/L) as seen in **Figure 4**. However,

after 30 minutes' sodium hypochlorite sanitizer maintained its free residual chlorine levels observed at 30 seconds (**Figure 14. A & B**); whereas both Pure Bright™ Germicidal Bleach and Clorox® Germicidal Bleach levels were lower than those observed in **Figure 14.A & B**. Sanitizers showed no significant difference amongst each other at 700 mg/L 30 seconds after the addition of each. However, after 30 minutes' sodium hypochlorite sanitizer maintained its free residual chlorine levels the same); whereas both Pure Bright™ Germicidal bleach and Clorox® Germicidal bleach levels were lower than those observed in **Figure 14.A& B**.

Even though both Pure Bright™ Germicidal bleach and Clorox® Germicidal Bleach showed a more rapid depletion of free residual chlorine at 700 mg/l after 30 minutes in comparison to the concentrated sodium hypochlorite solution, all sanitizers were able to inactivate 7 logs CFU/ml of *E. coli* O157:H7 after 30 minutes, results shown in **Table 11**. However, when looking closely at their disinfecting performance in reducing 7 log CFU/ml of *E. coli* O157:H7 over a period of 30 minutes, at 0 mg/l there was a significant difference between Clorox® Germicidal Bleach and both Pure Bright™Germicidal bleach and sodium hypochlorite. Both Pure Bright™Germicidal bleach and sodium hypochlorite were able to reduce 7 log CFU/ml of *E. coli* O157:H7 after 30 seconds, whereas it took Clorox® Germicidal Bleach 30 minutes to reduce the 7 log CFU/ml of bacteria in solution. At 700 mg/l all three sanitizing solutions were able to reduce the 7 log CFU/ml of bacteria in solution after 30 minutes. Organic load and TSB solution used as a growth medium can play a protective role on the bacteria itself (Virtro, et al, 2005) causing the sanitizers activity to take longer in order to eliminate the microbial load present.

Table 8: Comparison of reduction times of microbial counts of *E. coli* O157:H7 using commercially available chlorine based antimicrobial

Antimicrobial Solutions	Time 1 (Before sanitizer)		Time 2 (30 seconds)		Time 3 (30 minutes)	
	0 COD	700 COD	0 COD	700 COD	0 COD	700 COD
Pure Bright™	6.7	6.9	nd	6.5	nd	nd
Clorox®	7.0	6.9	5.1*	6.4	nd	nd
Sodium Hypochlorite	6.8	6.8	nd	6.5	nd	nd
No Sanitizer	7.1	7.0	7.0*	7.0	7.0*	7.0*

Organic load solutions were treated with 25ppm of chlorine based sanitizer and 7 log CFU/ml of *E. coli* O157:H7.

PB: Pure Bright Germicidal Bleach, **GB:** Clorox Germicidal Bleach, **SH:** Concentrated Sodium Hypochlorite, **NS:** No sanitizer/Control

Time 1: Before sanitizer, **Time 2:** After sanitizer, 30 seconds, **Time 3:** After sanitizer, 30 min

* Significant difference (p<0.05)

nd: Not detected

Both Pure Bright™ Germicidal bleach and Clorox® Germicidal Bleach are labeled as germicidal bleach and both fall under the same regulations by the EPA where a maximum of 25ppm can be used for fruit and vegetable washing (EPA, 2007; EPA 2010). Both of them use sodium hypochlorite as an active ingredient and both state yield 5.75% free residual chlorine. Such similarities would suggest a similar disinfectant activity, however it took Clorox® Germicidal Bleach up to 30 minutes to inactivate the microbial load in solutions, whereas Pure Bright™ Germicidal bleach was able to do it in just after 30 seconds. The MSDS for both products do not contain information about the other ingredients used, which makes it challenging to identify the specific differences between the products and how the other ingredients may play a role in the disinfecting capacity. Sodium hypochlorite concentrations for both were labeled as trade secret, where Pure Bright™ Germicidal bleach states it contains 5-7% of sodium hypochlorite, and

