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Chlorine Decay And Pathogen Cross Contamination Dynamics In Fresh Produce Washing Process

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CHLORINE DECAY AND PATHOGEN CROSS CONTAMINATION DYNAMICS IN FRESH PRODUCE WASHING PROCESS

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DEDICATION

To my Father, who was an ocean of love, kindness and forgiveness

To my Mother, who defines resilience, sacrifies and hope

To my Brother Omid, who is brave and patriot

And to my beloved wife, Asal

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CHLORINE DECAY AND PATHOGEN CROSS CONTAMINATION IN FRESH PRODUCE WASHING PROCESS

MOHAMMADREZA DEHGHAN ABNAVI

ABSTRACT

In this study, we developed a comprehensive mathematical model to predict the free chlorine (FC) concentration and bacterial cross-contamination during produce wash processes. A second-order chemical reaction model for FC decay which utilizes a proportion of chemical oxygen demand (COD) as an indicator of organic content in the wash water was employed, yielding an apparent reaction rate of 9.45 $\pm$ 0.22 $\times$ 10^{-4} $\mu M^{-1}.min^{-1}$. Using a proportion of successive changes in COD in the wash water due to produce washing, typically ranging from 6 to 11% across produce types, the model was able to consistently predict experimental FC levels, however, we note that while the FC level drops, the COD level stays constant.

Therefore, we established the total amino acids concentration as an alternative indicator of organic load, and modified our model based on modeling the reaction kinetics of chlorine and amino acids. Apparent reaction rate between FC and amino acids was in the range of 15.3 – 16.6 $M^{-1}.s^{-1}$ and an amplification factor in the range of 11.52 - 11.94. This study also presents a modified disinfection kinetics model to evaluate the potential effect of organic content on the chlorine inactivation coefficient of *Escherichia coli* O157:H7 in produce wash process. While the chlorine inactivation coefficient of *E. coli* was 70.39 $\pm$ 3.19 $L.mg^{-1}.min^{-1}$ in the absence of organic content, it dropped by 73% for a COD level of 600 - 800 $mg.L^{-1}$.

Finally, the mechanisms by which FC inactivates *E. coli* was studied. Results showed that at low levels of FC and shorter exposure times, cell surface became rough and plicate; however, holes and wrinkles formed on the cell surface at higher FC concentrations or at longer exposure times, causing significant damage to the cell membrane. The cellular permeability changed due to chlorination, resulting in a significant decrease in the number

of viable cells. Besides, around $3.45\% \pm 0.62$ of cells lost their culturability and transform to viable but not culturable (VBCN) state during the disinfection process in low CT values ($> 0.2\ mg.min.L^{-1}$). Our studies have implications in the design of disinfection protocols relevant for produce wash industry.

Keywords: Fresh produce wash process, Free chlorine decay, Pathogen cross-contamination, *Escherichia coli* inactivation model, bacterial disinfection mechanisms.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Foodborne illnesses affect millions of people in the United States every year. According to the Center for Disease Control (CDC), each year roughly 800 foodborne disease outbreaks occur in the United States, resulting in 15,000 illnesses, 800 hospitalizations, and 20 deaths (Fig. 1.1) [1, 2]. Outbreak-associated foodborne disease are only a small portion of estimated 9.4 million foodborne illnesses from known pathogens that happen in US [3]. From 2011 to 2017, 5,896 outbreaks reported by CDC - Surveillance for Foodborne Disease Outbreaks – accounting for 99,660 illnesses, 6,176 hospitalizations, and 157 deaths (Fig. 1.1) [2]. Among outbreaks with a single confirmed etiology, norovirus was the most common cause of outbreaks with 2,000 outbreaks (34%) and outbreak-associated 39,202 illness cases (39%), followed by *Salmonella* with 948 outbreaks (16%) and 22,774 illnesses (23%) [2]. *Escherichia coli* (STEC) also was responsible for 191 outbreaks (3.2%) and 3,825 illnesses (3.8%). The food categories responsible for the most outbreak-associated illnesses were land animals (41%), aquatic animals (30%) and plants (26%) (Fig. 1.2) [2]. Among foods that could be classified into a single food category, fish had the highest records of outbreak (16%), followed by fruits (13%), and dairy (12%) [2].

One source of these outbreaks is fresh produce contamination. Vegetable and leafy greens associated outbreaks were 5% of all outbreaks [2]. Bacterial outbreaks of *E. coli* and *salmonella* have been linked to the consumption of fresh-cut produce [4, 5]. The fresh

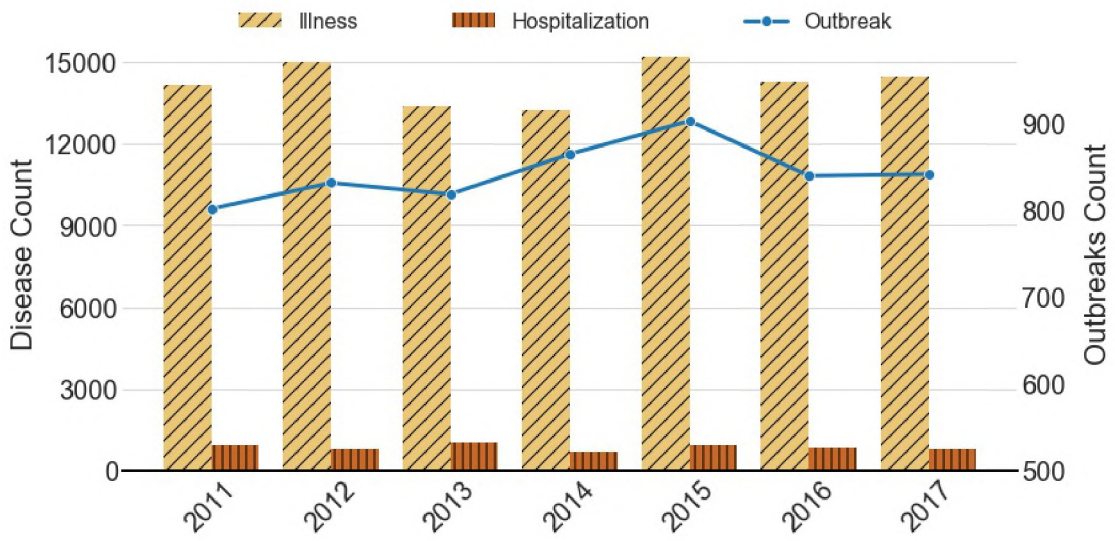

Figure 1.1: Foodborne disease outbreaks and outbreak-associated illnesses and hospitalization from 2011 to 2017—Foodborne Disease Outbreak Surveillance System, United States.

cut produce goes through the processes of harvesting, chopping, washing, and finally packaging. Before fresh-cut produce reaches the shelves of a supermarket, they must be washed and sanitized which is a crucial sanitization step in the produce industry. When the produce is washed, there is a possibility of bacterial cross-contamination from bacteria-infected batches to uninfected ones that were subsequently washed in the same water. Washing produce in water alone is insufficient; usually, some sanitizer must be used. Chlorine is a commonly used water-disinfectant in the produce washing industry as it is a readily available strong disinfectant. Chlorine is widely credited with virtually eliminating outbreaks of waterborne disease in the United States and other developed countries.

However, maintaining a stable free chlorine (FC) concentration during washing process is challenging [6]. The washing phase could be a major source of bacterial cross-contamination due to the decrease in the efficacy of chlorine-based sanitizers with increasing organic matter content in water [7, 8]. Chlorine will bind with organic matter that has been released by the fresh-cut produce instead of binding to the bacteria. If the organic matter levels are too high, the sanitizing effect of chlorine is reduced [9]. Likewise, chlorine levels (measured as free chlorine (FC)) must be continuously regulated to remain at

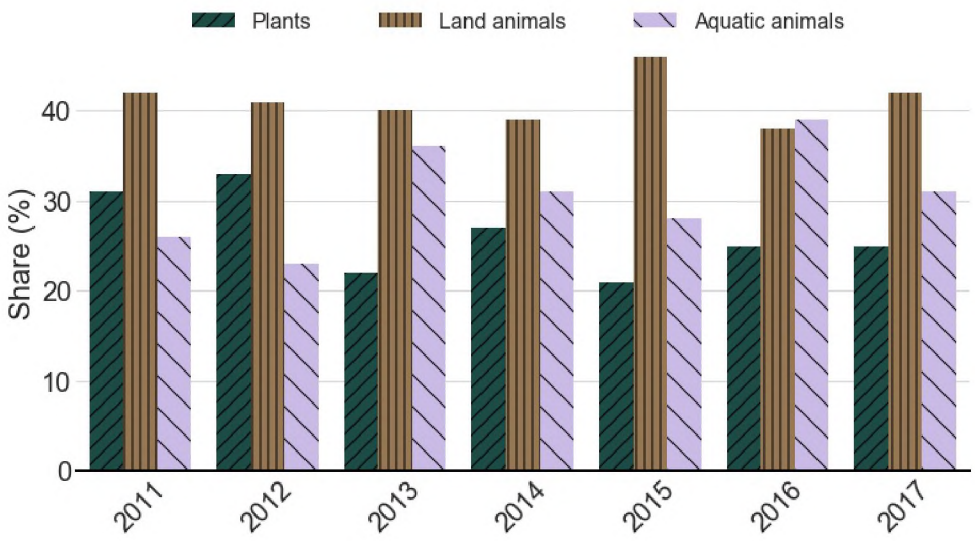

Figure 1.2: Percentages of foodborne disease outbreaks by food category—Foodborne Disease Outbreak Surveillance System, United States, 2011-2017

higher concentrations to allow better sanitation [10]. So, there is a critical need to minitor in real-time, the dynamic changes in organic matter concentration, FC concentration, and inactivation rate of pathogens during fresh-cut produce wash operations. Also, there is a need to maintain critical levels of FC in water during washing cycles to prevent the possibility of cross-contamination. However, it should be noted that too high levels of chlorine could be dangerous to the operating personnel in the washing plant as well as to the produce itself. Based on experimental data of washing operation, a quantitative mathematical model should be developed to quickly predict FC and bacteria levels based on the mass of produce washed, which allows for better control of FC level in the wash water.

1.1 Hazards of fresh produce and their sources

Fresh fruit and vegetables are recognized to be an important part of a healthy diet, as they provide necessary nutrients such as vitamins, minerals, fibers and antioxidants [11]. The consumption of fresh produce has increased considerably in the U.S. in recent decades, especially for pre-packed, ready-to-eat fresh-cut produce [12]. The average per capita use of fresh vegetables increased 67% from 86.9 lbs in 1970 to 145 lbs in 2017 [13]. Produc-

tion of fresh-cut and value-added produce has also grown significantly. Sales of bagged salad in U.S. supermarkets surged from $197 million in 1993 to $3.7 billion in 2015 [14]. Fresh produce after harvesting goes through various processing stages including storage, trimming, chopping, washing or sterilizing, packaging, cold storage and distribution. The most important and challenging step among these is washing. The main goal of washing the produce is to remove microorganisms and any source of dirt. Since none of these other processing steps involves inactivation of microorganisms, washing plays a very important role in sanitation of produce because it is the only step that removes the microbial load from the produce [15]. Leafy greens are relatively susceptible to pathogen microbial contamination [7] and fresh produce are a common source of microbial outbreaks [7, 16]. The demand for a wide choice of fruit and vegetables has increased the number of potential commodities implicated in foodborne outbreaks. In addition, consumers tend to buy fresh produce often at large supermarkets instead of local shops, enabling a batch of pathogen-infected produce to simultaneously reach a larger number of consumers [12, 17]. There are a multitude of pathogens that have been associated with fresh produce including bacteria *Salmonella spp.*, *Listeria monocytogenes* (*L. monocytogenes*), *E. coli* O157 and non O157 VTEC, *Shigella spp.*, *Campylobacter spp.*, *Yersinia enterocolitica*, *Clostridium spp.*, *Bacillus cereus.*, *Staphylococcus aureus.*), *protozoa* (*Cyclospora cayetanensis*, *Cryptosporidum spp.*, *Giardia spp.*), viruses (*Hepatitis A, norovirus*) and helminths [18, 19, 20, 21, 22]. Among fresh produce, leafy greens were primary food group of concern, while *Salmonella spp.*, *E. coli* O157:H7 or norovirus are the pathogens of greatest concern, due to their major occurrence [19, 12]. In 2018, an outbreak caused by *E. coli* linked to Romaine Lettuce has sickened almost 60 individuals and killed two in the U.S. and Canada. Around 173 produce-associated outbreaks occurred from 1996 to 2014, bringing about 17,212 instances of ailment and 69 deaths. Among these outbreaks, 25.43% were caused by contaminated leafy greens and 46% were connected to fresh-cut produce [1].

Pathogen contamination on fresh produce can be due to contaminated seeds and seedlings,

manure and soil, animals, and water [23]. The used water needs to be of enough initial microbial quality. Water can serve as source of or as a vector for transport of pathogens to plants and crops [18, 20, 21]. Several outbreaks have been traced back to the use of contaminated irrigation water [24, 25, 21]. Contamination occurring on-farm can persist and spread during post-harvest processing [26]. A major part of post-harvest processing of leafy greens involves washing with water in flumes or tanks where a large quantity of produce mingles [27, 23, 28]. Pathogenic microorganisms present in individual leaves can be dislodged and spread to other leaves via the wash water. As produce wash water is often reused, bacteria present in the water could further spread to subsequent batches of produce and result in widespread contamination. The potential for batch cross-contamination during post-harvest washing of fresh-cut leafy greens has been demonstrated [7, 28]. Industry and government rules have proposed the utilization of sterilizing chemicals in wash water to avoid microbial cross-tainting and have suggested that the level of antimicrobials in wash water be checked at a recurrence adequate to keep up clean conditions [29, 30, 31]. Washing produce by tap water will reduce the microbial load to some degree. This process can be improved by adding a sanitizer to the water in order to disinfect the produce or decontaminate [28]. Naturally-present microorganisms are not removed from the produce by using only tap water. The success of this process is limited to 1 or 2 log reduction of microbial load (Fig. 1.3) and the total removal of microorganisms cannot be achieved [7]. This is because part of the microorganisms is firmly attached to the surface, sometimes in hard to reach crevices or irregular surface structures. In addition, they might form biofilms, or become internalized within the plant tissues through stomata, cut surfaces or other tissue wounds, or in the preharvest stage via the root system, although the significance of the latter is far from confirmed [32, 23]. Also, the washing process can be a potential pathway of infection among produce [28] and utilizing extensive amounts of water do not minimize the danger of cross-contamination [33].

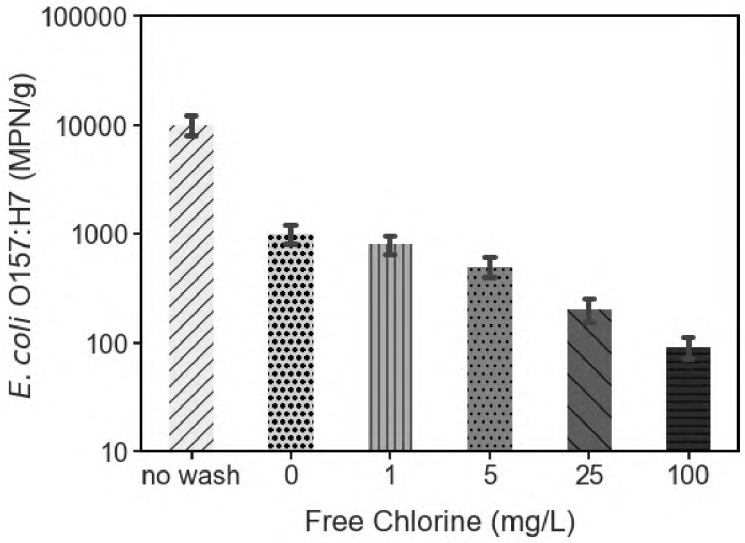

Figure 1.3: *E. coli* O157:H7 populations recovered from inoculated fresh-cut romaine lettuce after washing in solutions containing 0 to 100 mg/L free chlorine. Data presented are the means of five replicate experiments with duplicate samples per replication (adopted from Luo *et al.*, 2011).

1.2 Cross-contamination in fresh produce washing process

Washing is a critical step in fresh produce industries for the removal of dust, soil, debris, pesticides and above all, pathogenic microorganisms. But the washing step, if not properly managed, can not only reduce the microbial load of the product but also be a source of contamination [34, 35]. At the industrial scale, the washing management varies depending on: the type of product (leafy greens, carrots, fruits) and the format (whole product, fresh-cut, microgreens). Even with the same produce and cut size, there can be differences between management practices and washing equipments like sanitizer and washing types (pre-wash, single-wash, double-pass).

Microbial cross-contamination in fresh produce systems is the microbiological contamination of produce through contact with contaminated materials (e.g. water, produce, machinery) [36]. Recycling the water is crucial in fresh produce facilities to increase the sustainability of the industry process, but it can lead to a cross-contamination risk if inadequate practices are performed [37]. Also, cross-contamination can happen within the washing tank from contaminated produce to uncontaminated produce via the water. So, the

proper use of antimicrobial treatments is a crucial strategy to avoid cross-contamination via process water at industrial settings [38, 39]. Apart from the using of sanitizers, other factors of washing procedures that can impact the risk of microbial cross-contamination are produce to water ratio, produce type and cut size, and washing types. In addition, particulate material present in the wash water is a potential vector for cross-contamination with pathogens [40].

Moreover, when cross-contamination has happened, rewashing the recently contaminated produce with a chemical oxidant solution is not efficient enough to remove all microorganisms from the newly infected produce, even very soon after the infection [33, 7]. The efficiency of inactivation process of microorganism is not only restricted by the issue of obscure disinfection process but also is limited by the mechanism in which the chemical oxidant reacts with organic matter present in washing solution [32]. Identifying process parameters that may affect the efficacy of a produce wash in preventing pathogen cross-contamination helps to recognize microbial cross-contamination that can occur as a result of the competition between how fast a dislodged pathogen can travel from a contaminated leaf through wash water to other leaves and how fast the pathogen can be completely inactivated in the sanitized water during transit [41]. To effectively prevent microbial cross-contamination, the conditions used for washing need to maximize the rate of microbial inactivation in the wash water while minimizing the chance of pathogens moving from contaminated leaves to other leaves in the batch [23].

1.3 Fresh produce disinfection management

Food safety is one of the hottest and most developing research areas with more than 36,000 published papers in 2020 alone (Fig. 1.4). One third of the published papers in food safety is on fresh produce and leafy greens. Chlorination is a standout amongst the most broadly utilized procedures for microbial control [23] in drinking water and wastewater treatment, and in fresh produce washing industries [7]. Acoording to the web of science, 2.5% of

all published papers in food safety area were on the use of chlorine for disinfection (Fig. 1.4). Chlorine is a great antimicrobial because of its oxidizing properties. There are various utilizes for chlorine in food safety area, including removing of microbials on the surfaces of agricultural products, and sanitation of food processing equipment in food industries. The most critical drawback of chlorine is the formation and accumulation of disinfection by-products [42]. In terms of disinfection by-products formation, peracetic acid (PAA) can be the best alternative as a less controversial agent [39, 43]. PAA is efficient in preventing cross-contamination of pathogenic microorganisms during fresh produce wash [44]. Banach *et al.* (2020) [45] reported the suitability of PAA in fresh-cut lettuce washing at the industrial scale. But PAA is 4-8 times more expensive and has slower microbial inactivation kinetics compared to chlorine [46]. Although PAA has recently been more studied, the number of published papers is still less than 100 per year (Fig. 1.4), indicating the concern amongst some users. Ozone and UV are the most studied disinfection agents after chlorine (Fig. 1.4). However use of UV is highly cost-effective and good only for small disinfection processes. Ozone is also not good for produce wash industries since it needs complicated process with expensive equipment and facilities.

In order to achieve a fast removal of microorganisms, high concentration of disinfectants such as chlorine is required. At such high concentration, most of the bacterial load will not survive, but it can cause two major disadvantages: first, as mentioned previously, high concentration chlorination increases the risk of hazardous by-product formation [47, 48] and second it can compromise the texture of produce and make off-tastes and smells [46]. On the other hand, in case of using low chlorine concentration, there is more risk of cross-contamination [28]. Apart from the type of sanitizer and disinfection methods, using the appropriate Standard Operating Procedures (SOPs) for the correct practices is critical in fresh produce industries. SOPs are required to guaranty the safety of the products and also consider economic and environmental aspects. For example, the sanitizer concentration should be monitored regularly and its demand should be predicted as inaccurate

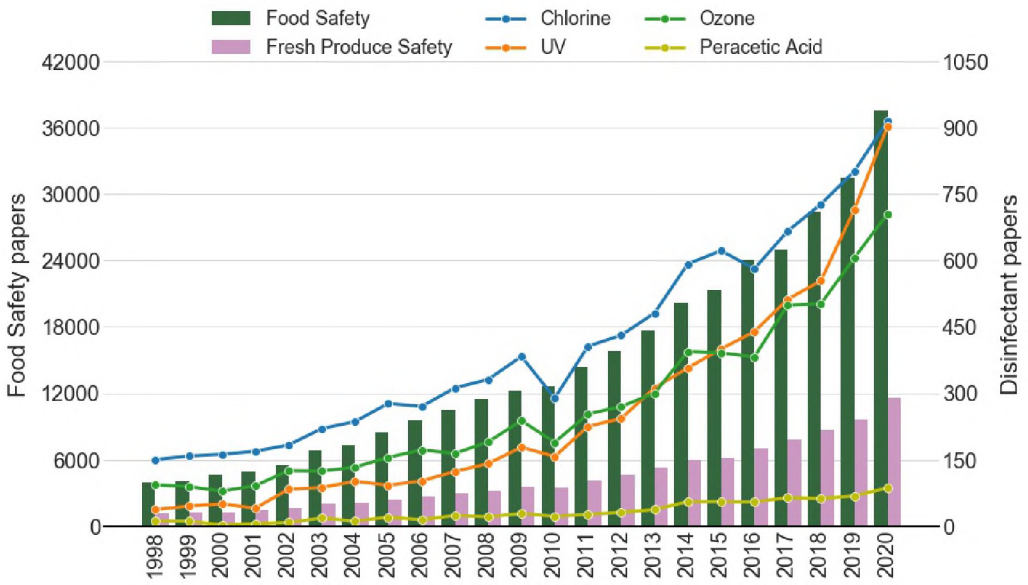

Figure 1.4: Number of published papers in Food safety and related papers to fresh produce safety and disinfectants used in fresh produce wash systems

measurements can lead to overdosing with high cost implications and health concerns or underdosing with unsafe washing practices. For instance if the used sanitizer is free chlorine (FC), the concentration of free chlorine as well as pH should be regularly monitored. Different free chlorine concentrations have been reported by different studies. Gombas *et al.* (2017) [49] and Luo *et al.* (2018) [14] reported that a minimum of $10\,mg.L^{-1}$ free chlorine for leafy greens. However, the operational limits depend on the type, cut size and produce to water ratio. Based on Tudela *et al.* (2019) [50], high concentration of FC ($> 30\,mg.L^{-1}$) should be used for baby leaves; shredded lettuce and cabbage need medium levels ($20 - 25\,mg.L^{-1}$), and diced onion can be washed in low levels ($10\,mg.L^{-1}$).

The maintenance of disinfectant during the produce wash depends on decay of disinfectant and how to exactly measure it and how to accurately predict its consumption. Sensors can be very helpful in measuring the concentration of sanitizers but for predicting the sanitizer demand, mathematical models are needed. The concentration of sanitizer reduces as more produce is washed due to the reaction with compounds that released to the wash water from produce. So, measuring parameters that are related to the physicochemical properties

of wash water is critical. For example oxidation-reduction potential (ORP) is a parameter that has been studied for the real-time measurement of chlorine by Van Haute *et al.* (2019) [51]. They concluded that multiple factors could affect the ORP and hence predicting free chlorine concentration by only ORP is not a good choice. Other studies reported that there is no correlation between free chlorine concentration and ORP [52]. Ghostlaw *et al.* (2020) [53] suggested that organic load, temperature and pH are factors that can be useful for predicting the PAA concentration and should be monitored to ensure that water quality and safety are maintained. For use of free chlorine as sanitizer regulating pH is critical since the microbial inactivation efficacy of free chlorine is higher when the pH is below 6.5 [54]. Accumulation of organic matter in the process water can reduce the sanitizer efficacy and also increase the formation of disinfection by-products. Teng *et al.* (2018) [55] reported that the presence of organic load reduced the efficacy of chlorine in inactivation of *Escherichia coli* O157:H7 even when the fluctuating chlorine level was avoided. In another study by Van Haute *et al.* (2018) [56] the use of UV absorbance for the real-time estimation of the chlorine demand of fresh produce wash water is assessed. However, the authors concluded that the application of this parameter can be hindered by factors such as the variability in the correlation between chlorine demand and UV absorbance among crops of the same vegetable and due to the possible interference by the pH regulators. Li *et al.* (2019) [52] suggested that total dissolved solids (TDS) is a promising parameter for predicting organic load and chlorine decay. Qi *et al.* (2020) [57] also investigated the effect of organic matter and concluded that UV254 is the primary indicator of the organic load effect. Measuring the demand for PAA in wash water is difficult because of the organic nature of this sanitizer, that is detected by common organic matter measurements (e.g. chemical oxygen demand, COD). In some cases, the measurement of turbidity could help for the assessment of the physicochemical quality of PAA-treated wash water [39].

1.4 chlorine in fresh produce washing process

The rate of microbial inactivation in a produce wash is dependent on the pathogen population, the sanitizer concentration and other factors that may influence pathogen survival or the sanitizer activity [33, 58]. Sodium hypochlorite (source of free chlorine) is the most commonly used antimicrobial for produce washing. Chlorine reacts through oxidation, addition and electrophilic substitution reactions with organic substances in the water; usually, electrophilic substitution (chlorination) is the predominant mechanism [59, 58]. Reactions with double bonds, alcohols and ketones are rather slow, whereas reaction with ammonia, aliphatic amines, amino acids and peptides (N atom of terminal amino-function is the target) occurs fast. Reaction rates with compounds containing reduced sulfur moieties are especially fast, and this includes the amino acids cysteine and methionine, proteins containing these amino acids, and the reducing compound gluthatione [60]. Chlorine can also react with several inorganic species present in water, such as reduced iron, arsenic and manganese, halides, and sulfide, etc. [60]. Inorganic chloramines and organic chloramines can be formed, due to reaction with ammonia and organic nitrogen compounds (particularly organic amino compounds) respectively [60].

Free chlorine (FC) is mainly present as hypochlorous acid (HOCl) in aqueous environments with neutral pH, which is the best form of chlorine for removing bacteria [50]. At higher pH, chlorine converts to the hypochlorite ion, OCl- which is less efficient disinfectant, however acidic pH forms hypochlorous acid. Since hypochlorous acid has higher disinfection efficiency than hypochlorite ion, it is better to have an acidic environment for washing produce [54]. However, working in the situations with pH $\approx$ 5 is dangerous because corrosion will be increased and forms gaseous form of chlorine (Cl2), which will in general be discharged from washing solution as off-gas in considerable amounts [61, 62]. A few studies have demonstrated that the pathogen removal viability of chlorine is subject to the convergence of hypochlorous acid and the residence time of produce in the wash solution [46]. But, a significant number of those studies haven't considered the impact of

organic matter in washing efficacy. Chlorine is very reactive and oxidizes organic matter rapidly, prompting quick decrease in its concentration and disinfection adequacy. This is particularly tricky for fresh cut produce wash, as considerable amount of organic matter enters the wash solution from cut area as tissue exudates, soil, and trash. So, in order to reimburse the loss of chlorine, it should be frequently added to the system. However, there is an absence of logical data relating to the planning and the capacity of the mediation to keep up the sanitation quality of the wash arrangement without acquiring the inconvenient reactions of overdosing.

The amount of free chlorine within wash tanks is dependent on several factors. For example, the pH influences the equilibrium between hypochlorite ion and hypochlorous acid, with neutral pH favoring the latter [49]. However, the major factor that governs the free chlorine concentration is the reactivity of the FC with organic and non-organic (ammonia) constituents to generate disinfection byproducts [63]. A diverse range of disinfection byproducts can be generated by reaction with chlorine, with trihalomethanes being predominant [9]. The formation of disinfection byproducts is of concern due to being carcinogens and negative impacts on the sensory characteristics of the product [63, 47, 64]. Of more concern is the significant decrease of free chlorine concentration due to the reaction with organic load [49]. Organic load impacted the sanitization not only through depleting free chlorine but also by reducing the inactivation efficacy of chlorine [55].

1.5 Microbial inactivation kinetics and mechanism

Inactivation and removal of microorganism depend on the sanitizer dose and contact time. A few models have been built to depict the inactivation kinetics of microorganisms. How quickly an unstuck pathogen moves starting with one leaf onto the next can be influenced by the disturbance rate of the wash water, and furthermore by the separation between leaves [49]. Subsequently, wash water stream rate, produce-to-water proportion (or load of produce), and setup configuration are critical elements to think about while assessing the effi-

ciency of a washing framework. While setup configuration might be settled for individual handling units, the wash water flowrate and leaf-to-water proportion are process parameters that can be balanced and controlled in the washing process. As the sanitizer level can change because of reaction with organic matter, disinfectant consumption should be considered in active models.

The bacterial cellular membrane is known to be the first target in the food processing treatments [65]. Various types of treatment are used in food industries including chemical preservatives, heating, chemical sanitizers, UV and etc. Some of these treatments like heating and chemical disinfectant procedure have shown the ability to change microbial cell permeability, which in turn results in releasing of macromolecules from the interior [66, 67]. Chemical sanitizers specially destroy the membrane structure and membrane proteins resulting in deformation of membrane organization and functionality [66]. Conventionally, to assess the quality of a disinfection treatments the bacterial survival is measured by the enumeration of viable cells using a standard plate counting method. However, this technique provides results limited to the ability of some cells to recover and grow after the disinfection process [68]. Bacteria can change to the state of viable-but-nonculturable (VBNC) as a result of disinfection process [69]. VBNC cells are not able to grow on non-selective agar medium but still represent certain vital processes indicative of life like respiratory enzymes or maintenance of membrane integrity, which means that the bacteria still has the potential to cause infection [68]. Some parts of microorganisms may be injured during treatment and then repair the cellular damage and recover [70]. Knowing the mechanisms of survival and the nature of injury/repair of the bacterial cells during chlorination is important which can help us perform the required actions against specific strain of microorganisms. Although there are studies which investigated the mechanisms by which chlorine deactivates cells, none of them could comprehensively explain the mechanism(s) of chlorine inactivation of microorganism.

1.6 Dissertation organization and outline

During the washing process of fresh produce, organic matter is released into the washing solution which reacts with chlorine and consume it. So, free chlorine management is needed in fresh produce wash systems. Ideally, free chlorine levels would be managed through dosing strategies based on in-line measurements to be adjusted rapidly for each specific case. The concentration of free chlorine can be monitored regularly using appropriate sensors but the prediction of chlorine demand is also crucially needed. On the other hand there is a direct relation between the survival rate of pathogens and chlorine concentration. So, in order to quantify cross-contamination and the inactivation rate of microorganisms, the first step is to predict the chlorine level in the washing solution. For this purpose, a mathematical model which could predict the chlorine decay rate in the washing process would be helpful. This work consisted of both simulations and experimental work to understand the decay of chlorine, cross-contamination dynamics and inactivation mechanism of microorganisms by free chlorine in fresh produce washing process and simulate them by mathematical model.

Chapter II presents a produce-specific (for cut carrots, cut green cabbage, and cut iceberg lettuce) mathematical model of FC dynamics that is consistent regarding FC replenishment as well as across multiple experimental scales (lab to pilot). In this chapter, we first developed a mathematical model to predict FC levels in dynamic wash of various produce types, based on the chlorination reactions and using COD as an indicator of organic load.

In **Chapter III**, we expanded our model to be commodity-specific and improved chlorine management strategies across multiple experimental scales (lab to pilot-plant). However, COD measurements do not account for the various organic matter which specifically react with chlorine and consume it. Moreover, while the FC levels are lowered due to various reactions, there is no corresponding decrease in COD levels.

In **Chapter IV**, we addressed another goal of this research, i.e., to find a reliable in-

dicator for organic load. So in this chapter, we demonstrated that total amino acids (AA) concentration is a more accurate indicator of FC levels for various produce types as AA concentration decreases proportionately with FC levels. So, we determined the relation between FC levels and AA concentration for three common produce types with different cut-sizes, and replaced COD by AA in the developed model for predicting FC. Finally, a discussion is presented on how the developed FC model could play an important role in validating FC compliance as well as in aiding experiments at the commercial-scale geared towards collecting key data for developing mathematical control strategies to optimize chlorine management during washing.

Chapter V presents a modified disinfection kinetics model to evaluate the potential effect of organic content on the chlorine inactivation coefficient of *Escherichia coli* O157:H7 in fresh produce wash processes. Several studies have shown that the efficacy of sanitizers is significantly reduced in the presence of organic matter [9, 57, 55]. However, a model for chlorine inactivation of pathogens in the presence of organic load is not presented yet. Therefore, in this chapter, we model chlorine decay in the presence of organic matter and investigate the effect of organic load on chlorine inactivation of *Escherichia coli* O157:H7 in a model system of iceberg lettuce wash process. To investigate how chlorine inactivates pathogens in the presence of organic load, we have presented a disinfection kinetics model. We utilized chlorine to inactivate a three-strain cocktail of non-pathogenic *E. coli* O157:H7 and calculated the maximum chlorine inactivation coefficient in the absence of organic load. Then, using our developed model for FC decay (chapter II) and pathogen cross-contamination dynamics, we quantified the chlorine inactivation coefficients in a multi-run wash process of fresh iceberg lettuce. Finally, we demonstrate how chlorine loses its sanitizing efficacy by comparing chlorine inactivation coefficient in the presence and absence of organic content.

Chapter VI presents investigations on the chlorination deactivation mechanisms of *E. coli*. The objectives of this chapter are to 1) investigate the disinfection efficiency and the

inactivation kinetics of *E. coli* by free chlorine; 2) examine the effect of chlorine concentration on inactivation; and 3) explore the mechanisms of chlorine-driven inactivation of *E. coli* by observing the destruction of cell membrane, the morphological changes, and understanding of ATP behavior as well as the bacteria metabolic changes. This work provides suggestions for the choice of an appropriate method for the detection of microbial inactivation or bacterial viability.

Chapter VII presents some key observations regarding fresh produce washing strategies, cross-contamination dynamics, and the influence of organic matter on disinfection efficiency. Furthermore, the usability of the developed models - for predicting free chlorine concentration and microorganism inactivation rate - as a management tool for preliminary decision making is discussed, and finally some future perspectives are explained.

CHAPTER II

MODELING OF FREE CHLORINE CONSUMPTION AND *ESCHERICHIA COLI* O157:H7 CROSS-CONTAMINATION DURING FRESH-CUT PRODUCE WASH CYCLES

Controlling the free chlorine (FC) availability in wash water during sanitization of fresh produce enhances our ability to reduce microbial levels and prevent cross-contamination. However, maintaining an ideal concentration of FC which could prevent the risk of contamination within the wash system is still a technical challenge in the industry, indicating the need to better understand wash water chemistry dynamics. Using bench-scale experiments and modeling approaches, we developed a comprehensive mathematical model to predict the FC concentration during fresh-cut produce wash processes for different lettuce types (romaine, iceberg, green-leaf, and red-leaf), carrots, and green cabbage as well as *E. coli* O157:H7 cross-contamination during fresh-cut iceberg lettuce washing. Fresh-cut produce exudates, as measured by COD levels, appear to be the primary source of consumption of FC in wash water, with an apparent reaction rate ranging from 4.74 $\times 10^{-4}$ - 7.42 $\times 10^{-4}$ $L.mg^{-1}.min^{-1}$ for all produce types tested, at stable pH levels (6.5 - 7.0) in the wash water. COD levels increased over time as more produce was washed and the lettuce type impacted the rate of increase in organic load. The model parameters from our experimental data were compared to those obtained from a pilot-plant scale study for lettuce, and similar reaction rate constant (5.38×10^{-4} $L.mg^{-1}.min^{-1}$) was noted, supporting our hypothesis

that rise in COD is the main cause of consumption of FC levels in the wash water. We also identified that the bacterial transfer mechanism described by our model is robust relative to experimental scale and pathogen levels in the wash water. Finally, we proposed functions that quantify an upper bound on pathogen levels in the water and on cross-contaminated lettuce, indicating the maximum potential of water-mediated cross-contamination. Our model results could help indicate the limits of FC control to prevent cross-contamination during lettuce washing.

2.1 Introduction

Foodborne illnesses affect millions of people in the United States every year according to the Center for Disease Control [1]. The association of these outbreaks to food products varies from raw meat and fresh produce contamination, to the consumption of undercooked and poorly packaged foods. In terms of fresh-cut produce, outbreaks of *E. coli* and *Salmonella* have been linked to the consumption of leafy greens as well as fruit [5, 4]. While it has been suggested that the primary source of these outbreaks most likely occurred during pre-harvest, the post-harvest wash process has the potential to play a significant role in secondary contamination [71]. The critical issue here is that when produce is washed, there is a possibility of bacterial cross-contamination from a bacteria contaminated batch to other uncontaminated ones that was subsequently washed in the same water. Therefore, while the post-harvest wash step is limited in completely decontaminating produce (even with potentially high levels of sanitizer in the wash water), the current focus of washing operations is to prevent this secondary contamination or cross-contamination between produce lots [49, 71].

Chlorine is a commonly used water-disinfectant in the produce industry as it is an inexpensive and effective disinfectant [7]. However, maintaining a stable FC concentration during washing is challenging [6]. Chlorine can be consumed by organic matter released from the fresh-cut produce and the bactericidal activity will be reduced [72]. If the organic

load, measured as chemical oxygen demand (COD), is too high, the sanitizing effect of chlorine is reduced [9]. Likewise, chlorine levels (measured as FC) must be continuously regulated to remain at sufficient concentrations to prevent cross-contamination [10].

There are commercial systems that could maintain almost constant free chlorine levels at an industrial scale due to the relatively short lag-time between FC measurements and chlorine addition. However, to optimize these systems from predictive framework as well as to provide independent validation of the control of FC variability relative to specified set-points, the underlying mechanisms that dictate FC concentration (relative to organic load and pH dynamics) during fresh-cut produce wash operations must be more specifically quantified. Part of the problem is that the relationships between chlorine levels and such water quality parameters have only been described through experimental and correlative approaches [27, 73, 8, 9, 74, 58, 75]. While these results have clear value, they cannot be used to make precise predictions of free chlorine concentration and antimicrobial capacity in dynamically changing circumstances.

Connected with maintaining sufficient sanitizer levels in the wash water, there have been many observational studies directly examining pathogen cross-contamination at the lab scale [13, 23, 32, 7] and at the pilot scale [28]. Also a number of recent studies indirectly address pathogen contamination at different scales [32, 28, 71]. However, given the potential for wash water to promote pathogen cross-contamination, there remains a critical need to determine mechanisms involved with pathogen transfer during produce washing [49]. Furthermore, considering the diversity of experimental procedure and scale in these prior studies, as pointed out by [10], a standardized approach which can synthesize current results, providing both a unifying perspective as well as a direction towards increased predictive capacity is necessary to advance fresh-produce safety [10].

To address some of these limitations, this chapter aims to (a) assess the dynamic changes in water quality during the washing process of lettuce, carrots and green cabbage by measuring parameters such as FC, total chlorine, pH, and COD; (b) develop a mathematical

model to predict chlorine decay in the wash water as well as *E. coli* O157:H7 cross-contamination during a simulated batch lettuce wash procedure; and (c) utilize the developed model to investigate the reaction rate constant for the reaction between FC and organic matter (COD) and the *E. coli* transfer rate from water to lettuce.

Previously, using the data provided by Lou *et al.,* (2012) [28], we have developed a mathematical model to predict FC concentration and pathogen levels in pilot plant washing systems [41]. In this study, our aim is to test and develop the model mechanisms to justify that the relevant chlorine consumption and pathogen transfer mechanisms at the pilot scale are similar to the bench-scale during batch process washing for iceberg lettuce. Moreover, we also demonstrate that a similar mechanism, with a similar rate constant, is also valid for romaine/ green-leaf/ red-leaf lettuce, carrots, and green cabbage. We illustrate how mathematical modeling tools can provide a reference point to address how experimental scale affects FC dynamics and mechanisms of cross-contamination during fresh-cut produce washing. In terms of FC decay, we first determine the apparent reaction rate of FC with organics during washing by conducting bench-top experiments and develop a corresponding predictive mathematical model. In addition to quantifying the FC decay dynamics relative to four types of lettuce (romaine, iceberg, green-leaf, and red-leaf), carrots (imperator type), and green cabbage, we simultaneously validate our model and address scaling questions by comparing our results with experimental data at the pilot/commercial scale [28]. Next, we use our model in conjunction with *E. coli* O157:H7 data from lab and pilot scale experiments [32, 7, 28] to specify processing conditions in which water-mediated cross-contamination dominates the pathogen transfer dynamic during washing. Finally, we use our model to illustrate the effectiveness of FC control to prevent cross-contamination and highlight specific experimental needs to improve such control.

2.2 Materials and methods

2.2.1 Produce preparation

Four types of lettuce (romaine, iceberg, green-leaf, and red-leaf), carrots (imperator type), and green cabbage were selected for analysis. Pre-bagged produce was purchased from a local supermarket and stored at 4 $°C$ before the experiments and used within two days of purchasing. Exterior leaves of the lettuce and cabbage were trimmed out and discarded, and the rest were cut to 1"$\times$1" size using a chopper. Carrots were chopped into two different shapes - stick cut (0.25"$\times$0.25"$\times$1") and disk cut (thickness 0.25").

2.2.2 Fresh-cut produce washing system

Produce washing experiments were carried out using a bench-top wash system consisting of a container holding 3 L of tap water, using experimental conditions similar to those reported in literature. Before starting each experiment, 1.4 mL of concentrated (4.5%) sodium hypochlorite (BCS Chemicals, Redwood City, CA, USA) was added to the wash water to achieve approximately 20 $mg.L^{-1}$ free chlorine concentration in the wash water (20 $°C$). The pH was adjusted to 6.5 for all experiments using 1 M citrate buffer. The cut produce ($\sim$ 600 g) were washed through six consecutive batches, with each batch weighing 100 g and a dwelling time of 30 $sec.$ After washing each batch, the cut produce was removed from the washing solution using a sieve and drained above the container for 30 $sec.$ Samples (wash water aliquots) were taken for analysis after washing each batch (100 g) of produce. The total experimental time for washing lettuce was 45 min, and that for carrots and cabbage was 30 min.

2.2.3 Evaluation of water quality

Free chlorine was measured immediately after washing each batch, based on a DPD (N, N-diethyl-p-phenylenediamine) method, using Chlorine Photometer (CP-15, HF Scientific Inc., Ft. Myers, FL). The pH was measured right after each batch was washed using a dig-

ital pH meter (Orion™ 2-Star, ThermoFisher Scientific, Grand Island, NY). The chemical oxygen demand (COD) was determined using a reactor digestion method.

2.2.4 Mathematical model for free chlorine dynamics in the wash water

A variety of factors could affect FC concentration, but organic load is possibly the primary source of consumption of FC [41, 72]. As produce was introduced into the wash water, organic material from the cut produce enters the water and COD increases significantly in water. For all produce in our study we found that as more produce was washed, the COD in wash water increases linearly. So, the relation between COD and time could be written as:

$$\frac{dO}{dt} = k_0 \tag{2.1}$$

where t denotes time (min), and O $(mg.L^{-1})$ is the COD in the wash water, and k_0 $(mg.L^{-1}.min^{-1})$ is the slope of increasing COD over time. If the washing process is continuous and there is no stop between the batches, then the integrated form of equation 2.1 is:

$$O = k_0 t + O_0 \tag{2.2}$$

where O_0 is the initial COD concentration. However, this study was carried out within a batch process and there were pauses between batches for data collection. To account for this, a step function for the rate of change of COD is used. This function is a constant when the produce is not immersed in the wash water (time for sampling and measuring pH, free chlorine, and COD, between batches), and is linearly increasing when the produce is being washed. Therefore, there are two steps for modeling each batch of the washing system: wash 100 g of chopped produce for 30 sec and drain for 30 sec and collect aliquots to measure parameters. In this case, if we modify equation 2.1 to reflect this two-step batch

process, the integrated form of the model becomes:

$$O = \begin{cases} k_0 t + O_0 - (n-1)k_0\tau_2, & (n-1)(\tau_1+\tau_2) \leq t \leq n\tau_1 + (n-1)\tau_2 \\ nk_0\tau_1 + O_0, & n\tau_1 + (n-1)\tau_2 \leq t \leq n(\tau_1+\tau_2) \end{cases} \tag{2.3}$$

Here n is the batch number, τ_1 is the amount of time for which the produce is washed (0.5 min), and τ_2 is the time taken between the sample collection and measurements (7 min for lettuce, and 4.5 min for carrots and cabbage).

Using COD as an indirect measure for the organic load (R), we consider the following apparent reaction [60, 41]:

$$HOCl + R \rightarrow Products$$

which accounts for FC reduction in the wash water. It is assumed that this reaction is both first- and second-order [60], so the rate of change of FC is given by:

$$\frac{dC}{dt} = -\beta_c\, O\, C - \lambda_c\, C \tag{2.4}$$

where C ($mg.L^{-1}$) indicates the FC concentration in the wash water, λ_c is the natural decay rate of chlorine in tap water, and β_c is the reaction rate of the above reaction. Typically, β_c is a function of pH and temperature, but since the temperature was held constant during the experiments and the change in pH was small, we assume that β_c remains constant. On the other hand, there are different types of organic and inorganic matter in the wash system that react with chlorine (, produce extract, bacteria, soil, ammonia, humic acid). Because the reaction of chlorine with each of these materials has a different rate constant, β_c is considered to be the average of all these constants.

2.2.5 Statistical analysis and Parameter-fitting

All experiments were done in triplicate and the average $\pm$ standard deviation of the three independent runs was reported. The resulting data was used in order to find k0 and c

parameters. The *fminsearch* function in MATLAB R2016b (MathWorks Inc., Natick, MA, USA) was used for curve-fitting.

2.2.6 Data and modeling for *E. coli* O157:H7 cross-contamination

Following our earlier model [41] , we built a modified mathematical model that accounts for the batch-wash timing described in experimental procedure in section 2.2.2 with the focus of quantifying *E. coli* O157:H7 concentration in the wash water as well as cross-contamination dynamics at the lab scale.

we consider the following mechanisms connected to the pathogen level in the water X_W ($MPN.mL^{-1}$) during batch experiments: (i) pathogens on inoculated produce shed into the water during washing (which we assume occurs at a constant rate given that produce is inoculated with the same level of pathogens on average), (ii) pathogens in the wash water can transfer/attach to produce during washing, and (iii) pathogens can be inactivated by FC in the wash water. Incorporating these three mechanisms we build the following equation:

$$\frac{dX_W}{dt} = \hat{\beta_W} S - \hat{\beta_L} W \frac{L}{V} X_W - \alpha X_W C \tag{2.5}$$

In the above equation,

$$\hat{\beta_{WS}} = \begin{cases} \beta_{WS}, & \text{during washing of produce} \\ 0, & \text{stoppage time} \end{cases}$$

$$\hat{\beta_{LW}} = \begin{cases} \beta_{LW}, & \text{during washing of produce} \\ 0, & \text{stoppage time} \end{cases}$$

where β_{WS} ($MPN.mL^{-1}.min^{-1}$) is the entry rate of the pathogen into the water, β_{LW} ($mL.g^{-1}.min^{-1}$) is the rate of pathogen transfer from the water to the produce, $\frac{L}{V}$

($g.mL^{-1}$) is the produce to water ratio in during washing, α ($L.mg^{-1}.min^{-1}$) is the kill rate of pathogens via FC, and C ($mg.L^{-1}$) is the FC level in the wash water. Note that we assume that complete mixing occurs during washing and thus successful transfer of pathogens from water to produce occurs at a rate proportional to the amount of pathogen in the water X_W ($MPN.mL^{-1}$) and the amount of produce in the water L (g). To incorporate the batch-washing dynamic, $\hat{\beta_{WS}}$ and $\hat{\beta_{LW}}$ are defined to be "on" during washing and "off" in between washes.

In terms of quantifying cross-contaminated pathogen levels on produce batch n we have the following equation for $n \in \{1, 2, \ldots, N\}$:

$$\frac{dX_{L_n}}{dt} = b^n_{LW} X_W - \alpha X_{L_n} C \tag{2.6}$$

$$b^n_{LW} = \begin{cases} \beta_{LW}, & \text{during washing of produce} \\ 0, & \text{stoppage time} \end{cases}$$

where X_{L_n} ($MPN.g^{-1}$) represents the average pathogen level via cross-contamination on produce batch n during washing, the first term (on the right-hand side of the equation) quantifies pathogen transfer onto lettuce (again defined to account for the batch process) and the second term tracks the killing of pathogens via FC on the produce surface during washing.

To inform the new model's parameters and justify its mathematical forms at this scale, we utilize *E. coli* O157:H7 data from lettuce wash studies by Luo *et al.,* (2012)[28], and *E. coli* (CECT 471, 516, and 533) data from a lettuce wash study by Lopez *et al.,* (2010) [32].

2.3 Results and discussion

2.3.1 Water quality and free chlorine concentration dynamics during washing

Results from the batch-wash experiments indicate that as the cut produce was sequentially washed in the container, COD levels increased in the wash solution (Fig. 2.2). Under the operating conditions present in this experiment, the increase in COD linearly corresponded to the amount of produce washed, and hence the amount of exudates and other organic material from the washed vegetables released into the wash system. Although there were variations among different batches of produce, the overall trend remained similar for each produce type (Fig. 2.2). COD dynamic changes remained similar for all produce types, although COD increased with a higher slope for romaine lettuce.

As chopped produce was introduced into the washing system, the increase in COD was accompanied by a decline in residual FC concentration (Fig. 2.1a: lettuce; Fig. 2.1b: sliced and stick-cut carrots and cabbage). The initial concentration of 20-22 $mg.L^{-1}$ of FC was nearly depleted by the end of each wash trial, when $\sim$600 g of chopped produce was washed in 3 L of tap water.

Washing cut produce in chlorinated water also affects the water pH. In general, the pH of vegetable extract ranges from 6.1 to 6.3 [59]. Depending on the initial pH of the wash solution, washing lettuce gradually changes the solution pH towards that of the produce pulp pH [46]. The pH level of the wash water is also a function of the amount of produce extract and sodium hypochlorite added [76]. At the beginning of each experiment in this study, the pH of chlorinated water for washing was adjusted to 6.5 using citrate buffer. As the amount of produce introduced into the water increased, the organic material in the washing solution increased as well. The changes in pH and FC were impacted by both the wash water conditioning (adding chlorine and citrate buffer) and the washing processes (produce exudate and debris). The pH was relatively stable during washing, with a slight decrease in pH for all products tested. The pH was below the recommended upper limit of

7.0 for maximizing the concentration of hypochlorous acid, the form of chlorine with the highest efficacy against microorganisms [60, 49]. We expect no significant changes in our model parameters due to this pH change.

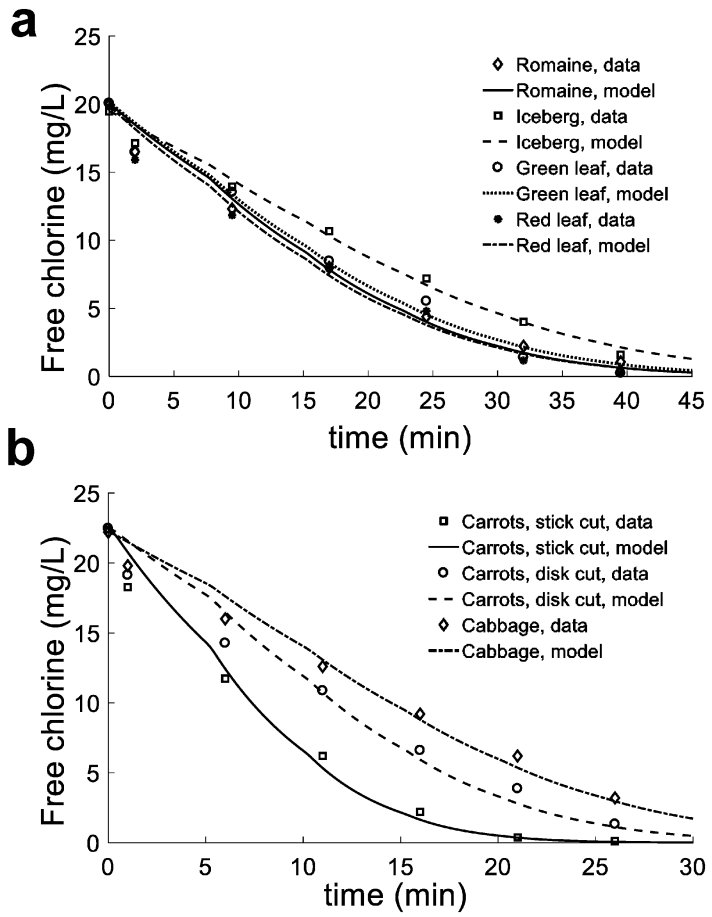

Figure 2.1: Free chlorine dynamics over time when (a) different types of lettuce were washed (pH level of 6.5), and (b) different cuts of carrots, and cabbage were washed (pH level of 6.5). The lines were from the model described in equation 5, and symbols represent experimental data.

2.3.2 Fitting the FC model to the data

We noted earlier (Figure 2.2) that the COD in the wash water increased linearly with the introduction of chopped produce to the wash-system. The curve-fitting for COD levels (Fig. 2.2) was performed using experimental data presented earlier as well as our model described in equation 2.3. In all experiments, the initial value of COD was approximately 32 $mg.L^{-1}$. As noted from the curve-fitting results in Table I, the model fitted the data well

Table I: Parameter values for different types of lettuce, carrots, and green cabbage, where the parameters O_0 and Cl_0 were measured in the experiments. The parameter k_0 was fitted to minimize the distance between equation 2.3 and measured values of COD levels for each produce type. This k_0 value was subsequently used to obtain the parameter β_c when fitted to minimize the distance with equation 2.4 and measured FC levels for each produce type.

Produce Type	O_0 $(mg.L^{-1})$	Cl_0 $(mg.L^{-1})$	k_0 $(mg.L^{-1}.min^{-1})$	β_c $(L.mg^{-1}.min^{-1})$	R^{2*} (COD)	R^2 (FC)	$RMSE$ (COD)	$RMSE$ (FC)
Romaine	31.8	20.0	76.62	5.62×10^{-4}	0.99	0.98	161.1	0.85
Iceberg	31.5	19.5	52.86	4.72×10^{-4}	0.98	0.99	116.6	0.61
Green leaf	31.5	20.1	59.36	6.17×10^{-4}	0.99	0.98	129.8	0.97
Red leaf	32.0	19.8	53.66	7.43×10^{-4}	0.99	0.98	120.1	1.02
Carrots, stick-cut	33.0	22.4	179.8	7.38×10^{-4}	1.00	0.98	343.2	1.05
Carrots, disk-cut	30.0	22.5	127.9	5.03×10^{-4}	0.99	0.97	247.2	1.33
Green cabbage	28.7	22.2	54.30	3.81×10^{-4}	0.98	0.96	111.1	0.95

* The coefficient of determination (R^2) measures the percentage of variability within the -values that can be explained by the regression model. Therefore, a value close to 1 means that the model is useful and a value close to zero indicates that the model is not useful. However R^2 alone is not sufficient for the goodness of the fit and analysis of residuals is also needed.

for each lettuce type. The R2 values for COD curve-fitting (Table I) suggest that the model we developed represented well the COD changes in wash solution as more produce entered the wash water.

Utilizing the calculated COD rate increase (k_0 values in Table I) together with equation 2.4 and FC data, the parameter β_c was determined for each respective produce type. The best fit curves corresponding to each produce type were shown in Fig. 2.1, and Table I lists β_c values for different produce types. Also, as noted in this table, the experimental data was well represented by the model for decaying FC levels, with only minor deviation between them, as indicated by the R^2 and RMSE values from the respective curve-fittings for all the produce types. This suggests that equations 2.1 and 2.4 adequately describe the decay dynamics of FC in the wash water.

Among the numerous reactions that could happen in this system, it was initially assumed that multiple reactions between free chlorine and organic matter might exist, and β_c is the average of all those reaction rate constants. Since the organic matter entering the wash solution for different types of produce was not the same, slight differences between β_c values could be expected. Our assumption that the main reaction in the wash system is of second-order between COD and FC gives an excellent fit for the data and yields very similar reaction rates not only for various produce types but also across different scales (as

illustrated in section 2.3.3. We take advantage of this by considering the same β_c value for all lettuce types. We used an average β_c value of 6 $\times 10^{-4}$ $L.mg^{-1}.min^{-1}$ from all produce types to predict the dynamics of FC in the wash water for each type of produce. The results show only a very minor deviation between predicted results and experimental data shown in Fig. 2.1, and the R^2 values were 0.95 or higher for each produce type. Moreover, deviation between predicted values by model and experimental data at each data point for all produce types was less than 5% in each case, showing that there is a very good match between the predicted FC and experimental data.