Clorox® Germicidal Bleach contains 5-10% sodium hypochlorite (Refer to **Figures 17** and **18** in appendix for label information). Clorox® Germicidal Bleach claims that it can eliminate *E. coli* O157:H7 within 5 minutes of contact, however based on our results this is more likely to be in the case in no organic load solutions (0mg/L), than in high organic load environments. At high organic load levels of 700 mg/l all three sanitizer showed the same efficacy behavior in reducing *E. coli* O157:H7 present in solution after 30 minutes. However, with on organic load (0mg/L) both PB and SH were able to eliminate the 7 log CFU/ml of bacteria present after 30 seconds, whereas GB was not able to achieve 7 log CFU/ml of bacteria present in the same time. Overall, results showed that Pure Bright™ Germicidal Bleach performed the best at reducing 7 logs CFU/ml of *E. coli* O157:H7 in high organic load solutions overtime when compared to Clorox® Germicidal bleach.

5.4 Conclusion

Data showed that the presence of organic load in in simulated wash water solutions had a significant effect on free residual chlorine levels on antimicrobial solutions studied. High organic load solutions at 700 mg/L showed a significant reduction of free residual chlorine levels at overtime (period of 2 hours). Sanitizer screening study, results showed all three antimicrobial solutions (Pure Bright™ Germicidal bleach, Clorox® Germicidal bleach and solution of sodium hypochlorite) effectively inactivated 7 log CFU/ml in both high and low organic load environments after a period of 30 minutes. In no organic load samples both Pure Bright™ Germicidal bleach and sodium hypochlorite were able to eliminate the 7 log CFU/ml of bacteria present after 30 seconds. However, Clorox® Germicidal bleach was unable to achieve a 7 log CFU/ml of within 30 seconds after the addition of the sanitizer. While both Pure Bright™ Germicidal Bleach and Clorox® Germicidal bleach are sanitizer options for both produce washing and food contact surfaces, results

showed that Pure Bright™ Germicidal Bleach performed better by inactivating the bacteria present within 30 seconds. Future studies can focus on evaluating the changes in performance of commercial sanitizing products over a long period a time by mimicking on farm conditions, like temperature variation and exposure to air, and how sanitizing capabilities withstand at high organic load environments.

CHAPTER 6

SUMMARY AND CONCLUSION

Simulated wash water preparation methods comparison study, no significant difference was observed between methods at on-farm observed conditions of COD and NTU, being 50 and 100 COD and 100 NTU respectively. However, at increasing levels of organic load there was a significant difference at high levels of organic load of 1,500mg/L where a blender method yielded a higher turbidity than the Stomacher both at the same COD concentration. These results showcase the need for a standard method of simulated wash water replication model in efforts to control variability introduced by the preparation method in efforts to compare sanitizer efficacy studies for produce washing systems.

Besides preparation methods, free residual chlorine detection methods were compared use. Two being standard titration method and one being a rapid commercial test kit. DPD and IOD titration methods when compared to a rapid test kit like HACH free chlorine test kit showed to be a better fit for laboratory studies by providing more accurate measurements of sanitizer concentration in organic load heavy solutions. However, when comparing titration methods side by side a significant difference was observed between methods at increasing levels of organic load of 100, 400 and 500 mg/L. The presence of high levels of organic load interfered with the formation of the Würster dye in the DPD titrations, inhibiting the titration method to adequately detect the endpoint, and thus potentially reporting inaccurate values.

When studying the depletion of free residual chlorine overtime, our results showed that the presence organic load had a significant effect on free residual chlorine levels when compared to those seen in no organic load solutions. Our results also showed that in the presence and absence

of bacteria can play a major role in the depletion of free residual chlorine in low organic load solutions like that of 100 mg/L (based on our studies).