2.3.3 FC model validation and predictability

All the experiments in this study were performed at a bench-scale (3-L water tank). In order to validate our model, we determined the parameters from the data by Luo *et al.,* (2012) and compared to those obtained in our work. Some of the operating conditions of that study were similar to our experimental conditions including the initial pH which was 6.5 in both studies, the use of sodium hypochlorite as the sanitizer agent, and cut iceberg lettuce as the produce washed. On the other hand, some conditions differed: the scale of the washing system (3200 L vs. 3 L), the type of process (continuous vs. batch), the amount of produce washed per liter of water per minute, and the initial COD level (307 $mg.L^{-1}$ vs. 32 $mg.L^{-1}$). However, because the differences between the two studies are accounted for as variables of our model, these changes do not affect the proposed mechanisms for consumption of free chlorine in the wash water. Therefore, we used the data from Luo *et al.,* (2012) to validate our model forms for these mechanisms.

Equations 2.3 and (2.4) are appropriate forms of our model to describe the dynamic changes of COD and FC concentration of the washing system and can be used even for continuous systems by just taking τ_1 to be the duration of the experiment, $\tau_2 = 0$, and $n = 1$. Using these versions of equations 3 and 5, and experimental data from Luo *et al.* (2012), we earlier reported the values of k_0 and β_c.

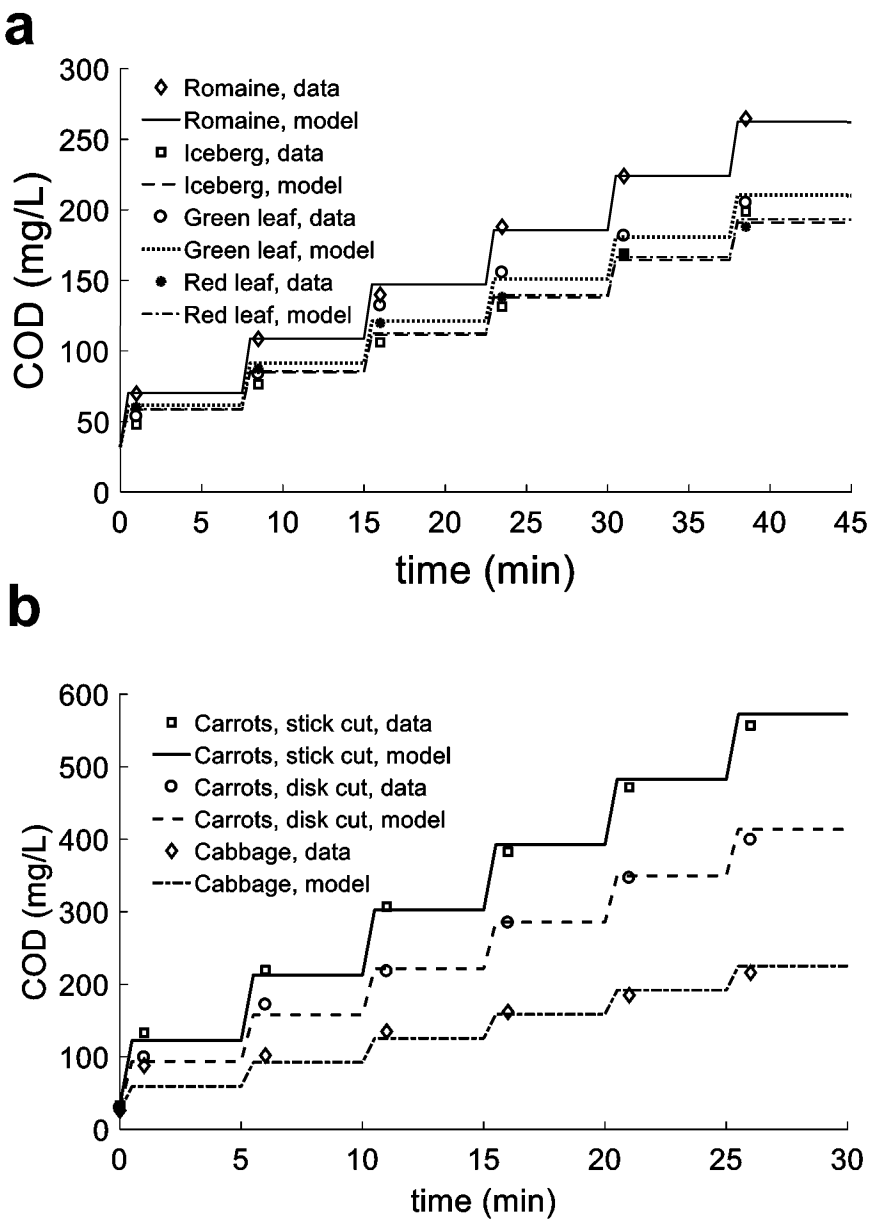

Figure 2.2: COD profile over time from washing (a) different types of lettuce, and (b) carrots and cabbage. The lines are from the model fit described in equation 2.3, and symbols represent experimental data.

The values of β_c found in the current study can be used to predict the FC concentration in experiments by Luo *et al.* (2012), and vice-versa. The R^2 values calculated for these predictions were shown in the last column of Table II and these curve-fittings were shown in Fig. 2.3. Figure 2.3a compares the fitting of data from Luo *et al.,* (2012), using the value of β_c from Munther *et al.,* (2015) (solid line) to this study (dashed line) as a predicting

model. Figure 2.3b compares the fitting from our current study using the β_c found in our study (solid line), and its value from Munther *et al.,* (2015) (dashed line) as a predicting model. We report $R^2 \geq 0.98$ in both the studies, indicating an excellent match between the fitted model and the experimental data. Moreover, the deviation between the predicting model and the curve fitting model was less than 5% for each data point, with a combined R^2 value of 0.95 for all the data points in both the cases. This suggests that the predictive model does very well in matching the experimental data from both studies.

Table II: Comparison of curve-fitting results in this study with that from Luo *et al.,* (2012), for iceberg lettuce processing. Using data from Luo *et al.,* (2012), we earlier calculated and reported parameters k_0 and β_c.

	O_0 $(mg.L^{-1})$	Cl_0 $(mg.L^{-1})$	k_0 $(mg.L^{-1}.min^{-1})$	β_c $(L.mg^{-1}.min^{-1})$	β_c $(\mu M^{-1}.min^{-1})$
Current study	32	20	52.86	4.74×10^{-4}	2.48×10^{-5}
Munther *et al.,* (2015) [41]	307	21	32.30	5.38×10^{-4}	2.82×10^{-5}

The numbers from Table II show a significantly lower value for k_0 from Luo *et al.,* (2012) compared to that in our work for iceberg lettuce. The main factor that affects the COD levels (and thereby the k_0 value) is the rate at which lettuce entered the washing water relative to the wash tank volume, which was lower in that study compared to ours. Other factors that may be relevant include the farm source of lettuce, its age, variations in cut sizes of the pieces, and season of the year [27, 8]. As may be expected, during different runs, we observed minor variations in k_0 values for the same lettuce type even under the same experimental conditions. Differences in the initial value of COD (O_0) and its rate of increase (k_0) are to be expected, as they depend on the produce used and the experimental setup. However, it is not clear if variations in c values are to be expected a priori due to potential scaling effects [77]. As can be seen in Table II, the value of β_c using data from Luo *et al.,* (2012) is close to the β_c in our study.

Our model also allows us to predict the FC levels by only knowing the initial value of COD in the wash water and its steady rate of increase. Our model could be extended to larger operation scales such as commercial wash systems, where the produce may not be

Table III: List of parameters and their values used in the complete model.

Parameter	Description	Values & Units	Reference
k_0	COD increase rate	52.86 $mg.L^{-1}.min^{-1}$	This study
λ_c	Natural FC decay rate	2×10^{-3} min^{-1}	(Hua, *et al.*, 1999)
β_c	FC apparent reaction rate	5.38×10^{-4} $L.mg^{-1}.min^{-1}$	(Munther *et al.*, 2015)
β_{LW}	*E. coli* binding rate: water to lettuce	0.38 $mL.g^{-1}.min^{-1}$	(Munther *et al.*, 2015)
β_{WS}	Effective *E. coli* input rate to wash water	[1,105] $MPN.mL^{-1}.min^{-1}$	This study
L	Amount of lettuce in wash tank	0.1 kg	This study
V	Volume of wash water	3 L	This study
α	Kill rate of *E. coli* via FC	0.75 $L.mg^{-1}.min^{-1}$	(Munther *et al.*, 2015)

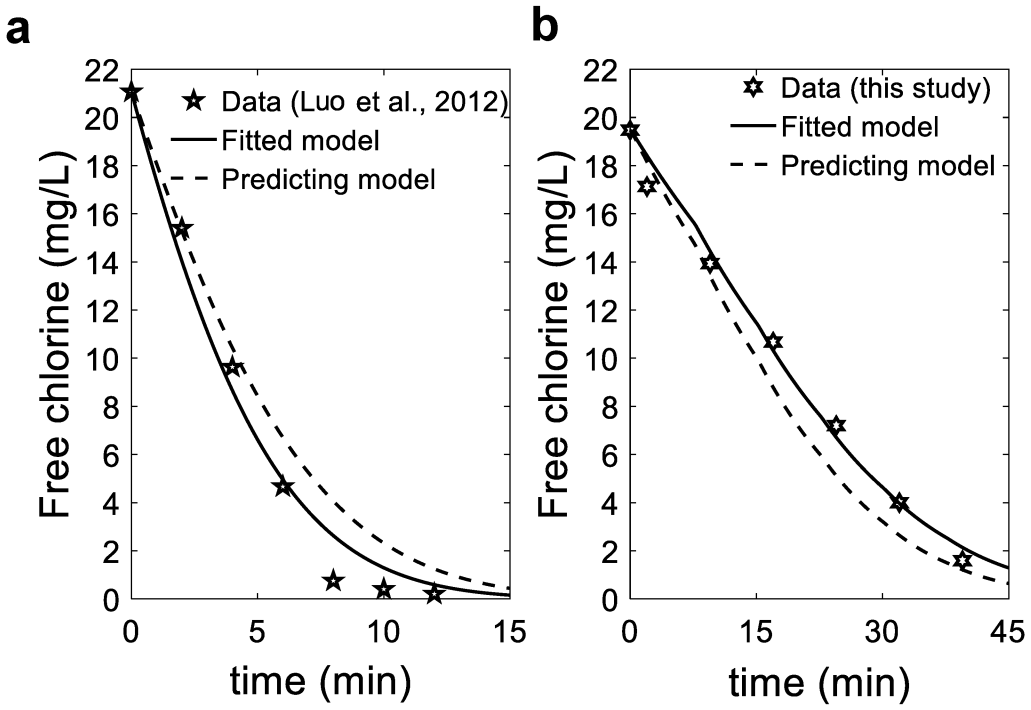

Figure 2.3: Free chlorine dynamics during washing of iceberg lettuce using data from (a) Luo *et al.*, (2012) and (b) the current study. The lines in (a) represent the fitting using a β_c value of 5.38×10^{-4} (solid line) from Munther *et al.*, (2015), and a value of 4.74×10^{-4} $L.mg^{-1}.min^{-1}$ (dashed line) as a predicting model from this study, and vice-versa for (b). The symbols represent experimental data.

introduced to the wash water at a nearly constant rate and requires constant monitoring of COD to accurately predict FC. Since real-time monitoring of COD is not feasible, fresh-cut produce processing companies could try to predict the COD present in the wash water by using product type and throughput and water replenishment data. In this context, our model could be used to help validate set points and test efficiency of FC dosing strategies via intermittent collection of COD data during specific washing durations.

2.3.4 Water-mediated cross-contamination dynamics

An important question regarding cross-contamination concerns which mode or modes of pathogen transfer significantly contributes to the dynamics during washing. Among the possibilities for cross-contamination, the top three suspects for mediation are: (i) by water, which involves pathogen transfer from water to the surface of uncontaminated produce; (ii) by particles in the water, i.e., pathogens attach to the surface of small produce debris in water and they in turn attach to the surface of uncontaminated produce; and (iii) by produce-to-produce contamination, i.e., contact between contaminated produce and uncontaminated produce in the same wash batch [49].

Note that we earlier [41] assumed that water-mediated cross-contamination played a dominant role in pathogen transfer to uninoculated iceberg lettuce. Using our batch model for pathogen dynamics, equations 6c-6d in section 2.7 as a gauge, and data from prior studies in this regard [32, 7, 28], we can justify this assumption by comparing relative values of the *E. coli* in water-to-lettuce transfer rate, β_{LW}. We earlier determined that $\beta_{LW} = 0.38\ mL.g^{-1}.min^{-1}$ [41]. Note that in the study by Lopez *et al.* (2010); hitherto referred as study A), produce-to-produce contamination during the pre-wash step was impossible as inoculated lettuce was dipped into the wash tank for one minute, removed, and then non-inoculated lettuce was dipped for one minute into the same water [32]. Using data from Figs. 1 & 4 of study A, we estimate that $\beta_{LW} = 0.19\ mL.g^{-1}.min^{-1}$. In contrast, Luo *et al.,* (2011); hitherto referred as study B) washed both inoculated and non-inoculated lettuce simultaneously and using data from Table II and III of study B, we estimate on average that $\beta_{LW} = 9.04\ mL.g^{-1}.min^{-1}$.

The above analysis concerning β_{LW} indicates a few points. First, considering the experimental procedure in study A, β_{LW} indeed represents the rate of *E. coli* transfer via contaminated water. Since the values of β_{LW} from study A and the pilot scale study by Luo *et al.,* (2012) are extremely close (note that this comparison is across 3 orders of magnitude in water volume), we conclude that water-mediated cross-contamination dominates

the bacterial transfer dynamic during the pilot scale experiment in the latter study. This is not to say that produce-to-produce contamination did not occur during the pilot scale study, but that its contribution towards the observed cross-contamination levels on the lettuce was minimal as compared with water-mediated transfer. This notion is reinforced by the fact that β_{LW} corresponding to study B, in which product-to-product contact was permitted during washing, is at least an order of magnitude greater. It is also interesting to note that in study A, the bacterial levels in the wash water are significantly higher, the water agitation/flow rate is much lower, and the produce mass/water volume ratio is significantly lower, compared to a study by *Luo et al.,* (2012); however, the β_{LW} values are remarkably similar. This suggests that the bacterial transfer mechanism described by the model is robust relative to experimental scale and pathogen levels in the wash water. Therefore, we propose that lab scale wash experiments for fresh-cut lettuce, in which product-to-product contamination is controlled against or at least limited (by lowering the produce to water ratio), may provide adequate representation of the cross-contamination dynamic via water that might occur in typical commercial wash processes.

2.4 Conclusions

In this study, the dynamic changes of water quality during the washing process of four different types of lettuce (romaine, iceberg, green leaf, red leaf), carrots and green cabbage were first studied. Results showed that COD levels increased over time as more produce was washed and, in particular, the lettuce type impacted the rate of increase in organic load. As the produce was introduced to the washing solution, FC concentration decreased mainly due to consumption of chlorine by organic matter. FC concentration is of high importance in washing of fresh-cut produce as it inactivates bacteria, and such depletion of FC might lead to cross-contamination in the washing water. Using data from a bench-top experimental setup, a mathematical model was developed for FC dynamics in the washing solution, to describe the mechanism by which FC changes in the washing of fresh-cut

produce. The reaction rate constant between chlorine and COD was calculated based on the experimental data. Results showed that the apparent reaction rate constant (β_c) ranges from 4.74 $\times 10^{-4}$-7.42 $\times 10^{-4}$ $L.mg^{-1}.min^{-1}$ for lettuce, carrots and cabbage, when the pH of the wash water is maintained at stable levels of 6.5. We compared the model parameters from our experimental data to those obtained from a pilot-plant scale study for lettuce [28, 41] , and observed very similar c value of 5.38 $\times 10^{-4}$ $L.mg^{-1}.min^{-1}$. This strongly supports our hypothesis that rise in COD is the main cause of consumption of FC levels in the wash water. Given the variety of produce types we have used in our study, we believe that our model and the value of β_c will be comparable to the range we have obtained in this study as long as the pH of the water is stable at ambient temperatures, and that the chlorine levels can be estimated well using our model by only knowing the rate of change of the COD levels under these conditions.

In addition to water quality dynamics, we used our complete model (CM) to not only confirm the utility of lab scale experiments in quantifying water-mediated cross-contamination, but also illustrate how our model can provide guidelines for future experiments to establish quantifiable guidelines for cross-contamination control. Further studies under a wider range of operational conditions need to be conducted to corroborate these results, so that they may be used to understand the dynamics of FC and cross-contamination during the wash process, and to potentially improve existing industrial practices.

CHAPTER III

TOWARDS ENHANCED SANITIZER CONTROL: MATHEMATICAL MODELING TOOLS FOR FREE CHLORINE DECAY KINETICS DURING FRESH-CUT PRODUCE WASHING

In this chapter, we developed produce-specific mechanistic models to predict free chlorine (FC) kinetics during washing of disk-cut carrots, cut cabbage, and cut iceberg lettuce, in 3 L and 50 – 100 L tanks, and of shredded iceberg lettuce in 3200 L pilot-plant trials. Using a fixed percentage of successive changes in chemical oxygen demand (COD) in the water due to produce washing, typically ranging from 6 to 11% across produce types, the models were able to consistently predict experimental FC levels, indicating a robustness of the apparent reaction rate constants across scales. Comparing sequential changes in COD with turbidity and total dissolved solids (TDS) relative to produce washing rates, our results also illustrate that turbidity and TDS are not consistent and reliable predictors of FC decay across produce types and experimental scales. In concert with future experiments, these models could serve as important tools aimed at developing optimal sanitization control strategies relevant for industry.

Practical Application: The results of this work can be applied in fresh producing washing industries in order to predict the concentration of free chlorine based on the rate of produce that is washed over time.

3.1 Introduction

As globalization has broadened the fresh produce supply chain and increased its complexity, more sophisticated methods of surveillance are needed to ensure the safety of fresh produce. Specifically, the produce washing juncture is a critical control point that has received much attention. Despite this, current understanding of the dynamics of sanitizer control during washing has still been limited. The most common sanitizer used to wash fresh produce in the United States is chlorine in the form of diluted sodium hypochlorite (NaOCl), which is used in over 75% of the industry [10]. Owing to the pH dependence of disinfectant effectiveness of hypochlorite via hypochlorous acid (HOCl) and its corrosive effects at low pH, produce wash plants typically maintain wash solution at $6.5 \leq pH \leq 7.5$, FC $\leq 200\ mg.L^{-1}$, and produce exposure to disinfectant at ≤ 2 min. While increasing the FC concentration in wash water offers a viable alternative to this problem, it results in the formation of disinfection by-products and unhealthy conditions for workers due to chlorine gas formation.

Considering the importance of maintaining a certain FC range during washing, many recent studies have provided correlative relationships between water quality parameters (dissolved solids (TDS), turbidity) and FC levels [[34, 14, 55, 37] and references therein]. In terms of chlorine sanitization, the residual FC concentration is critical in controlling pathogen inactivation and preventing cross-contamination [49, 9, 7, 58]. Maintaining such FC levels, however, is challenging as the continuous rise in organic load has been implicated as the primary consumer of FC during wash cycles. Thus, understanding the dynamic interactions between organic load and FC concentration is critical to develop practical sanitization strategies for maintaining safety of fresh-cut produce [10, 41, 58]. Studies have demonstrated that COD is a good indicator of organic load at both the lab and pilot scales [28, 41, 72, 78], but is impractical to measure during real-time processing [78].

Therefore, contemporary research has been focused on identifying parameters which can predict and monitor FC levels in real-time for specific produce commodities. While

many studies [27, 73, 79, 74, 72] have examined the correlative strength of various water quality parameters such as UV254, turbidity, and TDS, more insight is needed with regards to chlorine demand and produce type. This is because dosing and monitoring standards built on such correlations alone do not capture the scope of dynamic interactions between FC levels and organic material. Besides, considering the variations in scale of operation and applications in industry, they are difficult to generalize even when washing the same commodity.

In light of these issues, the objectives of this work are to: (i) develop and validate produce-specific (for cut carrots, cut green cabbage, and cut iceberg lettuce) mathematical models of FC decay that are consistent with regards to FC replenishment as well as across multiple experimental scales (lab to pilot); (ii) determine whether TDS or turbidity are consistent predictors of FC decay across experimental scales (lab to pilot) for cut carrots, green cabbage and iceberg lettuce; and (iii) discuss how the developed FC decay model can play an essential role in aiding experiments at the commercial scale geared towards collecting key data for developing mathematical control strategies to reduce variability of FC and ensure maximum efficiency during washing.

3.2 Materials and methods

3.2.1 Single-batch wash experiment and the Initial model

Carrots (imperator type), green cabbage, and iceberg lettuce were purchased from a local supermarket and stored at 4 $^{\circ}C$ and used for experiments on the same day of purchase. Exterior leaves of the cabbage and lettuce were trimmed out and discarded. Cut specifications for cabbage and lettuce were 1"$\times$1", and disk carrots were 0.25" thick.

Experiments for single-batch washing were carried out in a 3 L wash water system. Before starting each experiment, 1.5 mL of concentrated (4.5%) sodium hypochlorite (BCS Chemicals, Redwood City, CA) was added to the wash tank to achieve approximately 25 $mg.L^{-1}$ of FC in the wash water. The pH was simultaneously adjusted to 6.5 using 1 M

citrate buffer. 100 g of cut produce were put into a sieve, submerged in the 3 L of tap water (20 $°C$), and washed (via manual agitation) for 30 sec. The sieve was held over the 3-L wash water for another 30 sec. Water quality parameters (FC, pH, and COD) were measured just before and after washing, as well as periodically for 20 min following washing. This procedure was repeated three times for individual washes of disk carrots, cabbage, and lettuce, respectively.

Water quality parameters (FC, pH, and COD) were measured just before and after washing, as well as at 1, 2, 5, 10 and 20 min following the introduction of produce into the water. FC was measured via a DPD (N,N-Diethyl-p Phenylenediamine) method using a Chlorine Photometer (CP- 15, HF Scientific Inc., Ft. Myers, FL). The pH was measured using a digital pH meter. The COD was determined using a reactor digestion method.

Most chlorination reactions can be formulated as $HOCl + R \rightarrow Products$, where R is an organic or inorganic compound [60]. While there may be many constituents that react with chlorine during a produce wash, following the single wash experiment results for each produce type, we assume the chlorination reactions can be modeled via an averaged second-order reaction relative to the concentration of fresh-cut produce constituents in the wash water [60], and a first-order reaction describing FC decay in tap water [80]. Note that while we do not know the concentration of the produce constituents reacting with FC, we estimate this concentration as a fixed percentage (γ) of the increase in COD due to washing a single batch of produce (for 30 sec). Based on the single wash data and these assumptions, we model the FC decay as follows:

$$\frac{dC}{dt} = -\beta RC - \lambda C \tag{3.1}$$

$$\frac{dR}{dt} = -\beta RC \tag{3.2}$$

$$C(0) = C_0, R(0) = \gamma(\Delta COD) \tag{3.3}$$

where C $(mg.L^{-1})$ is the FC concentration in the wash water at time t, R $(mg.L^{-1})$ is the concentration of the reactant(s) coming from the produce at time t, $\frac{d}{dt}$ is the first derivative operator, β $(L.mg^{-1}.min^{-1})$ is the apparent second-order rate constant, λ (min^{-1}) is the first-order decay rate, ΔCOD $(mg.L^{-1})$ represents the difference in COD prior to and after washing a single batch of cut produce, and γ is the percentage of COD increase in the wash water due to washing one batch of produce. We used γ to determine the initial concentration (R_0) of the reactant(s) entering the wash water from the cut produce. Similar models in the context of produce washing were developed by [41] and in the context of drinking water by Kohpaei and Sathasivan (2011) [81] and references therein). We point out that the above model (equations (3.1)-(3.3)) is distinct from the model in [41] regarding the dynamics of FC consumption with respect to the reactant(s) from the produce. In particular, from equations (3.2) and (3.3), one can see that $R(t)$ $(mg.L^{-1})$, for each $t > 0$, is not proportional to ΔCOD.

3.2.2 Parameter determination

Using our model (equations (3.1)–(3.2)) and the assumption that reaction rate between R $(mg.L^{-1})$ and C $(mg.L^{-1})$ dominates FC consumption when R is present in the wash water, it follows that the change in FC concentration equals the change in reactant(s) concentration, $(\Delta C = \Delta R)$. Using this relationship, the assumption that R $(mg.L^{-1})$ is completely depleted within 5 min during the single wash experiments (see Fig. 1 and the discussion in section 3.1), and Eq. (3.3), the parameter γ can be calculated as $= \frac{C(0)-C(5)}{\Delta COD}$.

In order to determine the parameter β $(L.mg^1.min^1)$, let D_k represent the data measurements (FC level in $mg.L^{-1}$) at time t_k and let $C(t_k, X)$ signify the model output (FC level in $mg.L^{-1}$) given parameter vector $X = [\gamma, \beta]^\tau$ at time t_k (where γ is fixed by using the formula above). The calculated residuals are $e_{k,X} = D_k - C(t_k, X)$. To find the pa-

rameter β that provides the best model fit to the data, we use the *fminsearch* function, in MATLAB (MATLAB 2018b, The MathWorks, Natick, MA) to minimize the 2-norm of the function F defined as:

$$\|F(X)\|_2 = \left(\sum_k e_{k,X}^2 \right)^{1/2}$$

In order to account for the possibility of multiple minima, a multi-start procedure was utilized, dividing the interval $[1 \times 10^3, 1 \times 10^{-1}]$ into n = 30 subintervals to specify initial guesses for β. Note also that model outputs were calculated using the ode45 solver in MATLAB (MATLAB 2018b, The MathWorks, Natick, MA). Table IV gives the results in terms of parameter ranges for both parameters relative to produce type.

Recall that λ (min^{-1}) is assumed to be the first order decay rate of FC in tap water. It has been previously found that across a range of temperatures and time points, the bulk decay constant for FC in tap water is on average approximately 0.002 min^{-1} [80]. This value is similar to the range of values for λ across our single wash experiments for varying produce types (using data from Fig. 3.1 from 5 min onward). Due to this and because the model output governing FC concentration is not sensitive to λ at this order of magnitude, we fixed $\lambda = 0.002$ min^{-1} for the rest of the study herein.

Table IV: Parameter fit results for single-batch wash experiments. Parameters were presented as mean $\pm$ standard deviation.

Produce	β $(L.mg^{-1}.min^{-1})$	γ	λ (min^{-1})
Disc Carrots	0.102 ± 0.014	0.062 ± 0.012	0.00133 ± 0.00005
Cabbage	0.114 ± 0.041	0.096 ± 0.012	0.00122 ± 0.00005
Iceberg Lettuce	0.057 ± 0.007	0.113 ± 0.026	0.0015 ± 0.00031

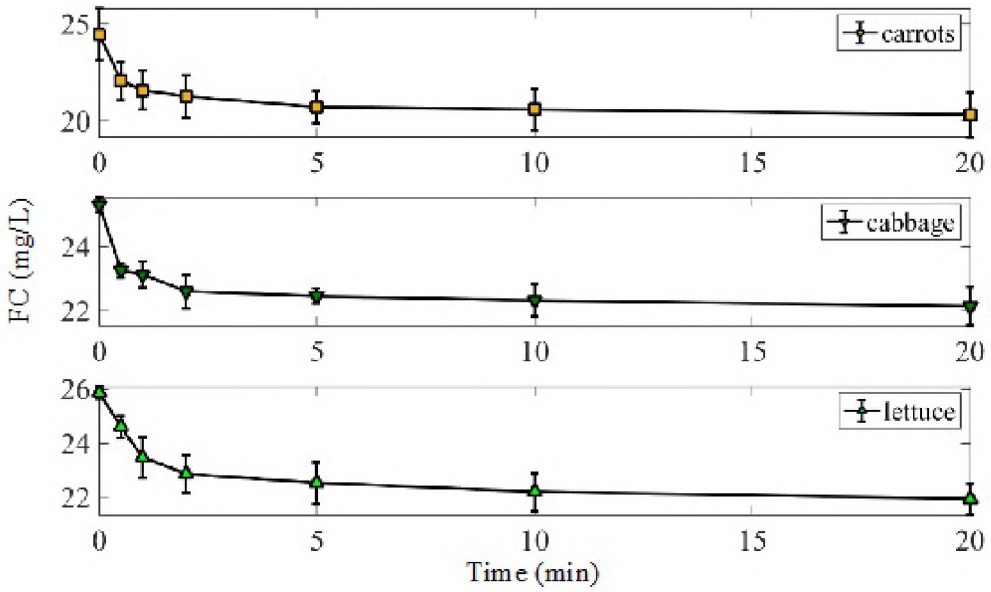

Figure 3.1: FC decay data from 100 g single wash experiments in 3 L of water for disk carrots, cut cabbage, and cut iceberg lettuce, respectively. Data presented were the mean $\pm$ standard deviation of 3 replicates (with respect to each produce type). Note that COD values were measured at the same times as the FC data presented; following the introduction of produce, after 0.5 min, the COD values did not change for respective produce types.