When comparing commercially available chlorine based sanitizers to a concentrated sodium hypochlorite solution, Pure Bright™ Germicidal Bleach performed the best when compared to Clorox® Germicidal bleach. While both commercial sanitizers took an average of 30 minutes to inactivate 7 log CFU/ml of *E. coli* O157:H7 at high organic load levels (700 mg/L), in absence of organic load (0 mg/L) Clorox® Germicidal bleach still took an average of 30 minutes to eliminate the bacteria present, whereas Pure Bright™ Germicidal Bleach was able to inactivate the bacteria present within 30 seconds after adding the antimicrobial solution. While both commercial products seemed almost identical in chemical composition (based on label information), future work can investigate the chemistry and kinetics of each sanitizer in simulated wash water solutions and how different microorganisms how the chemical composition changes can play a role in the disinfecting capabilities commercial sanitizers.

APPENDIX

SUPPLEMENTARY TABLES AND GRAPHS

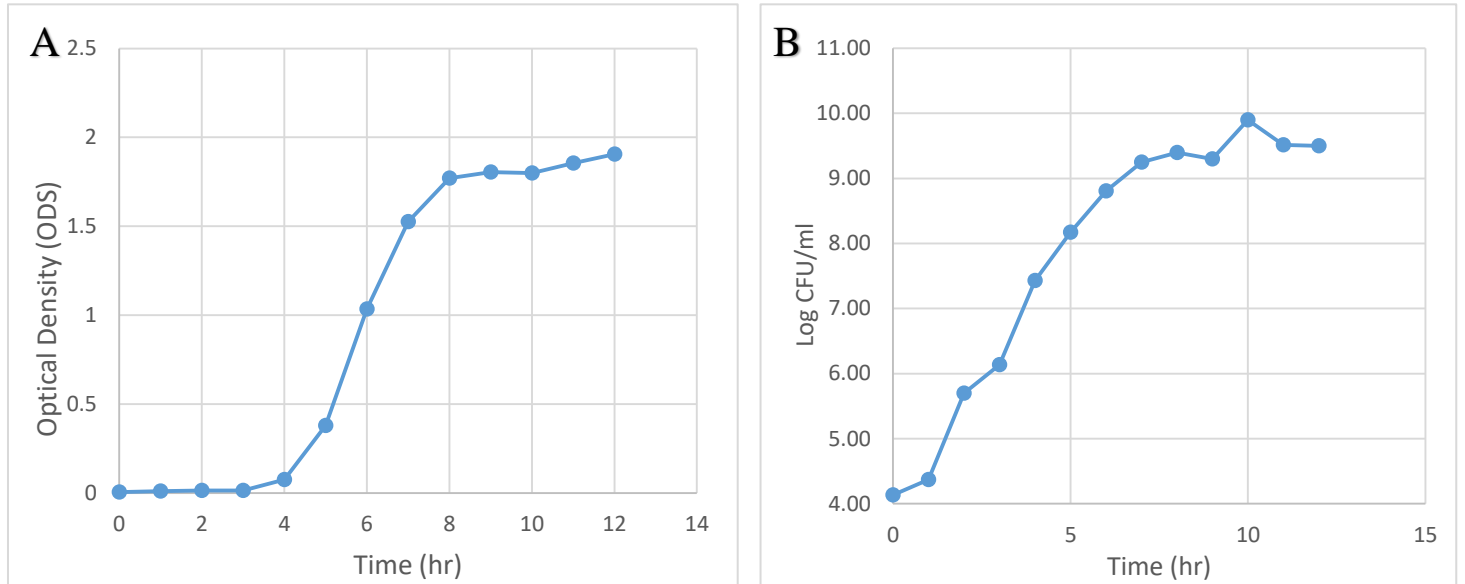


Figure 15: *E. coli* O157:H7 48934 Growth Curve of 100ug/ml of NAL expressed as ODS (left) and log counts (right) as a function of time

The growth of *E. coli* O157:H7 ATCC 43894 was evaluated over a 12-hour period testing both ODS and growth on TSA treated with 100ug/ml. Growth curve was replicated three times, and averages are represented in graphs above

A: Optical Density

B: Microbiological Growth on TSA

ACTIVE INGREDIENT:
SODIUM HYPOCHLORITE: 6.0%
OTHER INGREDIENTS: 94.0%
TOTAL: 100.0%
 (Yields 5.7% Available Chlorine.)
CONTAINS NO PHOSPHORUS