3.2.3 Multi-batch wash experiments and extended model

To test whether the model (equations (3.1)–(3.3)) captures the essential dynamics involved in FC decay, it was first calibrated using data from single-wash experiments. That is, parameter ranges for β and γ were determined as mentioned in section 3.2.2. In order to validate the model, two types of experiments were performed: (i) multi-batch produce washing with periodic FC replenishment at the 3 L scale, and (ii) multi-batch produce washing with periodic FC replenishment at 50100 L scale. In addition, published data from an experiment by Luo *et al.*(2012) [28] was used to validate the model for shredded iceberg lettuce washing at the pilot scale of 3200 L [28]. For each of these types of experiments, model inputs included only: (a) parameter values for β and β determined from single wash experiments, (b) COD increase rates, (c) the initial FC concentration before any produce was washed (time $t = 0$), and (d) sodium hypochlorite dosing information between respective runs, for carrots, cabbage, and iceberg lettuce, respectively.

Small scale experiments (3 L)

Experiments for periodic FC replenishment were carried out in a 3 L wash system. Essentially, the procedure followed that described in section 3.2.1, but for 3 separate runs. Briefly, sodium hypochlorite was added to the wash tank to achieve approximately 20 $mg.L^{-1}$ of FC (specific measurements presented in Fig. 3.2) in the wash water. The pH was simultaneously adjusted to 6.5 using 1 M citrate buffer. 600 g of the respective cut produce types were washed during each experiment through 6 batches, each consisting of 100 g. Directly after the first run of washing 600 g of produce, the FC was replenished (via the addition of sodium hypochlorite) to reach approximately 2030 $mg.L^{-1}$ (specific measurements presented in Fig. 3.2) and the pH was again adjusted to 6.5. The procedure above was followed until 1200 g of produce was washed batch-wise and then the FC was replenished a final time, the pH adjusted to 6.5, and the final 600 g of produce was washed. Thus, a total of 1.8 kg produce was washed for each trial with 3 separate runs.

Water quality measurements included FC and total chlorine, COD, turbidity, TDS, and pH. FC was measured immediately after each batch based on a DPD method using a Chlorine Photometer. The pH, turbidity, and TDS were measured on-site using a digital pH meter, turbidity meter, and TDS meter, respectively. The COD was determined using a reactor digestion.

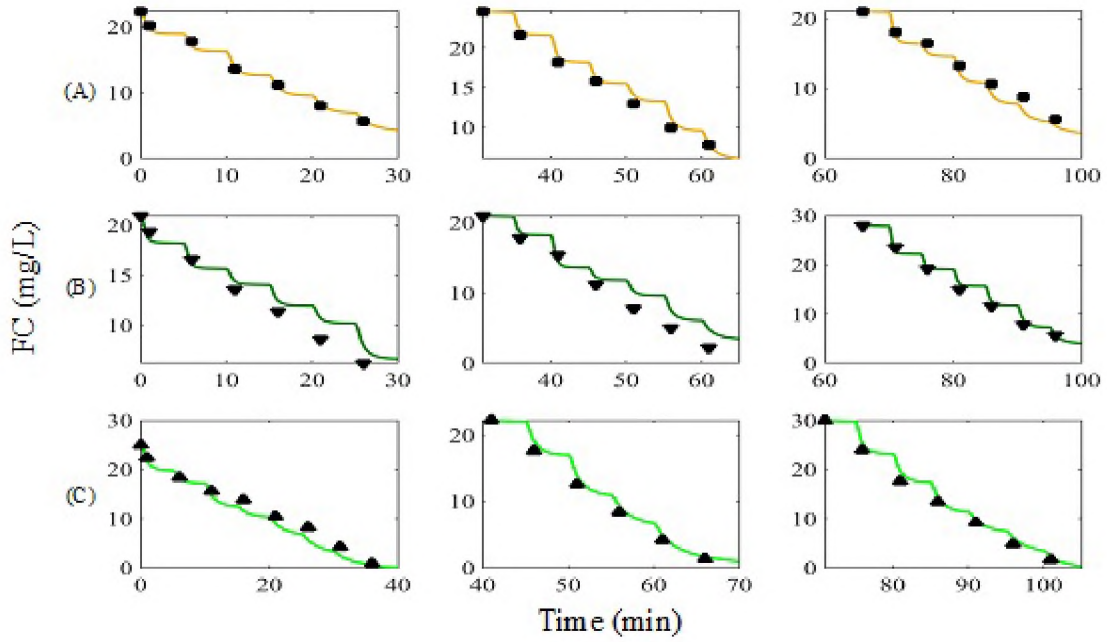

Figure 3.2: Model prediction of FC kinetics for disk carrots (A), cut cabbage (B), and cut iceberg lettuce (C), at 3 L (multi-batch wash experiments). Panel A indicates the predictions for disk carrots (trial 1, see Table 3) corresponding to three experimental runs with redosing of FC in between each run. Similarly, panels B and C correspond to cut cabbage (trial 1, see Table 3) and cut iceberg lettuce (trial 1, see Table 3), respectively. The solid lines are the model predictions and the data points are FC measurements. Refer to Table 3 for RMSE values.

Larger scale experiments (50100 L)

15 kg of produce were sliced using a mandolin slicer to $\frac{1}{8}$" thick for carrots and 1"$\times$1" pieces for lettuce/ cabbage, and kept in sterilized containers immediately prior to being discharged into a wash tank (tap water; 100 L for carrots and 50 L for lettuce/ cabbage) at a rate of 0.5 $kg.min^{-1}$ (0.25 kg batch every 30 sec). The experiment consisted of three 10 min runs, simulating a continuous wash operation with periodic replenishment of sodium hypochlorite. In each run, 5 kg of produce was washed. Before the start of the first run, 60 mL (for carrots wash) or 18–20 mL (for cabbage/ lettuce wash) of concentrated (4.5%) sodium hypochlorite was added to the wash tank to achieve approximately 21.5 $mg.L^{-1}$ FC (for carrots) and 13.5 $mg.L^{-1}$ of FC (cabbage/ lettuce) washing solution (specific measurements presented in Fig. 3.3). The pH was simultaneously regulated to 6.5 using citrate

buffer. During washing, the carrots pieces were submerged into the water manually, washed for 30 sec, and then removed via sieves. Water quality (COD, pH, Turbidity and TDS) and FC levels were measured every two minutes. After finishing the first run, the FC level was replenished by adding concentrated (4.5%) sodium hypochlorite and the pH was again adjusted to 6.5 by adding citrate buffer. The experiment resumed after 5 min, and samples were collected every two minutes for the second segment. Similarly, after finishing the second run, concentrated (4.5%) sodium hypochlorite was added to tank, pH readjusted to 6.5 using citrate buffer, and the same procedure was repeated for the third run.

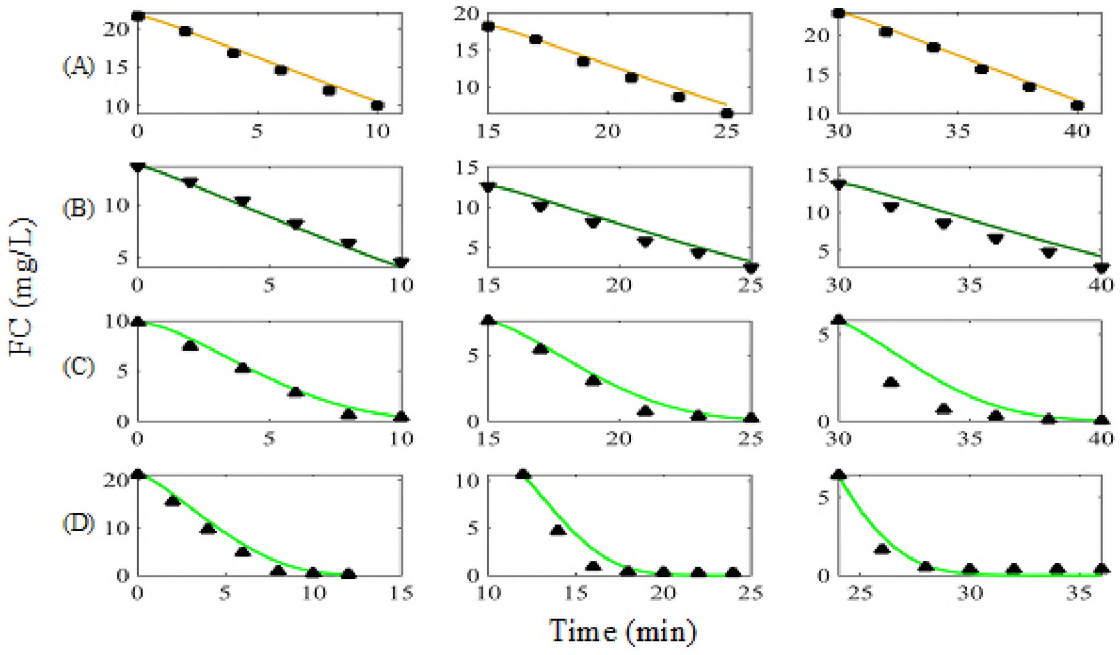

Figure 3.3: Model prediction of FC kinetics for disk carrots, cut cabbage and cut iceberg lettuce at 50–100 L and 3200 L (continuous wash experiments). Panel A indicates the predictions for disk carrots corresponding to three experimental runs (at 100 L) with redosing of FC in between each run. Similarly, panels B and C correspond to cut cabbage (50 L) and cut iceberg lettuce (50 L), respectively. Finally, panel D illustrates the FC prediction for washing shredded iceberg lettuce at the pilot scale (3200 L). The solid lines are the model predictions and the data points are FC measurements. Refer to Table 3 for RMSE values.

Model for multi-batch washing with chlorine replenishment

To account for a multiple batch wash process with FC redosing, equations (3.1)–(3.3) were naturally extended as follows:

$$\frac{dC}{dt} = D_n - \beta RC - \lambda C \tag{3.4}$$

$$\frac{dR}{dt} = \gamma K_n - \beta RC \tag{3.5}$$

$$K_n = \begin{cases} k_n & \text{for } t \text{ during batch } n \text{ wash time} \\ 0 & \text{else} \end{cases} \tag{3.6}$$

$$D_n = \begin{cases} d_n & \text{for } t \text{ during } n^{th} \text{ dose time} \\ 0 & \text{else} \end{cases} \tag{3.7}$$

$$C(0) = 0, R(0) = 0, \tag{3.8}$$

where d_n $(mg.L^{-1}.min^{-1})$ is the rate of FC increase depending on the addition of sodium hypochlorite to the wash water, and where k_n $(mg.L^{-1}.min^{-1})$ is the rate of COD increase during the n^{th} wash. Notice that for larger scale (> 3 L) washing experiments, $K_n = k_{avg}$, the average COD increase was used. We calculated d_n as follows: let S_0 be the initial volume of concentrated sodium hypochlorite added before any produce is washed, let S_n be the volume of sodium hypochlorite added at dose n, and let τ (min) represent the time to add sodium hypochlorite (usually about 2.5 sec), then $d_n = \frac{\frac{S_0}{S_n} C_0}{\tau}$.

3.2.4 Experimental procedure at the pilot plant scale

An experimental study by Luo *et al.,* (2012) [28] was conducted in a commercial pilot plant (New Leaf Food Safety Solutions, LLC, Salinas, CA). The setup consisted of a commercial double wash system, each tank with approximately 3200 L capacity, and equipped with rotating screens to ensure produce submersion, and air pumps to create turbulence in the wash water. While the larger goal of that study was to track *Escherichia coli* O157:H7 cross-contamination during lettuce washing, we considered here only the following components of that experiment relevant to tracking FC decay kinetics: (i) The entry rate of the shredded lettuce was approximately 45 $kg.min^{-1}$; (ii) While the process water was continuously screened and re-circulated, produce spent an average of 26 sec in each tank; (iii) The pH in the wash water was maintained at 6.5 using citrate buffer; (iv) Each test run involved three consecutive 12 min segments, simulating continuous processing with a periodic FC dosing scheme; (v) Sodium hypochlorite was added every 12 min (during a 2 min dosing period) with increasing dose volumes over a continuous wash period of approximately 40 min; and finally, (vi) pH, COD, turbidity and FC were monitored every 2 min.

In this Chaper, we utilize the COD data [from Fig. 2A (Luo *et al.,* 2012) [28]] to inform the parameter k_{avg} (increase in COD in the wash water relative to incoming rate of lettuce) and the parameter values for β and γ (determined via the procedure in section 3.2.2 relative to iceberg lettuce) as inputs into model (equations (3.4)–(3.8)) to predict the FC level data [from Fig. 3A in (Luo *et al.,* 2012) [28]].

3.2.5 Comparison of model predictions against data

Parameter values for β and γ (Table V), taken from the ranges determined from the single batch wash experiments for respective produce/cut types (as outlined in section 3.2.2), were used in the model (equations 3.4–3.8) to predict FC levels relative to each experiment conducted as described in sections 3.2.3 and 3.2.4. Note that λ is fixed to the same value

for all predictive simulations (see section 3.1 for more details). Root mean square error (RMSE) values were computed relative to each run/produce type to quantify the quality of the respective predictions and were reported in Table VI.

Table V: Parameter values used for model predictions.

Produce	β $(L.mg^{-1}.min^{-1})$	β $(\mu M^{-1}.min^{-1})$	γ
Disc Carrots	0.102	5.24 $\times 10^{-3}$	0.062
Cabbage	0.114	5.77 $\times 10^{-3}$	0.096
Iceberg Lettuce	0.057	2.99 $\times 10^{-3}$	0.113

HOCl molecular weight (52.46 $g.mol^{-1}$) is used for changing the unit of β.

3.3 Results and discussion

3.3.1 Parameter fits from single-batch wash experiments

FC decay data during single-batch washes, corresponding to the respective produce type, were presented in Fig. 3.1. Notice that for each produce type the decay pattern is bi-phasic, means that an initial fast decay (until about 5 min) followed by a transition to a relatively slower decay rate, similar to that observed by [55] as well as in the context of drinking/waste water by Kastl *et al.,* (1999) [82] and Clark, (1998) [83]. This observation as well as the fact that following the introduction of 100 g of cut produce into the water, after the first measurement (at 0.5 min), the COD values did not change for respective produce types, justify the assumption that the reactant(s) (denoted by R) must be depleted after the initial 5 min. Furthermore, the steady decay of FC after this point (Fig. 3.1) is assumed to follow purely first-order kinetics, which closely matched the decay rate of FC in tap water [80].

The results of the calculations for γ and the fitting procedure for, outlined in section 3.2.2 along with data to determine ranges for each parameter across the three experiments were presented in Table IV. The results for respective β values did not depend on the initial conditions administered during the minimization algorithm.

Table VI: Results of model prediction (quantified in terms of R^2 values) for multi-batch washing experiments at 3 L, continuous wash experiments at 50–100 L and continuous washing experiments at the pilot plant scale of 3200 L. Note that Trial i = 1 or 2 in the 3 L context, indicates separate repeat experiments (3 runs each).

Produce/Trial	Volume	R^2 (Run 1)	R^2 (Run 2)	R^2 (Run 3)	RMSE(exp.)
Disc Carrots1	3 L	0.99	0.98	0.95	0.89
Disc Carrots2	3 L	0.99	0.98	0.68	1.09
Cabbage1	3 L	0.94	0.92	0.99	0.93
Cabbage2	3 L	0.95	0.81	0.71	4.82
Iceberg Lettuce1	3 L	0.97	0.98	0.98	0.96
Iceberg Lettuce2	3 L	0.99	0.99	0.91	0.96
Disc Carrots	3 L	0.98	0.97	0.98	0.67
Cabbage	3 L	0.98	0.95	0.88	0.75
Iceberg Lettuce1	3 L	0.98	0.97	0.76	0.59
Iceberg Lettuce2	3 L	0.97	0.93	0.96	0.54

3.3.2 Model predictions at the 3 L and 50-100 L scale

Using commodity-specific values of the model parameters β, γ (Table V), and COD data [used to inform the change in COD, k_n , after each batch of produce washed for multi-batch washing , k_{avg} for larger scale washing experiments] as inputs and our model (equations 3.4–3.8) was able to successfully predict the FC decay kinetics at both the 3 L (multi-batch washing experiments) and 50100 L scales. Table V indicates the parameters used for predictions against all wash experiments for respective produce types and scales. Note that for each of the produce types, the values for β and γ fall within the ranges determined from the single wash experiments (Table IV).

Model results at 3 L

Table VI summarizes the performance of our model (equations 3.4–3.8) against FC decay data during the respective cut produce washing with FC replenishment at the 3 L scale, listing RMSE values for each respective run as well as a total RMSE value considering all three runs. Fig. 3.2 provides a clear visual of the success of this model in predicting FC decay for disk carrots (panel A), cut cabbage (panel B), and cut lettuce (panel C), respectively, at the 3 L scale. The model output for FC levels replicates a bi-phasic type decay pattern

between successive data points as washing is batch-wise (Fig. 3.2). Mathematically, this is accounted for via the on/off input function (Eq. 3.6). Furthermore, Fig. 3.2 indicates the dynamics of FC during the dosing interval, in between successive washing runs. For instance, Fig. 3.2(A) shows that sodium hypochlorite was added at $t = 27\ min$ with carrot washing restarted at $t = 35\ min$ for the second run. Sodium hypochlorite was additionally added at $t = 62\ min$ with carrot washing restarted at $t = 70\ min$. Dosing for washing the other produce types followed a similar pattern as seen in Fig. 3.2(B) and (C).

It is important to mention that the only chlorine related data the model takes as inputs are the FC measurement at time min and the amount of sodium hypochlorite added between runs. Thus, the results in Fig. 3.2 as well as those presented in Table VI (3 L experiments) illustrate the success of the model predictions in terms of both FC increase due to dosing and FC decay due to washing respective produce types. Notice that the total RMSE < 1.1 (Table VI last column) for each prediction except for cabbage trial 2, which had a total RMSE of 4.82. The issue here was that the model underestimated the FC decay during the second half of each washing run. The interesting part is that the data for cabbage trial 2 indicated faster FC decay rates at the end of each washing run with corresponding slower COD increase rates than during the first half of each washing run. It is not yet clear why this occurred, however, this inconsistency was not seen with any of the other experiments at 3 L or at the larger scale.

Model results at 50–100 L

For the large-scale multi-batch wash experiments, 50 L of water was used for lettuce and cabbage, and 100 L of water was used for disk carrots, respectively. We used 100 L of water for carrots vs. 50 L for cabbage and iceberg lettuce merely to make sure that similar sodium hypochlorite dosing amounts could be used for the experiments involving the three produce types. In particular, the water volumes were chosen so that rate of COD increase from washing the respective produce types would be similar. This ensured that the FC

would not be completely depleted half-way through a washing run.

Using the same parameter values listed in Table V for respective produce types, COD input (tracked as the average increase in COD, k_{avg}), our model (equations 3.4–3.8) was again able to successfully predict the FC dynamics (Table VI). Fig. 3.3 illustrates the model's prediction versus data for disk carrots at 100 L (panel A), cabbage washing at the 50 L scale (panel B), and for iceberg lettuce at 50 L (panel C).

The results in Table VI (and Figs. 3.2 3.3) suggest a few points. First, they indicate that the model (equations 3.4–3.8) captures the main mechanisms governing observed FC decay relative to the produce/cut type. Second, the model can consistently account for the FC levels in between runs relative to the amount of sodium hypochlorite added and the rate of decay of FC due to the amount of reactant(s) R ($mg.L^{-1}$) remaining at the start of the dosing period. Third, FC reaction rates relative to the produce/cut type appear to be robust with respect to scaling as the same rates used by the model at both the 3 L and 50100 L resulted in accurate predictions. Finally, the parameter γ, the fraction of the COD increase due to repeated produce washing, is a relatively consistent predictor of the associated produce/cut type organic load in wash water, and therefore a reliable predictor (via the model) of FC decay rates.

3.3.3 Model predictions for iceberg lettuce at a pilot-plant scale

Using the values of the model parameters γ, β (Table V), for shredded iceberg lettuce, and COD data from Luo *et al.,* (2012) [28] [used to inform the change in COD, $K_n = k_{avg}$, during continuous washing], as well as the volume/timing of 12.5% sodium hypochlorite additions as inputs, our model successfully predicted the FC dynamics at the pilot plant scale (3200 L). The comparison of the model output versus FC data were presented in the last row of Table VI and panel D of Fig. 3.3.

It is noteworthy that in the 3 L and 50 L iceberg lettuce experiments, the wash water temperature was 20 $°C$ and the lettuce cut size was 1"×1", whereas the water temperature

during the pilot scale experiments was about $5°C$ and the lettuce was shredded (6 mm shreds). Furthermore, the lettuce used in the 3 L and 50 L experiments was purchased from a local supermarket whereas the lettuce in the pilot plant study was washed within 24 h of harvesting. Our model results (Table VI) indicate that FC reaction rates associated with iceberg lettuce washing appear to be robust despite differences in produce supply-chain history, cut size, and experimental scale, as the same value for the apparent second order rate constant $\beta = 4.9 \times 10^{-2}$ $(L.mg^1.min^1)$ utilized in the model at the 3 L, 50 L and 3200 L resulted in accurate predictions. Note that for the 3200 L prediction, $\gamma = 0.135$ whereas for the 3 L and 50 L predictions, the model input used was $\gamma = 0.11$. While both values for γ are in the range determined from the 3 L single wash experiments, it may be that since the lettuce in the pilot plant study was used within a day of harvesting, soil and other such materials contributed to a slightly higher value of . These results also indicate that the temperature differences (20 $°C$ vs 5 $°C$, respectively) between the lab and pilot scale experiments did not significantly influence the observed FC decay rates. Furthermore, cut type seemed to not be significant as single wash 3 L experiments using shredded iceberg lettuce yielded almost identical ranges for γ and β as those for square cut lettuce (data not shown).

Additionally, Fig. 1A [from (Luo et al., 2012)] indicates that the average initial COD value in the 3200 L tank was about 300 $mg.L^{-1}$. This value is extremely high for a mixture of tap water, citrate buffer for pH control, and an initial dose of 700 mL of 12.5% sodium hypochlorite. If for instance some initial produce washing occurred before the start of the recorded runs, the initial condition for the reactant(s) concentration $R(0) > 0$. Notice that if we let $R(0) = 8mg.L^{-1}$, set $\gamma = 0.115$ and $\beta = 4.9 \times 10^{-2}$ $L.mg^1.min^1$, then the model predictions are similar to those in Fig. 3.3D, with an overall RMSE of 1.45.

Finally, even though the rate of iceberg lettuce coming into the wash tanks differed across experimental scale (and thus the COD increase rate differed across scales), the range for γ from the single wash lettuce studies at 3 L, used to represent the corresponding

increase in organic load, resulted in faithful FC predictions at all scales.

3.3.4 Predictors of FC decay

Note that the success of the model predictions (as discussed in sections 3.3.2 and 3.3.3) depends on the fact that the change in organic load (relative to produce/cut type) was quantified via a model that utilizes the successive increase in COD rather than current COD levels. More specifically, the consistency of the model (equations 3.4–3.8) predictions stem from two main assumptions (built from the single wash experiments), namely: (i) as cut produce enters the wash tank, the increase in constituents in the wash water that rapidly deplete FC [represented as the concentration of R in the model] is fairly well approximated as a fraction of the change in COD in the wash water (due to washing more and more produce), and (ii) the slower depletion of FC (via tap water or possibly other constituents) can be well approximated by a first-order decay term (see Eq. 3.4).

In terms of practically predicting FC levels, unfortunately, real-time COD measurements are not currently feasible during produce washing and therefore surrogate parameters which can be measured in real-time have been sought after. Many studies have examined the correlative strength of various water quality parameters such as UV254, turbidity, TDS, to name a few, with regards to chlorine demand and produce type [27, 73, 34, 74, 56, 72]. In particular, because of the ease of real-time measurements and because of their inclusion in multiple wash water studies, we chose to examine if the change in turbidity (ΔTUR) or the change in total dissolved solids (ΔTDS) could be consistently used to predict the change in COD (ΔCOD) relative to consecutive multi-batch and continuous washing of disk carrots, cabbage and lettuce. That is, we seek functions U and T such that $\Delta COD = U(\Delta TUR)$ and $\Delta COD = T(\Delta TDS)$. Furthermore, it is logical that these functions be monotonic (an increase in the input corresponds to an increase in the output).

To evaluate if such functions U and T can be determined in practice, wash data for carrots/ cabbage/ lettuce obtained at both the 3 L and 50100 L level (as detailed in section

3.2.3) was utilized as well as wash data for shredded lettuce at the pilot plant scale (as discussed in section 3.2.4). In particular, the Spearman's rank correlation coefficient (ρ) between (a) ΔTUR and ΔCOD, and (b) ΔTDS and ΔCOD, for respective produce types and experimental runs was calculated. Essentially, indicates how well the connection between two variables can be described by a monotonic function.

Using the *corr* function in MATLAB, the corresponding ρ value for each experimental run and across respective experiments was calculated. The results are presented in Table VII, Table VIII (3 L scale), Table IX, Table X (50-100 L scale), and Table XI (pilot scale 3200 L), providing strong evidence that there is no consistent relationship between ΔTUR and ΔCOD as well as between ΔTDS and ΔCOD for cut carrots, cabbage and iceberg lettuce across the scales considered. While a complicated mechanistic relationship that links real-time ΔTUR or ΔTDS with FC decay kinetics might exist, the analysis above illustrates that this relationship is not readily evident.

Table VII: Spearman's rank correlation coefficient (ρ) for ΔCOD vs ΔTUR data from experiments at the 3 L scale.

Produce	Experiment	Run 1	Run 2	Run 3	Total
Carrots	1	0.1490	0.3714	0.0290	0.1376
Carrots	3	0.4569	0.1218	0.5385	0.3659
Cabbage	1	0.3714	0.4928	0.1160	0.1894
Cabbage	3	0.3361	0.3810	0.2101	0.2699
Iceberg	1	0.3353	0.700	0.2319	0.1402
Iceberg	2	0.2224	0.7143	0.8469	0.4011

Table VIII: Spearman's rank correlation coefficient (ρ) for ΔCOD vs ΔTDS data from experiments at the 3 L scale.

Produce	Experiment	Run 1	Run 2	Run 3	Total
Carrots	1	0.1177	0.3381	0.6269	0.4054
Carrots	3	0	0.1674	0.2582	0.0181
Cabbage	1	0	0.8454	0.1791	0.3119
Cabbage	3	0.7307	0.2520	0.1967	0.0274
Iceberg	1	0.0782	0.2887	0.1852	0.2191
Iceberg	2	0.3203	0.0976	0.1456	0.0884

Table IX: Spearman's rank correlation coefficient (ρ) for ΔCOD vs ΔTUR data from experiments at the 50 L for cabbage and iceberg, and 100 L scale for disk carrots. * indicates significance where $p < 0.05$.

Produce	Experiment	Run 1	Run 2	Run 3	Total
Carrots	1	0.3000	0.100	0.100	0.2538
Cabbage	1	0.3000	0.600	0.100	0.1251
Cabbage	2	0.8208	0.100	0.500	0.0413
Iceberg Lettuce	1	0.6325	1.00	0.400	0.1053
Iceberg Lettuce	2	0.6669	0.300	1.00*	0.4293

Table X: Spearman's rank correlation coefficient (ρ) for ΔCOD vs ΔTDS data from experiments at the 50 L for cabbage and iceberg, and 100 L scale for disk carrots. * indicates significance where $p < 0.05$.

Produce	Experiment	Run 1	Run 2	Run 3	Total
Carrots	1	0.7071	0.7182	0.5798	0.4080
Cabbage	1	0	0.2887	0.8660	0.3233
Cabbage	2	0.8652	0.7379	0.2887	0.5911*
Iceberg Lettuce	1	NaN	0.4472	0.2582	0.1913
Iceberg Lettuce	2	0.1579	0.3536	1.00*	0.0088

Table XI: Spearman's rank correlation coefficient (ρ) for ΔCOD vs ΔTDS average data from pilot plant experiment [from Fig. 2A of (Luo *et al.*, 2012)].

Produce	Run 1	Run 2	Run 3	Total
Iceberg Lettuce	0.3714	0.4857	0.0286	0.0588

3.4 Conclusions

The wash model developed in this study applies to a variety of produce types in the context of predicting FC dynamics (decay and re-dosing) across various scales. The fact that its predictions of FC levels hold at multiple experimental scales and across three produce types strongly suggests that the model illustrates fundamental chlorine dynamics that occur during fresh cut carrot/cabbage and iceberg lettuce washing. In particular, these findings give validity to performing future lab scale experiments to quantify FC kinetics associated with different produce/cut types as well as experiments aimed at understanding the impact of continuous FC dosing on FC dynamics during produce washing. Specifically, the effect of pH dynamics should be explored in this context as the results from this paper were

obtained via wash water pH levels near 6.5.

In addition, it was shown that turbidity and TDS measurements may not be reliable in predicting FC levels (as opposed to chlorine demand) as there is no consistent, observable relationship linking the increase in organic load (in terms of change in COD) from cut carrots/cabbage/lettuce entering the wash tank (across various scales 3 L, 50–100 L, and 3200 L) and the corresponding increase in turbidity or TDS. Recall that the model's predictive success across scales and produce types provides a strong case that COD information is much more dependable than that of turbidity or TDS. In particular, the fraction (γ) of the COD increase due to washing produce was shown to be a consistent predictor of the associated produce/cut type organic load in wash water and therefore a reliable predictor (via the model) of FC decay rates.

Finally, the results from this work demonstrate the utility of using mathematical models as tools to elucidate fundamental mechanisms, like FC reaction rates associated with various produce cut types. To minimize expensive experiments at the commercial scale, the model developed herein, and particular lab scale experiments could aid in planning the logistics of how and what should be measured during commercial scale experimentation.

CHAPTER IV

TOTAL AMINO ACIDS CONCENTRATION AS A RELIABLE PREDICTOR OF FREE CHLORINE LEVELS IN DYNAMIC FRESH PRODUCE WASHING PROCESS

In this Chapter, We establish the total amino acids (AA) concentration in wash water as an alternative indicator of free chlorine (FC) levels, and develop a model to predict FC concentration based on modeling the reaction kinetics of chlorine and amino acids. Using single wash of iceberg lettuce, green cabbage, and carrots, we report the first *in situ* apparent reaction rate β between FC and amino acids in the range of 15.3 – 16.6 $M^{-1}.s^{-1}$ and an amplification factor γ in the range of 11.52 - 11.94 for these produce. We also report strong linear correlations between AA levels and produce-to-water ratio ($R^2 = 0.87$), and between chemical oxygen demand (COD) and AA concentrations ($R^2 = 0.87$). The values of the parameters γ and β of the model were validated in continuous wash experiments of chopped iceberg lettuce, and predicted the FC ($R^2 = 0.96$) and AA ($R^2 = 0.92$) levels very well.

4.1 Introduction

Produce-associated foodborne outbreaks have increased widely over the last two decades due to the steady rise in consumption of ready-to-eat fresh produce [55]. According to the Center for Disease Control and Prevention, 27% of all outbreaks in the United States were due to the consumption of fresh produce [8]. In order to reduce the risk of pathogen

contamination and enhance produce safety, it is critical to have an effective washing step towards the sanitation of fresh produce. While the aim of washing process is to reduce the microbial load, it could also be a source of contamination in the absence of a sanitizer, and spread pathogens from an infected produce to a clean produce [37]. So, the presence of disinfectants is essential to inactivate pathogens and prevent cross-contamination [13, 71]. Chlorine based sanitizers are the most common disinfectants in fresh produce washing industries because chlorine is cost-effective and inactivates pathogens quickly [9, 5]. However, our knowledge of sanitizer control is limited and there is a critical need for more studies on sanitizer decay kinetics during fresh produce washing process [55, 50].

When produce is cut and introduced to wash water, several organic compounds are added from the cut surfaces to the water. In the produce washing industry, a major portion of wash water is recycled and organic load accumulates in the wash water, which decreases the efficacy of chlorine [84, 9]. On the other hand, since chlorine is very reactive, it reacts with these organic compounds and gets depleted [56]. Maintaining sufficient levels of free chlorine is key to avoid pathogen cross-contamination in produce washing process [8, 13, 85, 50]. While presence of residual free chlorine (FC) is critical to ensure the safety of the washing process, high levels of chlorine could lead to carcinogenic, halogenated, disinfection by-products (DBPs), such as trihalomethanes [8, 54]. Therefore, developing models that can predict FC levels is crucial to maintain their levels in a range that prevents both cross-contamination and formation of hazardous DBPs [33].

Numerous efforts are currently ongoing to find methods and mathematical models for predicting chlorine demand in fresh produce washing process [56]. Various physicochemical characteristics of wash water such as chemical oxygen demand (COD), oxidation reduction potential (ORP), ultraviolet absorbance at 254 nm (UV254), turbidity, total organic carbon (TOC), total suspended solids (TSS) and total dissolved solids (TDS) have been quantified to predict chlorine demand (CLD) [13, 52, 55, 73, 75]. A recent study by Li *et al.,* (2019) [52] correlated CLD with COD, TOC, TDS, and turbidity for various

produce types and cut sizes, and concluded that there is a strong correlation between CLD and COD. Another study by Chen Hung, (2016) [73] looked at COD, ORP, and UV254 to predict CLD and reported that UV254 had the highest correlation with CLD. In yet another study by Weng *et al.,* (2016) [37] evaluating the origins of CLD input and chlorine decay, the authors concluded that COD and TOC were strongly correlated to CLD regardless of the type of produce. UV absorbance has been explored as a tool to estimate CLD during fresh-cut produce washing process [56], and both UVAmin and UVAmax (range 240–290 nm) were deemed necessary to predict CLD, although the exact wavelength depended on the produce type. Although ORP was explored as a potential parameter to predict FC [51], it is significantly affected by wash water properties and process conditions, rendering the FC predicted levels under certain conditions useless for other situations. Finally, it was reported that there is no significant correlation between FC levels and ORP [71].

Despite this progress, a comprehensive understanding of chlorination kinetics in a dynamic wash process is still lacking [50]. While predicting CLD in a single wash cycle or in process wash water was the focus of most of these studies, elucidating the mechanisms by which chlorine is dynamically depleted is more relevant for the produce wash industry, where chlorine is typically dosed several times during the continuous produce wash. Previously, we have developed a mathematical model to predict FC levels in dynamic wash of various produce types, based on the chlorination reactions and using COD as an indicator of organic load [86, 41]. We have expanded our model to be commodity-specific and improved chlorine management strategies across multiple experimental scales (lab to pilot-plant) [87]. However, COD measurements do not illuminate the various organic matter which specifically react with chlorine and consume it. Moreover, while the FC levels are lowered due to various reactions, there is no corresponding decrease in COD levels [28]. Therefore, the goal of this research is to demonstrate that total amino acids (AA) concentration is a more accurate indicator of FC levels for various produce types as their concentrations decrease proportionately with FC levels. In addition, we here (I) determined

the relation between FC levels and AA concentration for three common produce types with different cut-sizes, (II) developed and validated a mathematical model based on the reaction between FC and AA, and (III) utilized the AA concentrations in these produce types and scales to predict FC levels over time.

4.2 Materials and methods

4.2.1 Characterization of wash water

Wash water samples from experiments were taken at regular intervals and analyzed for COD, FC, and AA concentration. COD was determined using the reactor digestion method, while FC levels were measured immediately after taking the sample, using DPD (N, N-diethyl-p-phenylenediamine) method, with a Chlorine Photometer (CP-15, HF Scientific Inc., Ft. Myers, FL). The pH was continuously measured using a digital pH meter (Orion™ 2-Star, Thermo Fisher Scientific, Grand Island, NY).

4.2.2 Chlorine decay in 3 L batch experiments

These experiments were designed to investigate FC and AA concentration decay after adding sodium hypochlorite as the source of chlorine. Various produce types (iceberg lettuce, carrots, and cabbage) were purchased from the local supermarket, preserved at $4°C$, and used for experiments on the same day of purchase. The outer leaves of lettuce and cabbage were discarded, and fresh leaves were chopped and washed in 3 L of water maintained at 4 $°C$ (Table XII). This is comparable to wash water temperatures at large commercial facilities. The pH was set to 6.5 using 0.1 M citrate buffer. Varying amounts of produce (Table XII) were washed in the water for 30 sec and then removed by a sieve. Before adding sodium hypochlorite, samples were collected for measuring COD and AA concentration. Then 1.5 mL of concentrated (4.5%) sodium hypochlorite (BCS Chemicals, Redwood City, CA, USA) was added to reach an initial FC level of $\sim$25 $mg.L^{-1}$. Samples to measure COD, FC and AA concentration were taken every two minutes for the next ten

minutes after adding sodium hypochlorite. All experiments were repeated 3 times.

Table XII: Produce washed in 3 L batch experiments.

Produce	Amount (g)	Cut type	Cut size
Iceberg Lettuce	250	Shredded	Stripes of 5 mm
Carrots	160	Sliced	Discs of 2.5 mm
Cabbage	250	Shredded	Stripes of 5 mm

4.2.3 FC decay in the presence of carbohydrates

The most abundant constituents in leafy greens are carbohydrates (Fig. 4.1), and dextrose and fructose are the dominant types in iceberg lettuce (FoodData Central, USDA). We therefore investigated if carbohydrates consume FC during leafy green wash in chlorinated water. Initially, 1.5 mL of concentrated (4.5%) sodium hypochlorite was added to 3 L of water (4 $°C$, pH adjusted to 6.5 using 0.1 M citrate buffer). Water samples were collected for FC and COD measurements. Fructose or dextrose were then added to the water to reach 100 μM concentration and FC and COD levels were monitored over the next 30 min.

4.2.4 FC decay in the presence of amino acids and carbohydrates

To investigate if the presence of carbohydrates affects the reaction rate constant of chlorine and amino acids, experiments were designed in which fructose and dextrose (Sigma Aldrich) were added to 3 L of water (4 $°C$) to reach a combined concentration of 100 μM. Cell-free amino acid mixture (Sigma Aldrich) was added to the water to reach 45 μM of total AA concentration. Next, 1.5 mL of concentrated (4.5%) sodium hypochlorite was added to the water. Samples for measuring FC levels and AA concentration were taken every two minutes over the next ten minutes. Control studies were performed without adding fructose and dextrose.

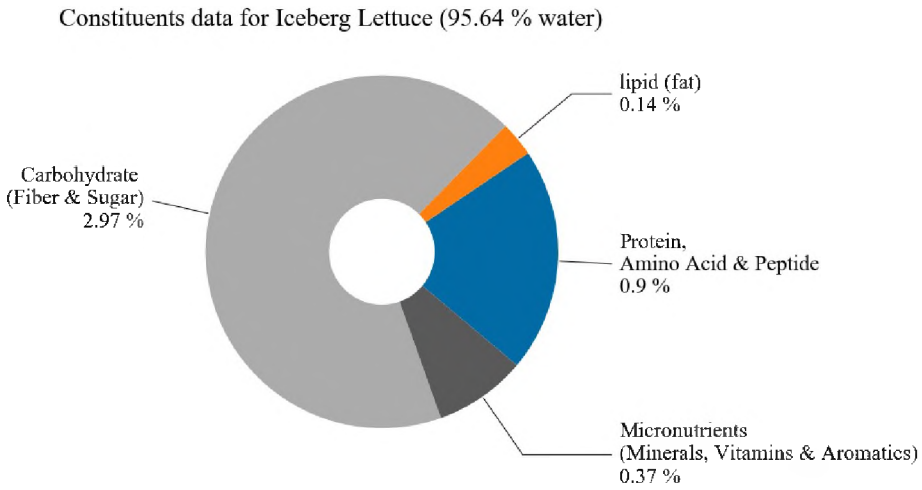

Figure 4.1: Iceberg lettuce constituents based on data provided by FoodData Central, USDA (Food Code: 11252; 2019)

4.2.5 Amino acid quantification assay

Total amino acids levels were measured using the L-Amino Acid Quantitation Kit (Sigma Aldrich), which measures the total L-amino acids concentration in the solution except for L-glycine. Samples collected for AA analysis were mixed with sodium thiosulfate (Sigma Aldrich; 1:5 w/v) to neutralize residual chlorine and stop the reaction between chlorine and amino acids. Briefly, L-amino acids standard solution was mixed with L-amino acids assay buffer (both provided with the assay kit) to generate 0, 0.8, 1.6, 2.4, 3.2, and 4 $nmol$/well standards solutions, which were added to the wells of a 96-well flat-bottom plate. Separately, 50 μL of each sample taken from experiments were directly added to the wells of 96-well plate, and mixed with 50 μL of master reaction mix (provided with the assay kit) by pipetting. This assay is based on the concept of coupled enzyme reaction at 37 $^{\circ}C$ for 30 min, which lead to a product measurable at an excitation $\sim$535 nm and emission $\sim$587 nm on a microplate reader (SynergyH1, BioTek, Vermont, USA).

4.2.6 Chlorine and amino acid concentration prediction model

As the produce was introduced to wash solution, FC concentration decreases. This is due to the reaction of chlorine with different organic and inorganic constituents that were released into the water when produce is washed. Among the various constituents of produce (Fig. 4.1), we observe that amino acids are the most likely to react with chlorine, and the reaction kinetics is of second-order [60]. However, in our measurements, the AA concentration alone does not fully account for the large reduction of FC levels, assuming second-order kinetics. Thus, we will use R to denote all the reactants that would react with FC. Based on the reaction between chlorine and amino acids, we developed a second-order reaction relative to the concentration of free chlorine and the reactants as follows:

$$\frac{d[R]}{dt} = -\beta[FC][R], \qquad [R](0) = \gamma[AA](0) = \gamma[AA]_0 \tag{4.1}$$

$$\frac{d[FC]}{dt} = -\beta[FC][R] - \lambda[FC], \qquad [FC](0) = [FC]_0 \tag{4.2}$$

where $[FC]$ $(mg.L^{-1})$ is the FC concentration in the wash water at time t, $[R]$ $(mg.L^{-1})$ is the concentration of all the reactants from the produce at time t, $[AA]$ $(mg.L^{-1})$ is the concentration of the total amino acids coming from the produce at time t, β $(L.mg^{-1}.min^{-1})$ is the second-order apparent reaction rate constant, λ is the decay rate of FC in water, and γ is an amplification factor of reactants not measured by the amino acid assay. While there is no a *priori* reason to assume that all the reactants have the same reaction rate β with FC, we will demonstrate that this is a surprisingly good approximation for describing the kinetics of FC and the reactants in the wash water.

4.2.7 Determination of model parameters

To reduce the number of parameters optimized, we will use the decay rate of FC in water to be 1.7 $\times 10^{-3} min^{-1}$ [80]. We independently conducted numerous experiments that

measure the FC decay in water in the absence of any produce and arrived at a similar value. Using the results of single batch experiments and the model presented in the previous section, we determined the parameters (γ,β) of our model for each specific produce type as follows: As the stoichiometric coefficient of the amino acids' chlorination reaction is the same for both amino acids and FC, we assumed that the change in the concentration of amino acids and FC should be the same. Thus,

$$\gamma\,(\Delta AA) = (\Delta FC) \tag{4.3}$$

where ΔAA and ΔFC show the change in total amino acids and FC concentrations, respectively. We calculated the value of γ based on this equation, and then using equations (1) and (2), we optimized β using the Levenberg–Marquardt algorithm.

4.2.8 Chlorine decay in 20 L continuous-wash experiments

These experiments consist of three 10-min runs, with 5 kg of chopped iceberg lettuce being washed in each run. Before the experiments, the outer leaves of iceberg lettuce heads were removed, and the heads were chopped into 1"$\times$1" pieces. To begin the experiment, concentrated (4.5%) sodium hypochlorite was added to a tank containing 20 L of water at 4°C, to approximately attain FC levels $\sim$12 $mg.L^{-1}$. The pH was regulated to 6.5 by adding 0.1 M citrate buffer. Within 5 min of adding chlorine, chopped iceberg lettuce was introduced at a rate of 0.5 $kg.min^{-1}$ and the dwelling time of lettuce was set at 30 sec, similar to prior experiments. Samples to measure COD and AA levels were collected every two minutes and stored at 4°C for quantification after the experiments, while FC levels were measured immediately after collecting samples. After washing 5 kg of chopped lettuce over the 10 min period, 18 mL of sodium hypochlorite was added to the tank within next 30 sec to replenish the FC levels, and citrate buffer added to restore the pH to $\sim$6.5. The next run started 5 min after the end of first run. Samples from the second run were collected and analyzed similar to their counterparts from the first run. At the end of second run, FC

levels were replenished by adding another 18 mL of concentrated sodium hypochlorite and the pH adjusted to 6.5, and the third run was performed similar to the previous two runs. A total of 15 kg of chopped iceberg lettuce were washed by the end of these three runs, and this experiment was repeated twice.

4.2.9 Model validation

The parameters of the model (presented in section 4.2.6) obtained from the single wash experiments in 3 L batches (detailed in section 4.2.2) were validated for continuous-wash experiments in 20 L tanks (section 4.2.8). As the iceberg lettuce is continuously added to the tank in this experiment, we need to extend the Equation 4.1 for the continuous wash and consider the adding rate of amino acids:

$$\frac{d[R]}{dt} = \gamma K_0 - \beta[FC][R] \tag{4.4}$$

$$K_0 = \begin{cases} k_0 & \text{continuous washing} \\ 0 & \text{batch washing} \end{cases} \tag{4.5}$$

where k_0 is the adding rate amino acids to the wash tank when the cut produce is introduced into the wash water. The equation for FC remains unchanged during the runs because FC is added to the tank only at the beginning of each run.

4.2.10 Correlating AA concentration to produce-to-water ratio and COD

As COD has been used in the past to study its effect on FC decay and CLD [73, 52, 28], we designed a set of experiments to study the correlation of AA concentration to produce to water ratio and COD. In these experiments, different amounts of chopped iceberg lettuce (1"×1" pieces), cabbage (1"×1" pieces) and sliced carrots (0.1" thickness) have been washed in 3 L of water at 4°C for 30 $sec.$ After washing, the samples were collected, and AA concentration and COD levels were quantified using procedures detailed earlier.

4.3 Results and discussion

4.3.1 Model training and parameter-fitting using single-wash experiments

The experimental results (FC and COD levels, AA concentration) of the single wash experiments (section 4.2.2) for various produce types (Fig. 4.2) indicates that the AA concentration reached almost zero after 10 min of adding chlorine to wash water. While the FC concentration decreased by $\sim$372 μM for iceberg lettuce, the corresponding drop in AA concentration over the same time frame was $\sim$32 μM (Table XIII). Similar reductions in the concentrations of FC and total amino acids were noted for carrots and cabbage. Recall that γ is an amplification factor and represents the ratio of the change in FC to the change in AA concentration. This factor accounts for all reactants in the wash water that are not measured by the amino acid assay used. The average value of γ across the three produce types studied here ranged between 11.52 to 11.94.

Over the 10 min of chlorine exposure, COD levels stayed nearly constant (shown as a horizontal line in Fig. 4.2). Comparing the COD levels for various produce types suggested that the total AA concentration that was released to the water is not related to the COD level. For example, while the COD level was higher for process wash water of carrots than process wash water of iceberg lettuce, the AA concentration for iceberg lettuce was higher. In other words, COD cannot be used as the only indicator to predict FC levels without considering produce type.

Table XIII: Results from curve-fitting and model training for single wash cycle experiments.

Produce	Cut-size	ΔFC (μM)	ΔAA (μM)	γ	β ($M^{-1}.s^{-1}$)
Iceberg lettuce	Chopped	372.47 $\pm$ 12.11	31.18 $\pm$ 3.08	11.94 $\pm$ 0.67	16.57 $\pm$ 1.08
Carrots	Sliced	289.74 $\pm$ 8.89	25.14 $\pm$ 2.11	11.52 $\pm$ 0.94	15.67 $\pm$ 1.22
Cabbage	Chopped	278.31 $\pm$ 9.91	24.01 $\pm$ 2.03	11.59 $\pm$ 1.02	15.33 $\pm$ 1.25

Using experimental data of FC and AA concentration along with the developed model (section 4.2.6), the fitting procedure was done using Levenberg–Marquardt algorithm and the results of fitting for the average apparent reaction rate constant β between FC and free amino acids present in the wash water for the three produce types are given in Table XIII,

and ranges between 15.3 - 16.6 $M^{-1}.s^{-1}$. The units $M^{-1}.s^{-1}$ used here for this reaction rate is done in a way that it can be directly compared to reaction rates reported in the literature. The absolute second-order rate constants for the reaction of hypochlorous acid with various individual free amino acids have been reported to be in quite a wide range of 10^{-2} – $10^{7} M^{-1}.s^{-1}$ at neutral pH, with the transfer of dissociated chlorine to the amino group nitrogen occurring very rapidly [60, 88]. Similar reaction rates could be expected if sodium hypochlorite is used instead of hypochlorous acid. From the experimental data obtained using the method outlined in section 4.2.10, we determined that the rate of FC decay was quite rapid when we only had amino acids and carbohydrates, the two most abundant components of iceberg lettuce apart from water (Fig. 4.2), present in the solution (Fig. 4.3). Using methods outlined in section 4.3.5, we eliminate the possibility that carbohydrates in the wash water hinder the reaction rate of FC with amino acids. As a result, we hypothesize that the reaction of FC in our system is primarily with protein backbone amides, whose reaction rate with hypochlorous acid is of the order of $10^{1} - 10^{-3}$ $M^{-1}.s^{-1}$ [88], and posstibly partly with the two most abundant amino acids in fresh produce, glutamic acid and aspartic acid. Chlorine reaction with amino acids and peptides (only terminal amines) is usually fast. For compounds containing no sulfur, it results in initial N-halo-(amino acids or peptides) formation [60]. In the case of a-amino acids, a decarboxylation and a desamination follows this initial chloramination step which leads to a carbonyl compound, ammonia and a nitrile. In the case of peptides, the initial N-chloramination would take place on the nitrogen atom at the amino-terminal function. No chlorine reactivity with the carboxy-terminal residue or the peptide bond was previously shown. Similar to a-amino acids, further decarboxylation and desamination mechanisms were shown for glycylphenylalanine and alanylphenylalanine [60].

We further hypothesize that the presence of a significant amount of backbone amides could be one of the primary reasons why the method used to determine the levels of AA concentrations in this study does not account for all the possible reactants with FC, as most

of these amino acids do not separate easily from protein backbones in order to be detected by our assay. However, we believe that testing such hypotheses detracts from the main aims of this work.

4.3.2 Interactions between carbohydrates and free chlorine

Carbohydrates are the most abundant constituent among all organic matter coming into the wash water from the produce exudates (Fig. 4.2), with fructose and dextrose dominating in iceberg lettuce based on the USDA nutrient data. We thus investigated if there is any reaction between free chlorine and these two types of sugars (Fig. 4.4). The FC levels stayed constant for 30 min in both these experiments, indicating that there is no reaction between chlorine and fructose or dextrose. The relatively stable COD levels in these solutions was also shown for comparison (horizontal dotted lines).

4.3.3 Correlating AA concentration with produce-to-water ratio and COD

Our model is based on the AA concentration in the wash water added by introducing the produce to the wash tank. Recent work by different groups [73, 52, 28, 41] have related FC levels and CLD to COD levels. Establishing a correlation between produce to water ratio or COD with AA concentration could help predict the adding rate of amino acids to the wash water as well as total amount of amino acids added to the wash tank. The correlation among produce to water ratio, COD, and AA concentration added to wash water for the three produce types (chopped iceberg lettuce, sliced carrots, chopped cabbage) are shown in Figure 4.5.

The strong positive linear correlation between COD and produce to water ratio is expected because, as more produce is being washed more exudates enter the wash tank. Similarly, the AA concentration was also strongly correlated to the produce to water ratio. Thus, it is not surprising that the AA concentration strongly correlated to COD levels for each produce type tested (Fig. 4.5), which we propose to use as a readout to assess the COD

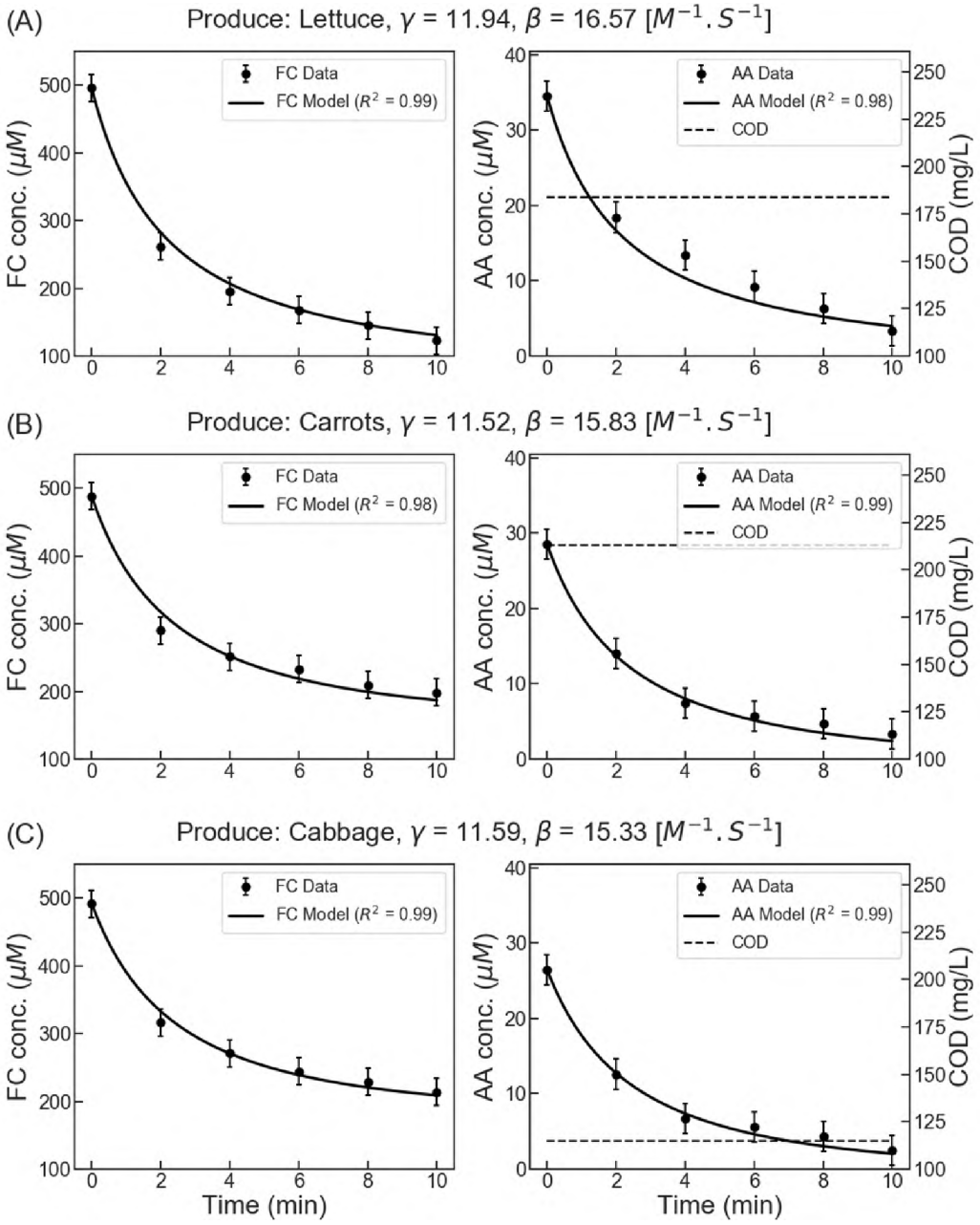

Figure 4.2: Time-dependent changes in free chlorine (FC), total amino acids (AA) concentration, and COD levels from single-wash studies of chopped iceberg lettuce (A), sliced carrots (B), and chopped cabbage (C). The experimental data (represented as filled circles $\pm$ standard deviation) was trained on the kinetics model to obtain the model parameters γ and β (reported above the figures, and along with their standard errors in Table XIII). It can be seen that the model fit to the experimental data were excellent ($R^2 \geq 0.98$), and the values of γ and β were very close in all cases.

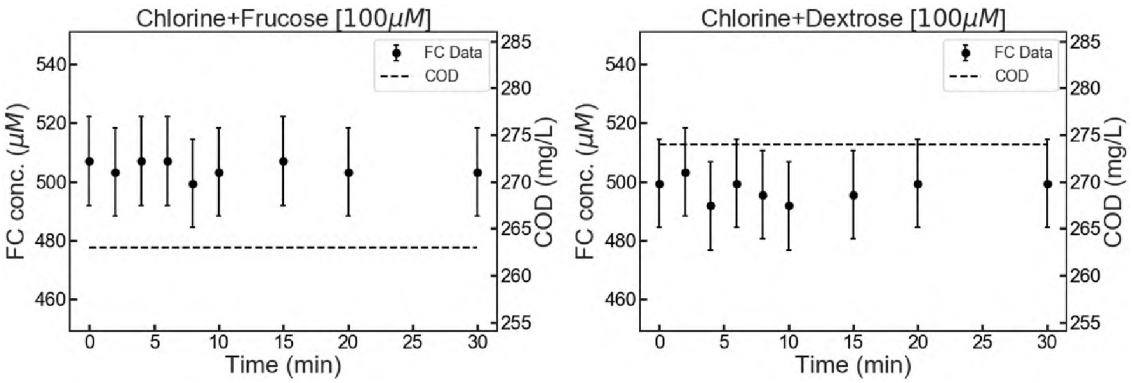

Figure 4.3: Changes in free chlorine (FC) and total amino acids (AA) concentration, in the absence (A), or presence (B) of externally-supplemented carbohydrates (50 μM of fructose and 50 μM of dextrose). These concentrations were chosen to closely mimic the conditions used in experiments for lettuce in Figure 4.2. The closed circles are experimental data represented as mean $\pm$ standard deviation, while the dotted lines are for visual aid only.

levels in respective wash solutions. Assessing AA concentrations in these produce wash solutions instead of COD measurements is advantageous as it is quicker to experimentally measure AA levels than COD levels for each sample solution.