INGREDIENTE ACTIVO:
HIPOCLORITO DE SODIO: 6.0%
OTROS INGREDIENTES: 94.0%
TOTAL: 100.0%
 (Proporciona 5,7% de cloro disponible.)
NO CONTIENE FOSFORO

PURE BRIGHT™
GERMICIDAL ULTRA BLEACH **BLANQUEADOR GERMICIDA ULTRA**
KEEP OUT OF REACH OF CHILDREN DANGER **CONSERVESE FUERA DEL ALCANCE DE LOS NIÑOS PELIGRO**

FIRST AID: Call a poison control center or doctor for treatment advice. Have the product container or label with you when calling a Poison Control Center (1-800-222-1222) or doctor, or going for treatment. **IF IN EYES:** Hold eye open and rinse slowly and gently with water for 15-20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. **IF ON SKIN OR CLOTHING:** Take off contaminated clothing. Rinse skin with plenty of water for 15-20 minutes. **IF SWALLOWED:** Have person sip a glass of water if able to swallow. Do not induce vomiting unless told to do so by the poison control center or doctor. Do not give anything by mouth to an unconscious person. **NOTE TO PHYSICIAN:** Probable mucosal damage may contraindicate the use of gastric lavage. See back panel for additional precautionary labeling.

PRIMEROS AUXILIOS: Consulte a un médico o a un centro de envenenamiento para obtener asesoramiento sobre el tratamiento. Cuando llame a un centro de envenenamiento (1-800-222-1222), o a un médico, o intente obtener tratamiento, tenga a la mano el envase o la etiqueta del producto. **SI ENTRA EN CONTACTO LOS OJOS:** Mantenga los ojos abiertos y enjuague con agua lentamente, con cuidado, durante 15-20 minutos. Si hay lentes de contacto, retírelos después de los primeros 5 minutos, luego continúe enjuagando los ojos. **SI ESTA EN LA PIEL O EN LA ROPA:** Quítese la ropa contaminada. Enjuague la piel inmediatamente con bastante agua por 15-20 minutos. **SI SE INGERIE:** Si la persona puede tragar, haga que beba a tragos un vaso de agua. No induzca el vómito a menos que así se lo indique un médico o un centro de envenenamiento. No administre nada por la boca a una persona que haya perdido el conocimiento. **NOTA PARA EL MÉDICO:** Probable daño de las mucosas puede contraindicar el uso de lavado gástrico. Vea el panel posterior para instrucciones adicionales de precaución.

NET CONTENTS 96 FL OZ (3 QT) 2.84 L **CONTENIDO NETO 96 FL OZ (3 QT) 2,84 L**

Figure 16: Pure Bright™ Germicidal Ultra Bleach label information

Active Ingredient:
Sodium Hypochlorite 6.0%
Other Ingredients: 94.0%
Total: 100.0%
 (Yields 5.7% available chlorine)

KEEP OUT OF REACH OF CHILDREN DANGER: CORROSIVE.
FIRST AID: IF IN EYES: Hold eye open and rinse slowly and gently with water for 15-20 minutes.

Whitens

Figure 17: Clorox® Germicidal bleach label information

Disinfecting Profile Comparison*	Clorox® Germicidal Bleach	Pure Bright® Germicidal Ultra Bleach (KIK)
Concentration	8.25%	6.00%
Number of disinfecting claims	39	27
Registered to kill multiple strains of MRSA, including CA-MRSA	✓	
Registered to kill norovirus (leading cause of foodborne illness, including stomach flu ¹)	✓	
Registered to kill 2 of the leading viruses that cause the common cold	✓	
Contact time required to kill hepatitis B and C viruses	5 minutes	10 minutes
Number of human influenza strains product is registered to kill	3	2
Contact time required to kill <i>C. difficile</i> spores	5 minutes	10 minutes

Figure 18: Comparison between Clorox® Germicidal Bleach and Pure Bright™ Germicidal Ultra Bleach

Source: Cloroxprofessional.com

* Based on master label comparisons of Clorox® Germicidal Bleach and Pure Bright® Germicidal Ultra Bleach (KIK) as of Oct. 2012.

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