4.3.4 Model prediction for continuous-wash process

We earlier fitted our model to the experimental data obtained from single-wash cycle (3 L experiments; Fig. 4.2) and the parameter values were reported in Table XIII. We evaluated the performance of our model in predicting FC levels in continuous-wash experiments of 20 L wash water batches (section 4.2.8). These experiments consist of three 10-min runs of washing iceberg lettuce with two 5-min stops between runs to replenish chlorine and regulate the pH. Results (Fig. 4.6) suggest that the chlorine decay increases in each successive run, most likely due to the residual amino acid levels in the wash water from the prior runs. This hypothesis was supported by experimental data (Fig. 4.6) which clearly showed the rise in the AA concentration in the wash water as more produce was washed.

Model evaluation was done by testing the ability of our model in predicting FC and AA concentrations in continuous-wash experiments using our model explained in section 4.2.9. For this, we have used the fitted parameter values of single-wash experiment for

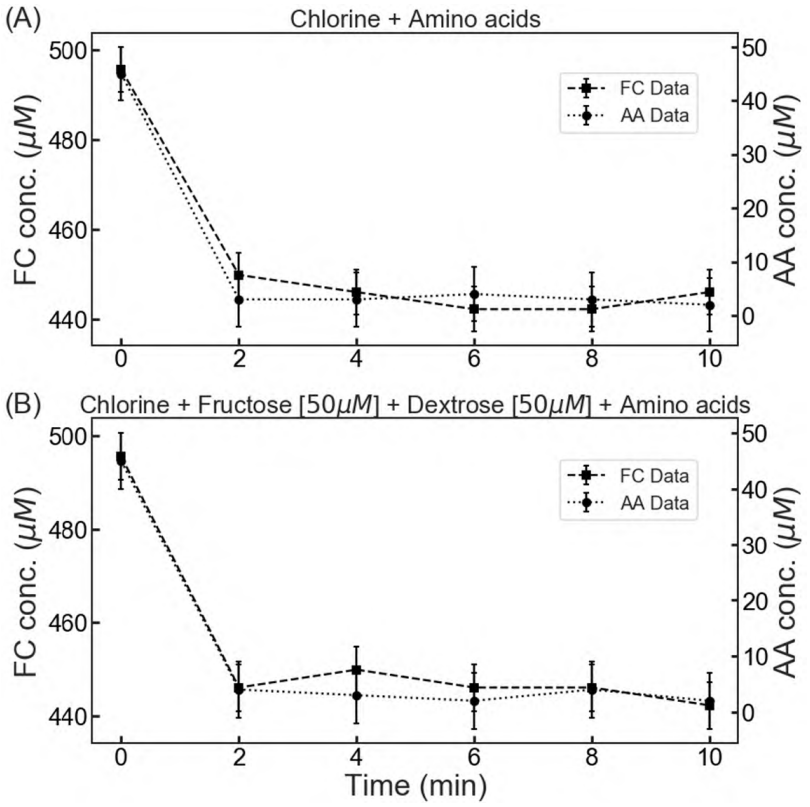

Figure 4.4: Relatively stable levels of FC concentration and COD were noted when fructose and dextrose were added to the wash solution, indicating the absence of any reaction between FC and fructose/dextrose.

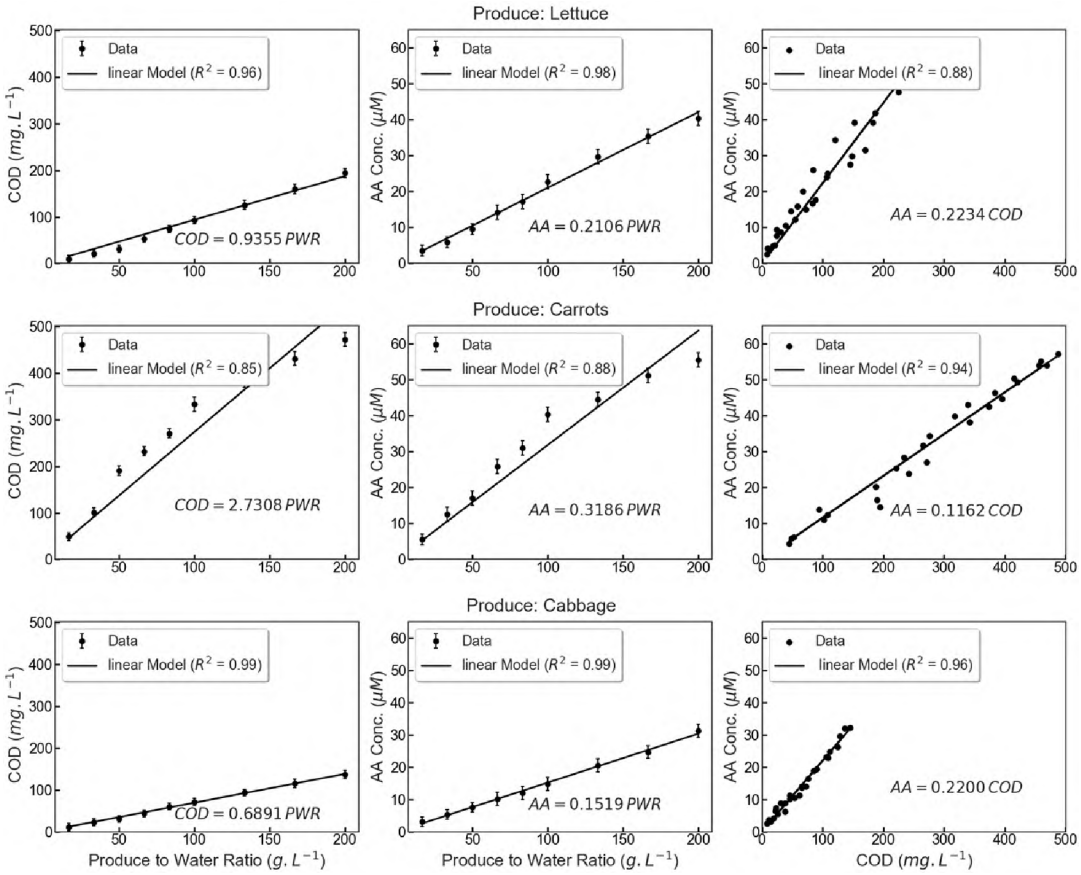

Figure 4.5: A positive linear correlation (solid line) was observed for the experimental data (closed circles) between COD levels and various produce to water ratios (left column), and between AA concentrations and various produce to water ratios (middle column), for the iceberg lettuce, carrots and cabbage. Similarly, the AA concentrations were found to be linearly correlated to the COD levels for these three produce types (right column). The strength of the correlation in each case was indicated by the relatively high R^2 values (≥ 0.85) in all cases. The initial COD and AA concentrations were set to zero in each case.

iceberg lettuce ($\gamma = 11.94$; $\beta = 16.57\ M^{-1}.s^{-1}$) as the fixed parameters of our model. Another parameter that is required to be calculated is k_0, which is the rate of increase of the amino acid concentration to the tank via the introduction of cut iceberg lettuce. We used linear correlation between produce to water ratio and AA concentration to predict k_0. Based on the experiment condition, our produce to water ratio is 25 $g.L^{-1}.min^{-1}$ for these experiments. Using data for lettuce (top row center, Fig. 4.5), k_0 was deduced to be 5.27 $\mu M.min^{-1}$ during the three wash cycles, and zero when there is no wash. Results showed that our model successfully predicted FC ($R^2 = 0.96$) and AA concentrations ($R^2 = 0.92$) in the continuous wash process (Fig. 4.6). While our model was fitted to the experimental data obtained from washing of chopped iceberg lettuce in small scale experiment (3 L), with produce to water ratio of 83 $g.L^{-1}$, it could also predict the FC decay and AA concentration for experiments performed at different scale (20 L), cut-size (chopped), and produce to water ratio (25 $g.L^{-1}$). This shows that our model captures the underlying mechanism of FC decay and indicates that our model is robust in predicting FC levels under different conditions.

We observe that in all our studies, we never encountered chlorine breakpoint – the effect when total chlorine and free chlorine do not always rise together (typical in pools and water treatment facilities), and our model does not account for it. However, it is possible that there are higher concentrations of disinfection by-products in commercial produce wash facilities that give rise to breakpoint chlorination, in which case our model will have to be modified in order to account for this effect. We observe that in all our studies, we never encountered chlorine breakpoint – the effect when total chlorine and free chlorine do not always rise together (typical in pools and water treatment facilities), and our model does not account for it. However, it is possible that there are higher concentrations of disinfection by-products in commercial produce wash facilities that give rise to breakpoint chlorination, in which case our model will have to be modified in order to account for this effect.

It could be deduced from the results presented here that measuring AA concentration in

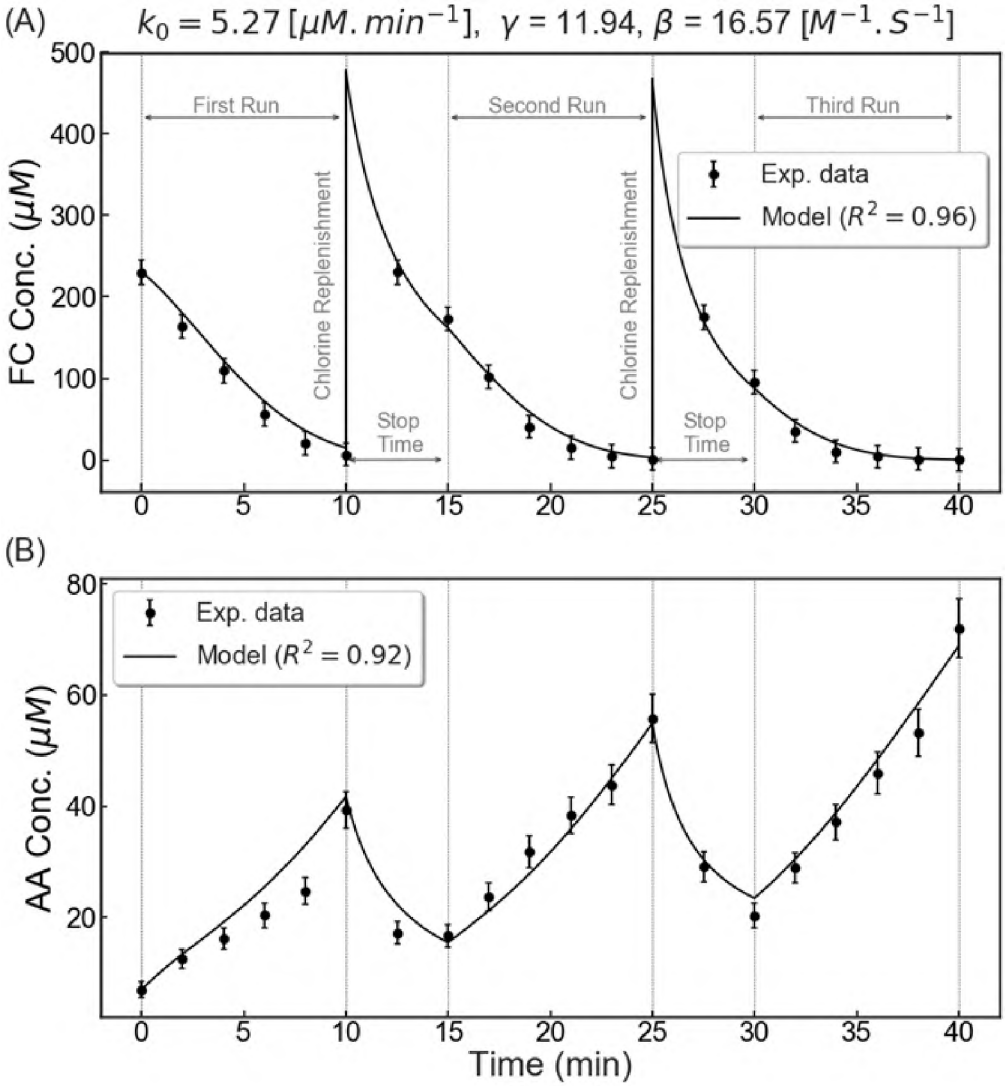

Figure 4.6: Experimental data (closed circles) and model prediction (solid line) of free chlorine levels (A) and AA concentration (B) for iceberg lettuce in continuous-wash cycle experiments with three wash cycles. FC decay during the first, second, and third runs were clearly demarcated (A) and lasted for 5 min each. The stop time between the cycles for produce loading was 5 min, and the total experiment lasted for 40 min. Three independent runs were done in similar fashion, and the results presented were experimental data average standard deviation. The parameters of the model used here are $k_0 = 5.25\ \mu M.min^{-1}$, $\gamma = 11.94$, and $\beta = 16.57\ M^{-1}.s^{-1}$, and were obtained from the single wash data (Fig. 4.2A). The predictive model was a strong fit to the experimental data, with $R^2 \geq 0.92$ in both the cases.

wash-water cycles could be an effective predictor of FC levels. This is particularly useful during industry-scale continuous washing, as the measurement of amino acid levels can be done in 1-2 hours, while obtaining the COD levels requires the reactions to run for 24 h. Upon proper calibration of industrial systems, this might also be useful in compliance protocols for both the industry as well as regulating agencies like the USDA and FDA.

4.3.5 Interactions between amino acids and FC in the presence of carbohydrates

We addressed the question whether the presence of carbohydrates in wash water hinders the reaction between amino acids and FC. The changes in FC and AA concentrations, with or without the presence of pure carbohydrates (no produce wash), are shown in Figure 4.3.

In the absence of any external carbohydrates or produce, almost all the amino acids appeared to have reacted with FC in a 1:1 stoichiometric ratio within the first two minutes, after which both the FC and AA levels remained relatively constant (Fig. 4.3A). Interestingly, the presence of 100 μM carbohydrates does not seem to have any effect on the reaction of FC with amino acids (Fig. 4.3B). It should be noted that in the single wash experiments of lettuce, carrots, and cabbage (Fig. 4.2), FC levels dropped by 38-48% whereas the AA levels dropped by $\sim$48% in the initial 2 min, while the COD levels remained relatively constant as no new produce was being introduced into the system. The drop in FC levels within the first two minutes (noted in Fig. 4.2) compared to the 10% drop in FC (noted in Fig. 4.3) could be due to the presence of other constituents in small quantities from produce wash exudates, which reacts with FC and depletes it. However, as the average apparent reaction rate β reported here does an excellent job in consolidating the overall reaction rate by predicting the FC levels accurately throughout, quantifying the type and amounts of these constituents and their individual reaction rates with FC were not the focus of this study.

4.4 Conclusions

Our results strongly suggest that AA concentration is a successful predictor of FC levels in produce wash water. We have developed the first mechanistic description of the interaction between AA and FC levels using second-order reaction kinetics. The proposed mathematical model is based on the measurement of AA concentration in the wash water, and accurately predicts FC levels. It is also very robust, and largely independent of the produce type. A comparison of our results when a proportion of COD change ($\Delta(COD)$) is used and when AA is used is shown in table XIV. these results suggest that our model parameters when AA is used do NOT depend on the produce type and cut size.

We propose that our results, when properly calibrated, could be of significant use in the produce industry to predict FC levels at regular intervals during the wash process, with implications not only for auto-regulation of appropriate FC levels in the wash water, but also for compliance purposes. Our model could also be potentially used to ensure that sufficient, but not excess FC concentrations are used, thus providing higher levels of safety during the washing process by the prevention of formation of higher concentrations of undesirable disinfectant byproducts – an unfortunate outcome from adding excessive amounts of chlorine-based sanitizers.

Table XIV: Comparison between model parameters when AA or Δ(COD) is used as indicators for organics

Indicator	k_0 $(\mu M.min^{-1})$	γ	β $(\mu M^{-1}.min^{-1})$	R^2
Δ(COD)	617 ± 70.4	0.12 ± 0.01	$9.45 \pm 0.22 \times 10^{-4}$	0.96
AA	5.27 ± 0.45	11.94 ± 0.67	$9.94 \pm 0.65 \times 10^{-4}$	0.96

CHAPTER V

CHLORINE INACTIVATION OF *ESCHERICHIA COLI* O157:H7 IN FRESH PRODUCE WASH PROCESS: EFFECTIVENESS AND MODELING

This study presents a modified disinfection kinetics model to evaluate the potential effect of organic content on the chlorine inactivation coefficient of *Escherichia coli* O157:H7 in fresh produce wash processes. Results show a significant decrease in the bactericidal efficacy of free chlorine (FC) in the presence of organic load compared to its absence. While the chlorine inactivation coefficient of Escherichia coli O157:H7 is $70.39 \pm 3.19\ L.mg^{-1}.min^{-1}$ in the absence of organic content, it drops by 73% for a chemical oxygen demand (COD) level of $600 - 800\ mg.L^{-1}$. Results also indicate that the initial chlorine concentration and bacterial load have no effect on the chlorine inactivation coefficient. A second-order chemical reaction model for FC decay, which utilizes a proportion of COD as an indicator of organic content in fresh produce wash was employed, yielding an apparent reaction rate of $(9.45 \pm 0.22) \times 10^{-4}\ \mu M^{-1}.min^{-1}$. This model was validated by predicting FC concentration ($R^2 = 0.96$) in multi-run continuous wash cycles with periodic replenishment of chlorine.

5.1 Introduction

Fresh produce and leafy greens play a role in approximately 27% of outbreaks in the United States, according to the Center for Disease Control and Prevention [8]. One critical step

in postharvest processing of most fresh produce is the wash with a sanitizing agent to inactivate the microbial load. When the sanitizer concentration drops below a certain level, not only do more pathogens survive, their potential for cross-contamination (, transfer from contaminated to uncontaminated produce) through water also increase [34]. Various types of sanitizers such as free chlorine [14, 50], chlorine dioxide [89, 46, 90] , peroxyacetic acid [39], activated persulfate [57], ozone [91, 92], and sodium acid sulfate [93] have been used for fresh produce sanitization. Despite their disadvantages, like the formation of harmful disinfection byproducts, and reduced efficiency in the presence of organic load [94], these sanitizers are relatively cheap, easy to use, and possess high bacterial inactivation efficacy [55, 50].

Chlorine based sanitizers are widely used in the fresh produce industry [14], as chlorine is very effective in inactivating pathogens due to its reactive nature [95]. On the other hand, chlorine also readily reacts with the organic content released from the exudates of fresh produce, and therefore gets depleted [84, 9]. Meanwhile, excessive levels of chlorine in wash cycles could lead to the formation of undesirable disinfection by-products (DBPs) [34, 43]. A critical real-time balance of chlorine levels is thus needed to prevent cross-contamination, as well as minimize the production and accumulation of hazardous DBPs. In this regard, we and others have developed mathematical models which simulate free chlorine (FC) decay in fresh produce wash process [41, 56, 34]. Though it is accepted that organic load consumes FC during the wash process, measuring these reactants directly is not simple or straightforward. Studies have linked chlorine decay and physicochemical characteristics of wash water such as chemical oxygen demand (COD), oxidation reduction potential, ultraviolet absorbance at 254 nm (UV254), turbidity, total organic carbon, total suspended solids, and total dissolved solids [52, 56]. These studies have afforded successful predictions of the chlorine demand in a single wash process or in pooled process wash water, but not in industry-relevant recycling wash processes with periodic chlorine replenishment. The mechanisms by which chlorine decays in a commercial wash process, and

how chlorine loses its efficacy in the presence of organic matter, remain unclear.

Several studies have shown that the efficacy of sanitizers is significantly reduced in the presence of organic matter [57, 55]. However, a model for chlorine inactivation of pathogens in the presence of organic load is not presented yet. Therefore, in this study, we model chlorine decay in the presence of organic matter and investigate the effect of organic load on chlorine inactivation of *Escherichia coli* O157:H7 in a model system of iceberg lettuce wash process. We have previously developed a model for chlorine decay in produce wash systems using COD as an indicator for organic load [41, 87]. To investigate how chlorine inactivates pathogens in the presence of organic load, we have presented a disinfection kinetics model. We utilized chlorine to inactivate a three-strain cocktail of non-pathogenic *E. coli* O157:H7 and calculated the maximum chlorine inactivation coefficient in the absence of organic load. Then, using our developed model for FC decay and pathogen cross-contamination dynamics, we quantified the chlorine inactivation coefficients in a multi-run wash process of fresh iceberg lettuce. Finally, we demonstrate how chlorine loses its sanitizing efficacy by comparing chlorine inactivation coefficient in the presence and absence of organic content.

5.2 Materials and methods

5.2.1 *E. coli* O157:H7 culture preparation

Three strains of *E. coli* O157:H7 (ATCC-35150, ATCC-43895, and ATCC-1428) were selected for this study. After opening lyophilized vials of each strain, one loop of frozen culture was transferred into Tryptic Soy Broth (TSB) and incubated in a shaking incubator (120 rpm) overnight (37 $^{\circ}C$). The incubated broth was further sub-cultured in TSB with nalidixic acid until the final broth had 50 $mg.L^{-1}$ nalidixic acid. After incubation, cells were harvested by centrifuging at 3000 g for 10 min, and the collected cells were washed twice with sterile phosphate buffered saline (PBS) and subsequently resuspended in 50 mL of PBS. Equal volumes of each strain were mixed to make the final *E. coli* cocktail

with approximately 9-$log\ MPN.mL^{-1}$. This bacterial cocktail was used to prepare 5- and 6-$log\ MPN.mL^{-1}$ solutions for disinfection experiments.

5.2.2 Lettuce preparation and inoculation

Iceberg lettuce was purchased from local grocery stores and used on the same day of purchase for experiments. After removing outer leaves, fresh leaves were chopped into 1"×1" pieces and stored at 4 $°C$ until the start of experiments. Separately, red leaf lettuce was purchased to be inoculated and added to the washing flume as inoculated produce. After removing outer leaves and chopping fresh red leaf lettuce leaves into 2"×2" pieces, 20 droplets of 5 μL *E. coli* cocktail with 6-$log\ MPN.mL^{-1}$ were placed per gram of chopped red leaf lettuce to obtain a final *E. coli* concentration of 5-$log\ MPN.g^{-1}$. Inoculated red leaf lettuce were kept refrigerated (4 $°C$) overnight to let the bacteria attach to the surface of leaves.

5.2.3 Inactivation of *E. coli* O157:H7 by chlorine

These experiments were designed to determine chlorine inactivation (lethality) coefficient of *E. coli* O157:H7 in the absence of organic load. All disinfection experiments were done in 500 mL flasks containing 250 mL of tap water. The pH was regulated to 6.5 by adding 0.1 M citrate buffer. After sterilizing flasks for 20 min at 121 $°C$, an appropriate density of cells from the *E. coli* cocktail were transferred to the flasks to yield a final *E. coli* concentration at 5- and 6-$log\ MPN.mL^{-1}$, and the flasks were refrigerated (4 $°C$). Then 0.7, 1.4, or 2.8 mL of 1000-fold diluted 4.5% sodium hypochlorite (BCS Chemicals, Redwood City, CA, USA) was added to the flasks to achieve 0.125, 0.25, or 0.50 $mg.L^{-1}$ initial free chlorine (FC) concentration solutions. Flasks were continuously mixed (200 rpm) using an overhead stirring apparatus equipped with sterile paddles. Samples (1 mL) were taken from the reaction vessels at the desired contact times and added to tubes containing 9 mL deionized water with 0.1% (wt./vol.) sodium thiosulfate (Sigma Aldrich) to

immediately neutralize residual chlorine. Chlorine concentrations were determined immediately after taking the sample, using the N, N-diethyl-p-phenylenediamine (DPD) method, with a Chlorine Photometer (CP-15, HF Scientific Inc., Ft. Myers, FL). Bacteria survival was measured by counting cells via modified Most-Probable-Number (MPN) method using 48-well deep microplates. All experiments were independently replicated three times.

5.2.4 Chlorine decay in single-wash (batch) experiments

The goal of these experiments was to model FC decay due to the reaction with organic matter released from produce exudate. Concentrated (4.5%) sodium hypochlorite was added to 3 L of tap water (maintained at 4 $°C$) to attain FC concentration ~26 $mg.L^{-1}$ (~498 μM). The pH was adjusted to 6.5 with 0.1 M citrate buffer. Before starting the experiment, samples were collected to measure FC and COD. Within 5 min of adding chlorine, 250 g chopped iceberg lettuce was washed in that water for 30 seconds and removed by a sieve. Aliquots of retained wash water were taken every two minutes after washing for the next 30 minutes.

5.2.5 *E. coli* O157:H7 inactivation in continuous lettuce wash process

These experiments were designed to investigate whether the chlorine inactivation coefficient of *E. coli* remains similar even in the presence of organic matter. Accordingly, these studies were done in a large tank containing 20 L of tap water maintained at 4 $°C$. Chopped iceberg lettuce with inoculated red leaf lettuce pieces entered the tank from one side and exit from the other side. Before adding chopped lettuce, concentrated (4.5%) sodium hypochlorite was added to the tank to achieve initial FC levels ~12 mg.L-1 (~230 μM). The pH was adjusted to 6.5 by adding 0.1 M citrate buffer. Within 5 min of adding chlorine, chopped iceberg lettuce was continuously introduced to the tank at a rate of 0.5 $kg.min^{-1}$, along with inoculated red leaf lettuce at the rate of 10 $g.min^{-1}$. The dwell time of the lettuce pieces was set to 30 sec, and each run lasted ten minutes.

Samples were taken every two minutes after starting the experiment to measure FC, COD, and bacterial load in the wash water (XW) and on the surface of iceberg lettuce (XL). COD was measured using the reactor digestion method. Wash water samples for bacterial analysis were treated with 0.1% (wt./vol) sodium thiosulfate to rapidly quench residual chlorine. To measure *E. coli* amounts transferred to the surface of uninoculated iceberg lettuce, two 25-g samples of iceberg lettuce slices exiting from wash tank were weighed and stored in sterile filter bags (WhirlPak, Nasco, Modesto, CA) and incubated with 125 mL of sterile tryptic soy broth (TSB) at 230 rpm for 2 min with a stomacher blender (Seward Stomacher 400, London, UK). The *E. coli* levels in the filtrate or water was enumerated using the modified MPN method.

After ten minutes and washing of 5 kg of iceberg lettuce, sodium hypochlorite was added to the tank to replenish chlorine and pH was adjusted to 6.5 using 0.1 M citrate buffer. The second run was started five minutes after the end of first run. Similarly, the third run was performed using similar procedures as the first and second runs. A total of 15 kg of iceberg lettuce were washed by the end of these three runs, and this experiment was independently repeated three times.

5.2.6 Chlorine decay and bacterial disinfection model in produce wash processes

We have previously reported that chlorine concentration could be predicted using a proportion of COD level change in wash water, which also serves as an indicator for organic load [87]:

$$\frac{dC}{dt} = -\beta RC - \lambda C \tag{5.1}$$

$$\frac{dR}{dt} = \gamma k_0 - \beta RC \tag{5.2}$$

where k_0 ($\mu M.min^{-1}$) stands for the rate of addition of organic matter to the washing flumes measured by the rate of change in COD levels. Namely,

$$k_0 = \begin{cases} \frac{dCOD}{dt} & \text{continuous washing} \\ 0 & \text{batch washing} \end{cases} \tag{5.3}$$

Equation 5.1 depicts the decay rate of chlorine with the FC levels at any time t given by C (μM), the concentration of organic load that reacts with chlorine given by R (μM), and the apparent reaction rate constant for the reaction of FC and organic matter in wash water given by β ($\mu M^{-1}.min^{-1}$). As the reactant concentration R introduced to the wash water by produce is not measurable directly, it has been replaced by a fixed proportion (γ) of COD change. It should be noted here that organic load is not proportional to the COD level, but to the change in COD level. Finally, λ (min^{-1}) denotes the first-order natural decay rate of chlorine. The second term on the right-hand side of equation 5.2 models the consumption rate of organic load by FC as a second-order reaction. The first term on the right-hand side of this equation is the rate at which the organic load is added to the washing flume. The initial conditions for equations 5.1 and 5.2 are $C(0) = C_0$ and $R(0) = \gamma(\Delta COD)$, where C_0 (μM) is the initial chlorine concentration and ΔCOD (μM) indicates the COD change (before and after wash) for a single wash experiment. We have used the fixed proportion of COD change before and after wash as an initial concentration of organic matter added to the wash vessel.

The concentration of pathogens in the process water, X_W ($MPN.mL^{-1}$), could be described as [41]:

$$\frac{dX_W}{dt} = \beta_W - \beta_{LW} X_W \frac{L}{V} - \alpha X_W C \tag{5.4}$$

$$\beta_W = \frac{m\left(X_{inoculated,in} - X_{inoculated,out}\right)}{V} \tag{5.5}$$

where β_W ($MPN.mL^{-1}.min^{-1}$) is the shedding rate of *E. coli* to washing system, m

($g.min^{-1}$) is the adding rate of inoculated produce, V (mL) is the tank volume, $X_{inoculated,in}$ and $X_{inoculated,out}$ are the respective concentrations of *E. coli* on the surface of inoculated produce entering and exiting the wash vessel. The second and third terms in equation 5.4 account for the removal mechanisms of pathogens from the wash tank. The second term of equation 5.4 represents the rate of binding of *E. coli* to uninoculated produce. This binding rate depends on the concentration of pathogens in the wash water (X_W) and the ratio of uninoculated produce in the wash tank to the wash tank volume ($\frac{L}{V}$ ($g.mL^{-1}$)), with β_{LW} ($mL.g^{-1}.min^{-1}$) being the proportionality constant. We model the inactivation of suspended pathogens by FC via the third term of equation 5.4 where C (μM) is the concentration of FC and α ($\mu M^{-1}.min^{-1}$) is the inactivation (lethality) coefficient of *E. coli* by FC.

The contamination dynamics for the uninoculated produce depends on the binding rate (, the rate at which pathogens in the water bind to lettuce), the FC inactivation of *E. coli*, as well as the average time lettuce spends in the wash tank [41]:

$$\frac{dX_L}{dt} = \beta_{LW} X_W - \alpha X_L C - c_1 X_L \tag{5.6}$$

where X_L ($MPN.g^{-1}$) is the pathogens level on the lettuce surface. The first term in equation 5.6 indicates the rate of *E. coli* transfer from water to lettuce, while the second term reflects the inactivation of pathogens on lettuce by FC. For the third term, we assume that the exit time of lettuce from the wash tank is exponentially-distributed whose mean is $1/c_1$, , $1/c_1$ (min) reflects the average dwell time for lettuce in the wash tank. It should be noted that we did not include produce to produce transmission of the pathogens in our model.

Equation 5.4 shows the bacterial concentration in the water in continuous wash of produce. In case of no produce wash (experiments of section 5.2.3), the first two terms are zero, and only the last term remains. In this case, the model reduces to the Chick-Watson

disinfection model [96]. Previous studies [89, 57, 55] have shown that organic load impacts sanitization by depleting as well as reducing the efficacy of the sanitizer. So, in order to consider the effect of organic load on the bactericidal efficacy of chlorine, the inactivation coefficient α is modeled as:

$$\alpha = \alpha_{max} \frac{k_M}{(k_M + R)} \tag{5.7}$$

Here, α_{max} ($\mu M^{-1}.min^{-1}$) is the maximum inactivation coefficient in the absence of organic load, R (μM) is the concentration of the organic reactants, and k_M (μM) is a constant. Analogous to Michaelis-Menten kinetics, the value of k_M is numerically equal to the concentration of organic reactants at which the inactivation rate is half of α_{max}. Equation 5.7 shows that when the concentration of organic load rises in the wash process, the sanitizer efficacy decreases. To account for this dependence of α on R, we use its form from equation 5.7 in equations 5.4 and 5.6.

5.2.7 Parameters determination and statistical analysis

All experiments were carried out in triplicate. MINITAB Statistical Software package (Version 17) was used to perform one-way analysis of variance (ANOVA) and Tukey's test. A p-value ¡ 0.05 between groups was considered statistically significant. Parameter optimization for ODE equations were done using the Levenberg–Marquardt algorithm. Three Python packages – *statsmodels*, *SciPy* and *lmfit* were used to optimize model parameters.

Maximum inactivation coefficient determination

Maximum inactivation coefficient (α_{max}) of *E. coli* by FC was obtained from inactivation experiments data with no organic load (5.2.3) and equation 5.4. For these experiments, there is no produce wash, and so the first two terms of equation 5.4 as well as the value of R equal to zero. So, equation 5.4 (replacing X_W with N) for this case will be:

$$\frac{dN}{dt} = -\alpha_{max} C N \tag{5.8}$$

$$\log_{10}\frac{N}{N_0} = -\alpha_{max}\log_{10} e\int_0^t Cdt \tag{5.9}$$

where N ($MPN.mL^{-1}$) is the density of surviving bacteria after time t (min), N_0 ($MPN.mL^{-1}$) is the initial concentration of bacteria, C ($mg.L^{-1}$) is the chlorine concentration, and α_{max} is the inactivation coefficient ($L.mg^{-1}.min^{-1}$) in the absence of organic matter. Equation 5.8 is Chick-Watson disinfection model. In order to be consistent with the literature, we have used $mg.L^{-1}$ as the unit for the chlorine concentration (C), so that the unit for α is $L.mg^{-1}.min^{-1}$. Others have reported the value $\alpha_{max}log_{10}(e) \approx 0.4343\alpha_{max}$ as the inactivation coefficient [97, 98].

FC decays over time due to the reaction with organic matter. Defining CT value ($mg.min.L^{-1}$) as $\int_0^t Cdt$ and given by the area under the chlorine decay curve (Fig. 5.1), we calculated CT for all experiments by applying Simpson's Rule.

The left-hand side of equation 5.9 is zero at $t = 0$ (, when $N = N_0$) and approaches a minimum when the surviving bacteria becomes undetectable. Assuming t^* as the time it takes for the bacterial concentration to become undetectable, equation 5.9 was modified as:

$$\log_{10}\frac{N}{N_0} = -\alpha_{max}\log_{10} e\int_0^t Cdt \qquad\qquad 0 \leq t < t^* \tag{5.10}$$

In our study, the lowest bacterial detection level is 0.12 $MPN.mL^{-1}$.

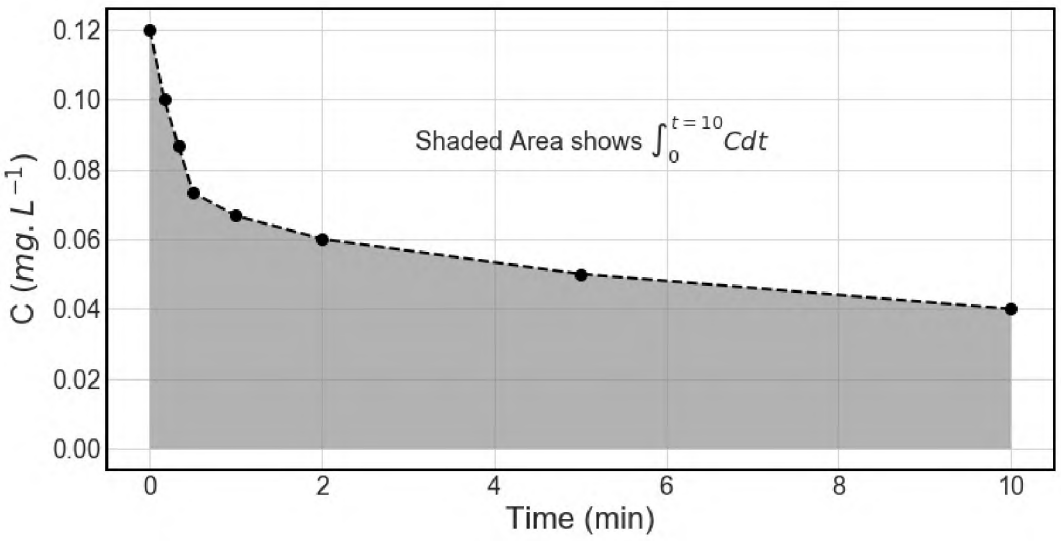

Figure 5.1: Schematic representation of CT value when initial FC concentration was 0.12 $mg.L^{-1}$. The shaded area shows the CT value (the integration part of equation 5.9) for ten minutes after adding chlorine to the solution

Apparent reaction rate constant determination

To find the optimal values for γ and β, we used the results from batch experiments (section 5.2.4). To begin with, the decay rate of FC in water (λ) is set to $1.7 \times 10^{-3}\ min^{-1}$ [41]. Since the stoichiometric coefficients of the reaction between FC and organic compounds (FC+R $\rightarrow$Products) are similar for both FC and organic matter [60], the change in the concentrations of FC and organic reactants should be similar, , $R_0 - R = C_0 - C$. At the end of the batch experiment when there is no change in FC, we assume that all organic matter has been consumed by FC and $R_{end} \approx 0$. Having this point and replacing $R_0 = \gamma(\Delta COD)$, we obtain

$$\gamma = (C_0 - C_{end})/(\Delta COD) \tag{5.11}$$

This equation for γ could thus be used to find the organic load at each time point:

$$R = \gamma(\Delta COD) - (C_0 - C) \tag{5.12}$$

Using the experimental data for FC, estimated organic content (R) from batch experiments, and the model described in equation 5.1 and 5.2 we obtained β via curve-fitting using the Levenberg–Marquardt algorithm.

Binding rate constant and inactivation coefficient determination in continuous wash

After determining γ and β, we validated our model by predicting the FC concentration in continuous experiments and comparing our results with experimental data. Finally, with the bacteria survival data from continuous experiments (section 5.2.5) and using the model explained in equations 5.4-5.7, we obtained the optimal values for k_M and β_{LW} with Levenberg–Marquardt algorithm.

5.3 Results and discussion

5.3.1 *E. coli* inactivation by free chlorine

The time-dependent drop in FC and *E. coli* levels (pH $\sim$ 6.5; 4 $^{\circ}C$) at 5- and 6-$log\ MPN.mL^{-1}$ of initial *E. coli* concentration and C_0 from 0.12 – 0.50 $mg.L^{-1}$ was shown in Fig. 5.2. The FC decay was higher in runs with 6-$log\ MPN.mL^{-1}$ *E. coli* within the first 2 minutes. Similar trends were noted at other conditions, and the change in FC levels was insignificant after the first two minutes. At $C_0 \geq 0.25\ mg.L^{-1}$, all bacteria were inactivated after the first two minutes. At $C_0 = 0.12\ mg.L^{-1}$, total inactivation of bacteria needed more time due to low FC concentration. Expectedly, these results also suggest that the higher the C_0, the faster the inactivation of *E. coli*. For example, a 3-log reduction in *E. coli* (99.9% inactivation) was achieved after 10 seconds when $C_0 = 0.5\ mg.L^{-1}$, while it took 30 seconds when $C_0 = 0.25\ mg.L^{-1}$ and even longer when $C_0 = 0.12\ mg.L^{-1}$. It took more than a minute to achieve 3-log reduction in *E. coli* levels when $N_0 =$ 5-$log\ MPN.mL^{-1}$, and two minutes when $N_0 =$ 6-$log\ MPN.mL^{-1}$.

The time-dependent *E. coli* reduction within the first minute of contact with FC was shown in Fig. 5.3. Regardless of initial bacterial load (N_0), if the chlorine level is enough,

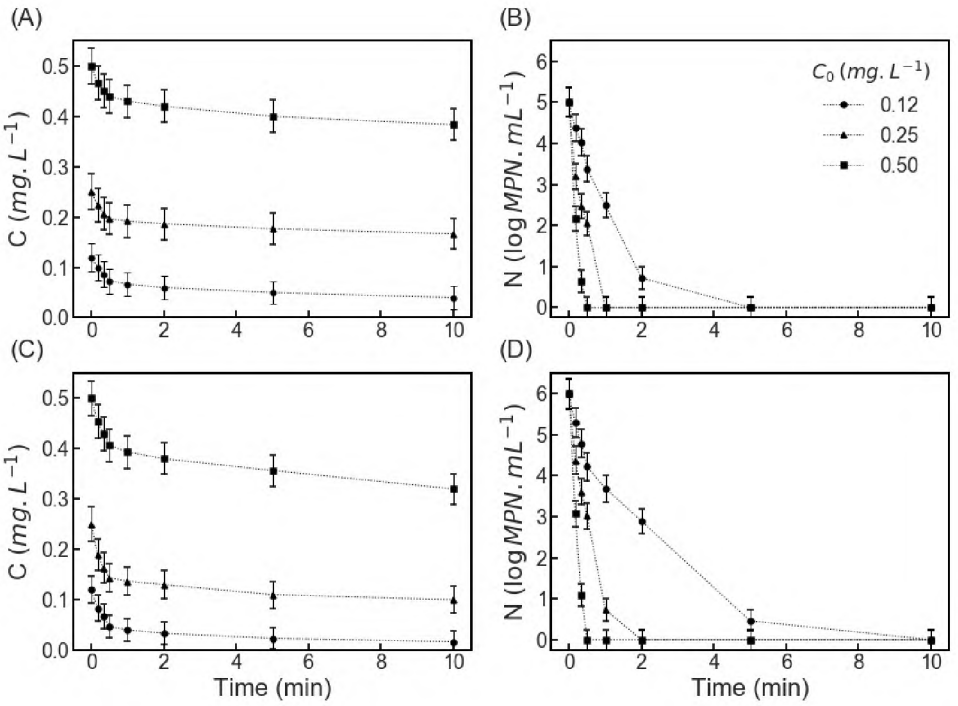

Figure 5.2: Free chlorine decay in the presence of initial *E. coli* levels at (A) 5-$log\ MPN.mL^{-1}$ and (C) 6-$log\ MPN.mL^{-1}$, at pH $\sim$ 6.5 and wash water temperature at 4 $°C$. Inactivation of (B) 5-$log\ MPN.mL^{-1}$ and (D) 6-$log\ MPN.mL^{-1}$ of *E. coli* at three concentrations of FC: 0.12, 0.25 and 0.5 $mg.L^{-1}$. The symbols are arithmetic averages of three independent experiments and the error bars indicate respective standard deviations. The dotted lines connecting symbols were for visual aid.

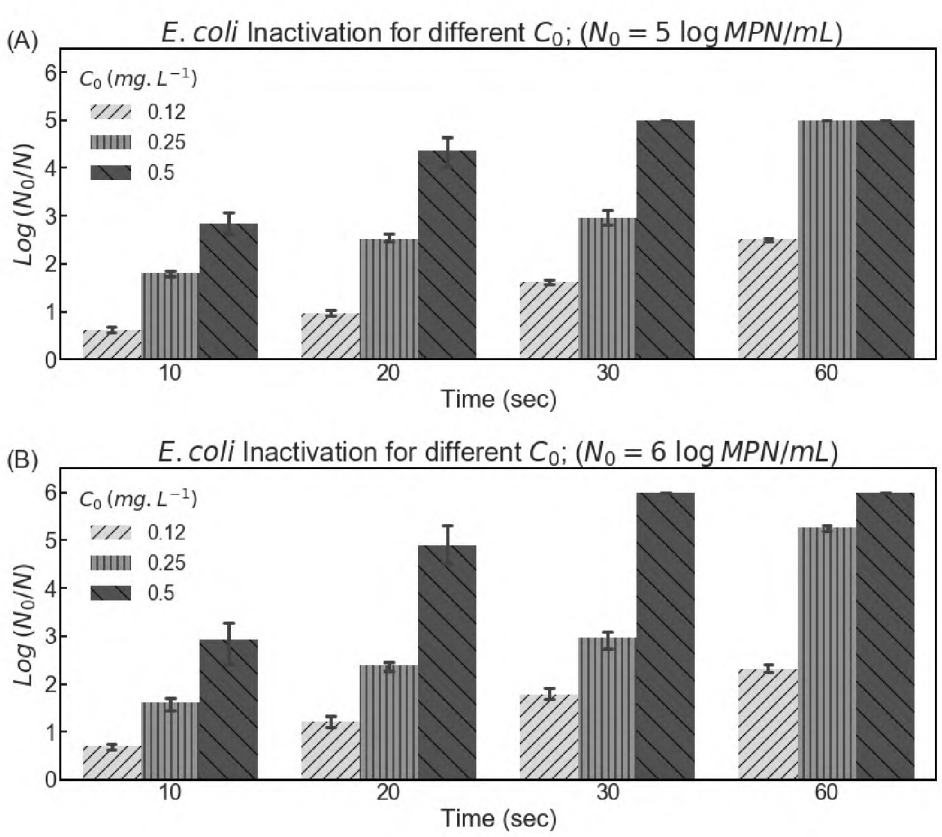

Figure 5.3: *E. coli* reduction by free chlorine at various initial FC concentrations (C_0: 0.12, 0.25, and 0.5 $mg.L^{-1}$) within the first one-minute of contact time (pH $\sim$ 6.5 and 4 $°C$). Error bars show standard deviation.

the *E. coli* reduction ratio is the same for all starting FC levels (C_0). Thus, it can be conjectured that *E. coli* inactivation coefficient is not a function of initial bacterial load and FC concentration. Equation 5.10 which explains the disinfection kinetics in the absence organic content (section 5.2.7) was used to calculate the maximum inactivation coefficients (α_{max}) at various C_0 and N_0, and in turn, the effects of C_0 and N_0 on maximum inactivation coefficient was investigated using ANOVA. ANOVA analysis showed that there is not enough evidence to prove that inactivation coefficient is dependent of the initial chlorine concentration (C_0) and bacterial load (N_0).

The first step towards finding the inactivation coefficient is to calculate CT values for

each experiment. CT value is the product of contact time and chlorine concentration which can be determined by integrating the chlorine decay profile (Fig. 5.2 - A, C). After obtaining CT values and results from bacterial disinfection experiments (Fig. 5.2 - B, D), the inactivation coefficients were calculated using equation 5.10 and tabulated (Table XV). The maximum inactivation coefficient (α_{max}) we attained was similar to that reported by others [97] using the Chick-Watson model, at 4 $°C$ and pH $\sim$ 8.5.

Table XV: Mean values of *E. coli* inactivation (lethality) coefficient (α_{max}) by FC under different initial FC level and *E. coli* concentration. Errors show 95% confidence interval.

N_0 ($log\ MPN.mL^{-1}$)	C_0 ($mg.L^{-1}$)	α_{max} ($L.mg^{-1}.min^{-1}$)	R^2
5	0.12	70.05 ± 3.65	0.99
	0.25	70.49 ± 22.68	0.97
	0.50	67.74 ± 20.89	0.98
6	0.12	76.73 ± 8.27	0.99
	0.25	78.38 ± 7.07	0.99
	0.50	76.35 ± 13.28	0.99

Results presented in Figures 5.4 and 5.5 suggest that the disinfection model, presented in equation 5.10, describes chlorine inactivation of *E. coli* very well. It is evident that there is no significant difference in the slopes of lines (, inactivation coefficients) at varying initial levels C_0 (Fig. 5.4). Similarly, bacteria initial load has no effect on their inactivation coefficient by chlorine (Fig. 5.5). The average inactivation coefficient (α_{max}) was 70.39 $\pm$ 3.19 $L.mg^{-1}.min^{-1}$ ($\approx$ 30.57 $L.mg^{-1}.min^{-1}/log_{10}(e)$ $\approx$ 3.69 $\mu M^{-1}.min^{-1}$). The CT values for 2- to 4-log inactivation of *E. coli* was 0.065 – 0.131 $mg.min.L^{-1}$. The CT values we noted for inactivation of *E. coli* were approximately 1000 times lower than the corresponding CT values for inactivation of Giardia cyst by free chlorine presented in the surface water treatment rule (SWTR) of USA (USEPA, 1991). Taylor *et al.* (2010) [99] reported CT values in the range of 0.034 - 0.05 $mg.min.L^{-1}$ for a 99% (2-log) reduction of *E. coli* by chlorine, while Helbing *et al.,* (2000) [100] noted the chlorine contact time for 3-log inactivation of *E. coli* as 0.032 $\pm$ 0.009 $mg.min.L^{-1}$, similar to our observations in the current study.

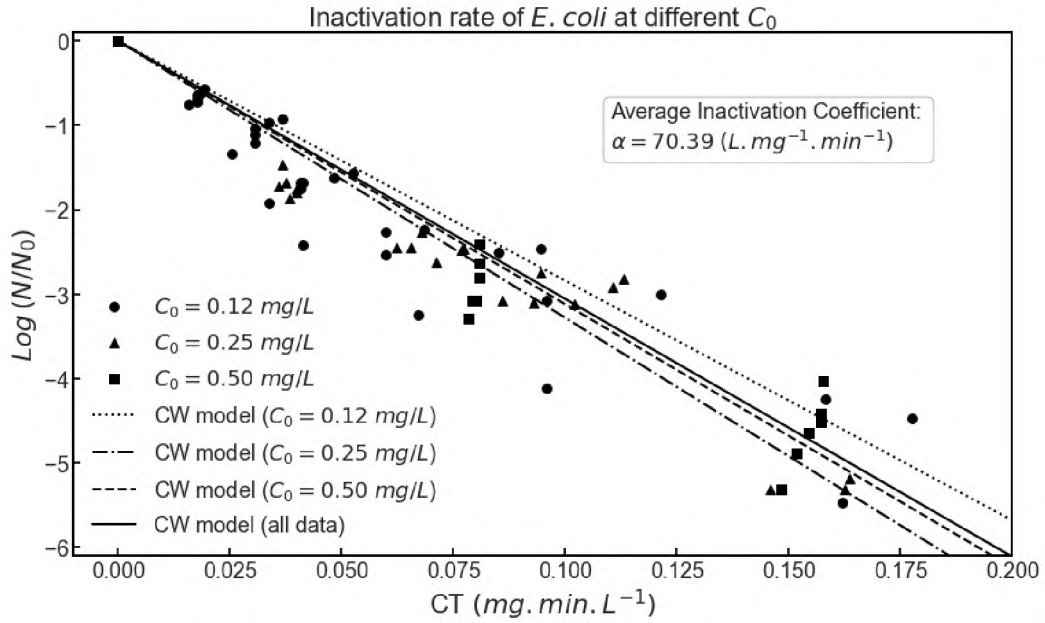

Figure 5.4: Inactivation of *E. coli* by free chlorine in the absence of organic load at different initial FC levels (0.12, 0.25 and 0.5 $mg.L^{-1}$). The symbols depict experimental data, and the lines represent the fits from the disinfection model (Eq. 5.10) for *E. coli* inactivation by free chlorine at different FC levels (pH $\sim$ 6.5 and 4 $°C$).

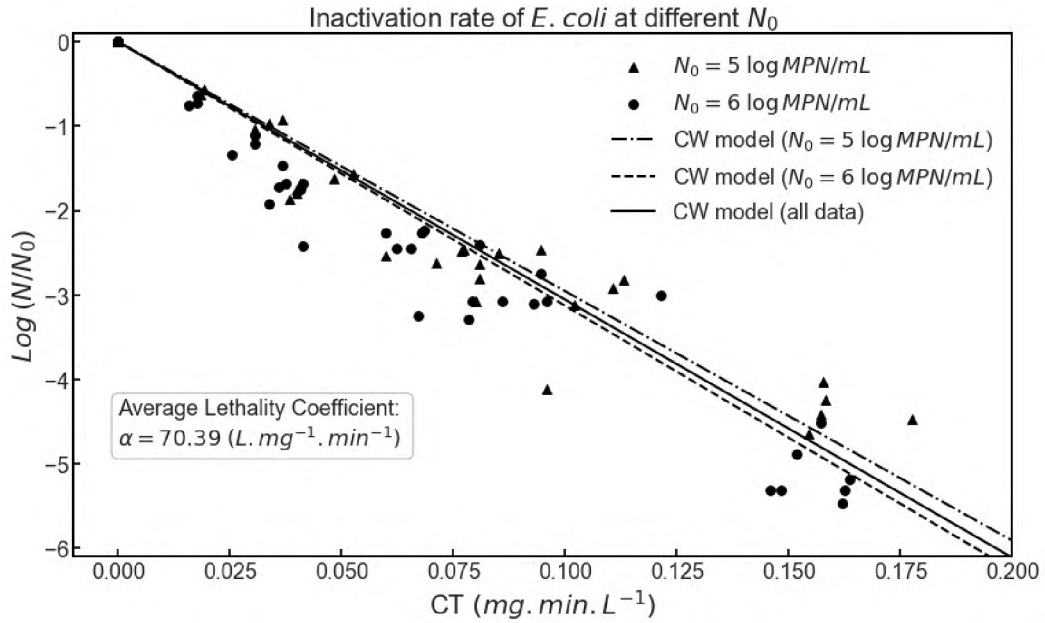

Figure 5.5: Chlorine inactivation of *E. coli* in the absence of organic load with starting concentrations of 5- and 6-$log\ MPN.mL^{-1}$. The symbols represent experimental data while the lines are fits from the disinfection model (Eq. 5.10) for *E. coli* inactivation by free chlorine (pH $\sim$ 6.5, 4 $°C$).

5.3.2 Establishing chlorine decay model using single-wash experiments

The changes in FC, organic matter, and COD levels in single-wash experiments (section 5.2.4) were shown in Fig. 5.6. A sharp drop (55%) at the beginning of experiment (first 10 min) was followed by a slow decay of FC (Fig. 5.6A). Since measuring organic content in real-time is not possible, we used COD levels from wash water as a proxy indicator. The drop in FC concentration was $\sim$ 296 μM after 30 min wash, and while the reaction between FC and organic matter most likely ended after the first twenty minutes. Assuming that the chlorination of most organic load released from produce into the water has the same stochiometric coefficient as FC [60], the change in organic load should reflect that in FC. Taking the changes in FC and COD levels into account and using equations 5.11 and 5.12, γ was determined as 0.12 $\pm$ 0.01 for iceberg lettuce (Table XVI). Recall that γ is the proportion of ΔCOD and represents the ratio of the change in FC to the change in COD level. While the COD level remained constant over the duration (30 min) of the experiment (horizontal line in Fig. 5.6B), the concentration of the organic load (R) will drop over the course of the experiment. This is the reason why the COD was not directly used in our model.

Table XVI: Results from curve-fitting and model training from single-wash experiments. Errors show the 95% confidence intervals.

COD_0^a $(mg.L^{-1})$	COD_{end}^b $(mg.L^{-1})$	ΔCOD $(mg.L^{-1})$	ΔCOD (μM)	ΔFC (μM)	γ	β $(\mu M^{-1}.min^{-1})$
28.0 $\pm$ 6.7	106.7 $\pm$ 5.8	78.7 $\pm$ 4.1	2458.3 $\pm$ 129.3	296.1 $\pm$ 10.0	0.12 $\pm$ 0.01	(9.45 $\pm$ 0.22) $\times 10^{-4}$

[a]COD before wash; [b]COD after wash

After calculating γ and estimating the organic content at each time point, equations 5.1 and 5.2 were fitted to the single-wash experimental data (section 5.2.4) and the apparent rate constant (β) for chlorination of organic load in iceberg lettuce wash process was determined using Levenberg–Marquardt algorithm (Table XVI). Based on the units used for FC concentration (μM) and time (min), β was obtained as 9.45 $\times$ 10^{-4} $\mu M^{-1}.min^{-1}$

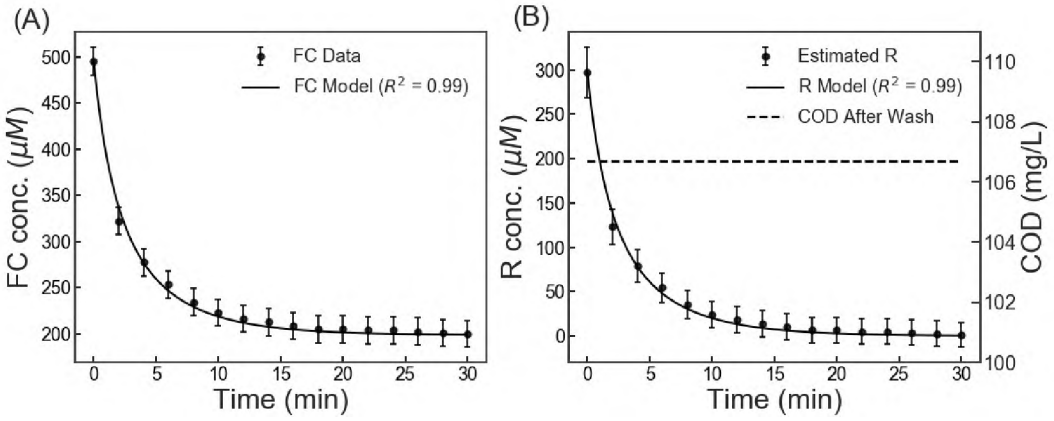

Figure 5.6: (A) Free chlorine (FC) decay, and (B) organic load consumption and corresponding COD levels obtained from single wash (batch) experiments of 250 g chopped iceberg lettuce. Data (filled circles) was reported as average $\pm$ standard deviation. The COD levels were shown as the dotted line in (B). The model was trained based on the experimental data to obtain the reaction rate constant, β (reported in Table XVI). $R^2 \geq 0.99$ proves that the model fits the experimental data well.

($\approx$15.76 $M^{-1}.s^{-1}$). The rate constant for the reaction of hypochlorous acid with different types of organic load that could possibly enter the wash water from produce exudate ranged from 10^{-2} $M^{-1}.s^{-1}$ to 10^{7} $M^{-1}.s^{-1}$ [60]. These organic compounds include amino acids, amides, and phenolic compounds, among others. The advantage of this model is that measuring each of these organic reactants involves complicated analytic methods while measuring COD is relatively quick and straightforward. Moreover, we and other researchers have reported the strong correlation between produce-water ratio and COD levels for various types of produce with different cut sizes [86]. Therefore, with careful calibration of commercial systems, it is possible that the measurement of COD is redundant.

Previously in chapter III, section 3.3.1, the γ and β values for iceberg lettuce is reported as 0.11 and 0.057 $L.mg^{-1}.min^{-1}$ respectively. In chapter III, FC and COD was used in $[mg.L^{-1}]$. Since our assumption in the current chapter is that the change in reactants concentration is equal to the change in FC concentration then we have used the molecular weight of HOCl to convert the unit of free chlorine to $[\mu M]$ and for the COD levels we have used oxygen molecular weight. Using the single-wash data for iceberg lettuce from chapter III and our approach in the current chapter (described in section 5.2.7) the γ and

β values were estimated as 0.09 and 13.4 10^{-4} $\mu M^{-1}.min^{-1}$. Table XVII compares the values for γ and β across the two chapters. The difference in γ can be due to different cut size. And the probable reason for the difference between the β values is using of two different brands of iceberg lettuce.

Table XVII: Model parameters for chlorine decay model (γ and β) across two studies from chapter III and chapter V.

	$\Delta(COD)$ $(mg.L^{-1})$	$\Delta(COD)$ (μM)	$\Delta(FC)$ $(\mu M))$	R_0 (μM)	γ	β $(\mu M^{-1}.min^{-1})$
Chaper III	30.33	947.92[a]	81.33[b]	81.33	0.09	13.4 $\times 10^{-4}$
Chaper V	78.67	2458.33	296.1	296.1	0.12	9.45 $\times 10^{-4}$

[a]Conversion factor for COD from $mg.L^{-1}$ to μM: $10^6/52460$
[b]Conversion factor for FC from $mg.L^{-1}$ to μM: $10^6/32000$

5.3.3 Chlorine decay prediction for continuous-wash process

Thus far, we presented a model for chlorine decay (equations 5.1-5.2), fitted to the single-wash experiment data (Fig. 5.6), and optimized the model parameters (Table XVI). To validate the accuracy of our model for large-scale experiments and various experimental conditions, we used our model to predict FC concentration in continuous wash of iceberg lettuce in a 20 L washing flume (section 5.2.5). These experiments consisted of 5 steps; three 10-min runs of washing iceberg lettuce and two 5-min stops to replenish chlorine and regulate pH. The decay rate of chlorine increased in each successive run due to the accumulation of organic compounds in the washing flume (Fig. 5.7A), which was supported by COD data. The COD level increases constantly over the three wash cycles, with a small dip in COD levels at the two 5-min intervals when chlorine was replenished (Fig. 5.7B).

We further tested the performance of our model by predicting FC levels in continuous-wash experiments using equations 5.1-5.3. The fixed parameters of the model were determined in the previous section using the experimental data from single-wash experiment (γ = 0.12; $\beta = 9.45 \times 10^{-4} \mu M^{-1}.min^{-1}$). The other required parameter is the rate of or-

ganic load entering the wash water (k_0) which could be determined from equation 5.3 and obtained via COD data presented in Fig. 5.7B. Our results (Fig. 5.7A) show that the model predictions for FC match the experimental data very well ($R^2 = 0.96$). While our model was trained with batch-wise experiments, it was able to accurately predict the FC levels for a continuous wash experiment, suggesting that the model can capture the underlying mechanism of free chlorine decay under different conditions.

5.3.4 *E. coli* inactivation in continuous wash: Modeling and parameter tuning

The bacterial concentration in the water (X_W) and on the surface of uninoculated produce (X_L) was shown in Fig. 5.7 (C - D). The bacterial load increases in successive runs due to the increase in organic load. While FC levels drop to near zero only at the end of the cycle in the first run, the chlorine levels depleted sooner in the second and third runs, leaving a greater chance for *E. coli* survival. Therefore, the *E. coli* levels rose to $\sim 122\ MPN.mL^{-1}$ in water by the end of the third run, while their levels were $\sim 6 - log\ MPN.mL^{-1}$ at the end of the first run. As noted in Fig. 5.7, as the COD level increases, FC decays faster, leading to rise of bacterial load in the water, and thus more bacterial transfer to iceberg lettuce.

Earlier (section 5.3.1), we determined the maximum chlorine inactivation coefficient of *E. coli* as ~70.39 $L.mg^{-1}.min^{-1}$ in the absence of organic load. We hypothesized that the organic load would affect the inactivation coefficient and chlorine will lose its sanitizing efficacy in the presence of organic load (equation 5.7). The inactivation coefficient could be determined from the continuous wash experimental data and the model explained for bacterial survival in produce wash systems (equations 5.4-5.10). However, to fit the model to the experimental data, we first need to measure the shed rate of *E. coli* to the system (β_W). As described in section 5.2.5, the inoculated produce (m) was added at a rate of 10 $g.min{-}1$ with 5-$log\ MPN.g^{-1}$ *E. coli* concentration. Results show a 1-log reduction in *E. coli* from collected inoculated produce after the wash. Taking the volume of the wash water

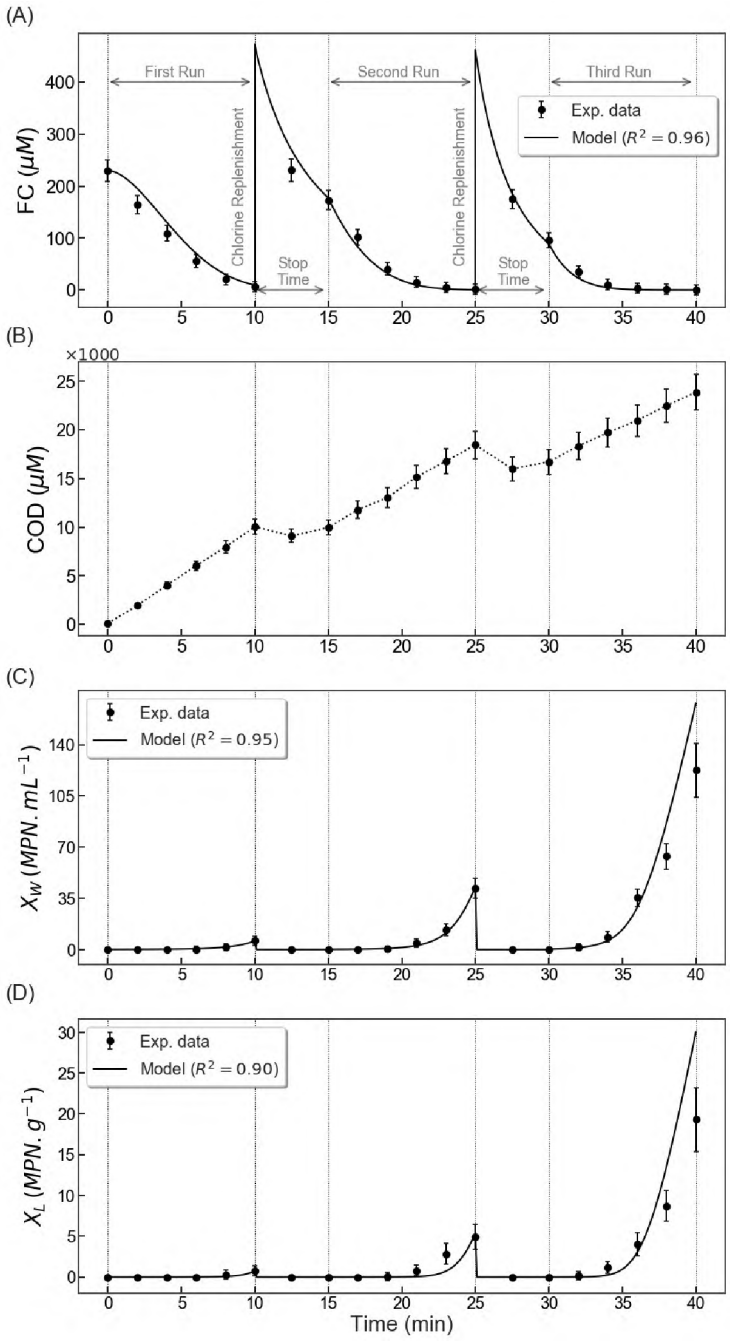

Figure 5.7: Free chlorine profile (A), COD (B), *E. coli* concentration in water (C), and *E. coli* concentration on iceberg lettuce post-wash (D). Solid circles represent the averages of experimental data with standard deviation, while solid lines are model predictions. Using experimental data from (C) and (D), the model parameters were fitted as $k_M = 524\ \mu M$ and $\beta_{LW} = 0.41\ mL.g^{-1}.min^{-1}$. The average inactivation coefficients for the three runs of the experiment were 45.05, 28.86, and 19.01 $L.mg^{-1}.min^{-1}$ respectively.

as 20 L, and using equation 5, β_W was obtained as 45 $\pm$ 1.8 $MPN.mL^{-1}.min^{-1}$. Finally, the model was fitted to the experimental data and the *E. coli* binding rate to iceberg lettuce (β_{LW}) and constant k_M were determined to be 0.41 $\pm$ 0.07 $mL.g^{-1}.min^{-1}$ and 524 $\pm$ 114 μM, respectively (Table XVIII). The average α for the continuous wash experiments was $\sim$ 64%, 41% and 27% of the α_{max} for the first, second, and third run, respectively. This confirms that as the organic content increases in the washing system, chlorine loses its efficacy to inactivate microorganisms. This could possibly be due to the competition between various components of organic matter reacting with available free chlorine. As the levels of organic reactants in the wash water increases, chlorine has higher chances of reacting with them and getting depleted, than deactivating *E. coli*. Others [55] also have reported that the organic load not only depletes FC but also reduces its sanitizing efficacy. Although the inactivation coefficient was not determined from their studies, a significant decrease in sanitizing efficacy of FC at higher levels of organic load has been reported. In this regard, the efficacy of other sanitizers such as activated persulfate and chlorine dioxide have also been reported to be affected by organic load [89, 57].

Taken together, such an integrated approach in using experiments and mathematical modeling significantly advanced our understanding of the mechanisms by which (I) FC is depleted in single and continuous wash cycles of fresh produce, (II) FC inactivates pathogens such as *E.coli* in the absence and presence of organic load, (III) depleting FC levels promote pathogen survival in wash water and their transfer to uncontaminated produce in continuous wash cycles, and finally (IV) determination of model parameters obviates limitations in real-time data collection (, organic load) during continuous produce wash cycles in industrial settings.

Table XVIII: Model parameters for the continuous wash experiments. Errors show the 95% confidence intervals.

k_0 ($\mu M.min^{-1}$)	β_W ($MPN.mL^{-1}.min^{-1}$)	K_M (μM)	β_{LW} ($mL.g^{-1}.min^{-1}$)
716 $\pm$ 70.4	45 $\pm$ 1.8	524 $\pm$ 114	0.41 $\pm$ 0.07

5.4 Conclusions

The current study shows that the organic load has a negative effect on the sanitizing efficacy of free chlorine during produce wash, and the chlorine inactivation coefficient for *E. coli* inversely varies with the organic load. Organic load not only consumes free chlorine in produce wash but also decreases chlorine disinfection efficacy (at the levels tested), possibly due to competing consumption of FC by the organic matter released from produce cut surface and internal organelles of *E. coli*, although more studies are needed to prove these hypotheses. The disinfection model we developed in this study shows promise in predicting *E. coli* inactivation in the presence of organic load in produce wash processes. Our approach in predicting free chlorine concentration using only a proportion of COD level as an indicator for organic load in continuous wash of iceberg lettuce shows remarkable improvement in the prevailing knowledge of this process. Although the proposed mathematical model was trained on single-wash (batch) experiments, it predicted the free chlorine decay in continuous wash quite accurately. Our proposed model could have significant industrial applications in predicting free chlorine decay during continuous produce wash, usage of minimally appropriate chlorine concentrations to achieve sanitization goals and preventing formation of undesirable disinfection products. Our model, when appropriately calibrated, can predict bacterial survival in wash water as well as help prevent cross-contamination in washing flumes.

CHAPTER VI

INACTIVATION MECHANISMS OF *ESCHERICHIA COLI* BY FREE CHLORINE

6.1 Introduction

Chlorine is the most used sanitizer in microbial control processes like drinking water and wastewater processing. Chlorine has a strong oxidizing capacity and low investment and operating cost. Other than drinking water, there are also other industries that use chlorine as a disinfection agent. For example, in food industry, chlorination is widely used for sanitation of raw foods like fresh produce and fruits. In fresh produce wash systems, chlorine is usually used to prevent the cross-contamination and reduce the risk of pathogens. There are many studies focusing on finding the minimum chlorine concentration and contact time to ensure the high quality of sanitation [7, 9, 58, 13]. Almost all of these studies rely on measuring the viability of pathogens by using the traditional culture-based methods (CFU, MPN, etc). Chen *et al.,* (2018) [101] have reported that disinfection performance can not always be obtained by culture-based methods such as plate counting. For instance, when lower concentrations of chlorine were used, some bacteria lose their ability to proliferate but they are still viable and contribute to the spread of disease [70, 102, 103]. Under certain circumstances, affected cells can self-repair their damage over time and recover [104, 70]. So, the traditional culture-based methods could overestimate the sanitization efficacy of disinfectants and also underestimate the tolerance of pathogens to sanitizers [101].

Since the conventional plate count methods are not appropriate for the evaluation of

disinfection efficiency, there is a need for alternative methods to assess the disinfection quality. Various methods have been proposed, building on their success with eucaryotes, to explain and evaluate the disinfection performance by distinguishing between the viable and dead bacteria cells. These methods work based on assessing the changes in membrane integrity, metabolic activity, membrane potential, damage to deoxyribonucleic acid (DNA), and messenger ribonucleic acid (mRNA) expression [102]. One powerful tool to quantitatively analyze the changes in cellular organelles or function, and thereby the performance of disinfection process, is flow cytometry (FCM) [103]. Most chemical sanitizers have been shown to damage the bacterial cell wall [70, 102] and FCM is useful to quantify the effects of chemical disinfectants on the integrity of cellular membrane [105]. With the help of various fluorescent dyes, FCM is able to assess the total and intact cell count. Also the available functional fluorescent stain combinations such as calcein AM/propidium iodide (PI) and SYBR Green/SYTO enables us to determine the membrane integrity of cells in disinfection process [106]. Another method to measure cell viability is to quantify the changes in Adenosine triphosphate (ATP) molecule, which stands as an indicator of the microbial metabolism activity. Monitoring the ATP concentration can show how the microorganism responds to the sanitizers and thereby quantification of the number of active cells. However, ATP detection is not applicable when the total number of cells are lower than 10^4 [107]. FCM analysis and ATP measurement are two rapid and accurate methods that have the potential for automation or high-throughput analyses [106].

The antimicrobial effect of a number of chemical disinfectants has been studied by other researchers including the effect of ozone [106], chlorine dioxide, [108, 109], IPA [65], slightly acidic electrolyzed water [110] and free chlorine [102, 103, 70]. Chemical agents interfere with cellular membrane and alter the cell permeability and/ or membrane potential [65]. Virto *et al.,* (2005) reported that the exposure of bacterial cells to chlorine caused extensive permeabilization of the cytoplasmic membrane, but they detected no relation between the occurrence of membrane permeabilization and cell death. Cheswick *et*

al., (2020) concluded that treatment with chlorine led to a reduction in intact cell count, while prolonged exposure caused both a reduction in the total cell count and fluorescence intensity indicating breaking down of the cell membrane. Similar results were reported by Xu *et al.,* (2018): low doses of chlorine ($<5\ mg.L^{-1}$) affected membrane permeability and consequently increased extracellular ATP levels indicating the leakage of intracellular ATP. However, there is still a need for extensive studies on how free chlorine affects cells over time.

The objectives of this study are to i) investigate the efficacy of free chlorine on inactivation of *Escherichia coli* and ii) identify the mechanisms by which free chlorine inactivates *E. coli*, using both quantitative and qualitative techniques. Immunolabeling approaches were used to quantitatively detect time-dependent and FC-concentration dependent changes in membrane potential, ATP, and cell survival, while conventional cultivation-based cell count was applied to quantify cell density in these cultures. Electron scanning microscopy and fluorescence microscopy were used to qualitatively observe the morphological changes in *E. coli* during the inactivation process by free chlorine. Finally, options and limitations of the current FCM and ATP methods for assessing and monitoring disinfection efficiency during chlorination of *E. coli* were discussed.

6.2 Materials and methods

6.2.1 Bacterial strains and preparation of suspension

E. coli O157:H7 (ATCC-1428) strain was used in this study. After opening lyophilized vial, one loop of frozen culture was transferred into Tryptic Soy Broth (TSB) and incubated in a shaking incubator (120 rpm) overnight (37 $^{\circ}C$). The incubated broth was further sub-cultured in TSB with nalidixic acid until the final broth had 50 $mg.L^{-1}$ nalidixic acid. After incubation, cells were harvested by centrifuging at 3000 $\times$ g for 10 min, and the collected cells were washed twice with sterile phosphate buffered saline (PBS) and subsequently resuspended in 50 mL of PBS. Equal volumes of each strain were mixed to make the final

E. coli cocktail with approximately 9-$log\ MPN.mL^1$. This bacterial cocktail was used to prepare 6-log $MPN.mL^1$ solutions for disinfection experiments (section 6.2.2). All the chemicals and bacterial mediums used in this study were purchased from Sigma-Aldrich or otherwise mentioned in the text.

6.2.2 Chlorine disinfection experiments

These experiments were designed to determine the mechanisms by which chlorine inactivates *E. coli* in the absence of an organic load. All disinfection experiments were done in 500 mL flasks containing 250 mL of tap water. The pH was regulated to 6.5 by adding 0.1 M citrate buffer. After sterilizing flasks for 20 min at 121 $°C$, an appropriate density of cells from the *E. coli* suspension were transferred to the flasks to yield a final *E. coli* concentration of 6-$log\ MPN.mL^1$, and the flasks were refrigerated (4 $°C$). Then 0.7, 1.4, or 2.8 mL of 1000-fold diluted 4.5% sodium hypochlorite (BCS Chemicals, Redwood City, CA, USA) was added to the flasks to achieve 0.125, 0.25, or 0.50 $mg.L^1$ initial free chlorine (FC) concentration solutions. Flasks were continuously mixed (200 rpm) using an overhead stirring apparatus equipped with sterile paddles. Samples were taken from the reaction vessels at the desired contact times and added to tubes containing sterile deionized water with 0.1% (wt./vol.) sodium thiosulfate (Sigma Aldrich) to immediately neutralize residual chlorine. Chlorine concentrations were determined immediately after taking the sample, using the N, N-diethyl-p-phenylenediamine (DPD) method, with a Chlorine Photometer (CP-15, HF Scientific Inc., Ft. Myers, FL). Bacteria survival was measured by counting cells via modified Most-Probable-Number (MPN) method using 48-well deep microplates [111]. All experiments were independently replicated three times.

6.2.3 Methods to uncover disinfection mechanisms of *E. coli* by free chlorine

Experiments were carried out to investigate the bactericidal mechanisms of free chlorine in terms of the effect on the culturability, morphology of the cells, metabolism and the

permeability of the outer cell membrane.

Scanning Electron Microscopy (SEM)

The changes in bacteria morphology was assessed using a SEM. Bacteria suspensions before and after adding free chlorine (at specific time points) were first centrifuged at 5,000 rpm for 5 min, then the supernatant was discarded, and 1 mL of glutaraldehyde (2%) was added into the tubes. Samples were placed in a refrigerator overnight at 4 $^{\circ}C$, then washed with PBS and ethanol with gradient concentration from 70% to 100% to remove excessive glutaraldehyde (Sigma Aldrich). Then 1 mL of isoamylacetate (Sigma Aldrich) was added into the tubes. After 1 hour, isoamylacetate was removed and samples were air-dried for 2 days under vacuum in a desiccator. Samples were coated with gold prior to imaging . These gold-coated samples were imaged using a Field-emission SEM (FEI Company Model Inspect F50) to assess the changes in cell morphology and visible damages to cells in each sample. The resulting images were analyzed with NIH ImageJ software to determine changes in bacterial size.

Fluorescence Microscopy (Fluorescent staining)

A LIVE/DEAD BacLight^{TM} Bacterial Viability Kit (Thermofisher, L7007) composed of two separate fluorescent dyes (SYTO 9 and Propidium iodide (PI)) was used to test the viability of free chlorine-exposed cells. SYTO 9 is a green-fluorescent nucleic acid stain and PI is a red-fluorescent nucleic acid stain. The diffusion strength of these two dyes differ in terms of penetrating into cells. SYTO 9 is able to penetrate all cells, given its small molecular weight, and labels them as fluorescent green. In contrast, PI can only penetrate into bacteria with damaged membranes and stain them fluorescent red. So, when appropriate amount of both stains are present in bacterial solutions, cells with intact membranes stain for fluorescent green and cells with damaged membranes stain fluorescent red. The Live/Dead assay was performed immediately after collection of the samples according to

the manufacturer's instructions: briefly, 3 μL of the stained mixtures was added to 1 mL of the bacterial samples from disinfection experiments and mixed thoroughly and incubated in the dark at room temperature for 30 min. Then, the fluorescence images were taken with a Zeiss AxioVert A1 fluorescent microscope under both phase contrast and fluorescent channels, using Axiocam C1 digital camera and Axiovision data acquisition software. At least five images were taken per condition in each well (n=3 wells/condition) at random locations.

Fluorescence spectroscopy (Fluorescence Microplate Readers)

We used a LIVE/DEAD BacLight^{TM} Bacterial Viability Kit (Thermofisher, L7007), described above, to measure the ratio of live to dead cells. The dead cell stock was made by exposing cells to 0.25 $mg.L^{-1}$ FC for 15 minutes. First suspensions of live/dead cells were made based on table XIX. These suspensions were used to make the standard curve (Fig. 6.1) which was subsequently used to determine the live/dead cell ratio in samples from disinfection experiments. The assay was done based on the manufacturer's instructions: 100 μL of disinfection samples and the standard live/dead suspension were added to a 96 well-plate and 100 μL of the 10 times diluted stained mixture was added to each well. Stained samples were incubated at room temperature in the dark for 15 minutes. Fluorescence measurement was done by setting the excitation wavelength at 485 nm and fluorescence intensity was measured at 530 nm (emission 1; FI_{green}) for all wells. One more time with the same excitation wavelength at about 485 nm, the fluorescence intensity was measured at 630 nm (emission 2; FI_{red}). The live/dead ratio is defined as:

$$Ratio_{G/R} = \frac{FI_{green}}{FI_{red}}$$

To obtain the standard curve, the $Ratio_{G/R}$ vs standard live/dead percentage was plotted (Fig. 6.1) and the live/dead percentage of disinfection samples were measured using the standard curve.

Table XIX: Volumes of live and dead cell suspensions to mix to achieve various proportions of live/dead cells for fluorescence microplate readers.

Ratio of Live Cells (%)	mL Live-Cell Suspension	mL Dead-Cell Suspension
lightgray 0	0	1.0
10	0.1	0.9
lightgray 25	0.25	0.75
50	0.50	0.50
lightgray 75	0.75	0.25
90	0.90	0.10
lightgray 100	1.0	0

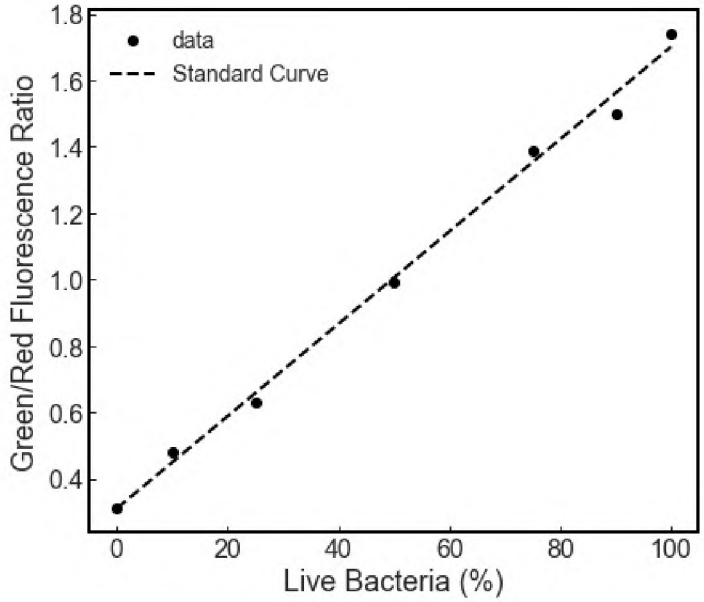

Figure 6.1: Standard curve for various proportions of live:dead cells for fluorescence microplate readers.

Determination of Total Adenosine Triphosphate (ATP)

Total ATP concentration was measured using an ATP Determination Kit (Thermofisher, A22066) and a luminometer (Synergy^{TM} 4, BioTek, USA). The standard reaction solution was prepared based on the kit protocols by mixing different reagents of the kit. Then some standard ATP solutions with different concentrations were prepared to obtain the standard curve (Fig. 6.2). After that, 90 μL of the standard reaction solution (provided with the assay kit) was added to the wells of a 96-well plate and the background luminescence was measured. Then 10 μL of each standard solution and samples from disinfection experi-

ments were added to the wells and luminescence was read again. After subtracting the background luminescence noise, first the standard curve was generated (Fig. 6.2), followed by the measurement of ATP concentration of disinfection samples.

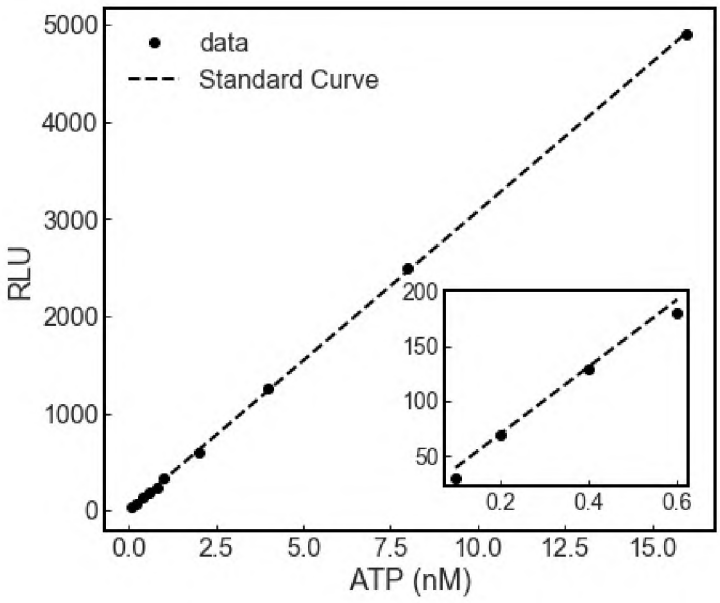

Figure 6.2: Standard curve for detection of ATP using the ATP Determination Kit.

MTT colorimetric method for cell proliferation inhibition

One method for measuring metabolic activity is to incubate cells with a tetrazolium salt such as WST-1, which is cleaved into a colored formazan product by metabolically active cells. Formazan is purple and the change in the color of the solutions is an indicator of cellular metabolic activity. So, quantifying the absorbance of the cell broth containing the MTT enables us to measure the metabolic activity of the cells. The negative effect of free chlorine on proliferation of *E. coli* was assessed through a CytoSelect™ MTT Cell Proliferetion Assay (Cell Biolabs, inc., CBA-252) and based on a method described by Ye *et al.,* (2017) [110]. Samples (1 mL) taken from disinfection experiments at each treatment time poins (section 6.2.2) were added to 9 mL of 0.01 M PBS solutions with 0.1% (wt./vol.) sodium thiosulfate to quench the residual chlorine. After 5 min of neutralization, samples were centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and cells re-

suspended in 1 mL of sterile tryptic soy broth. Then 100 μL of this bacteria liquid was added into 96-well cell culture plates. Afterwards 10 μL of the MTT reagent was added to each well, which was then incubated at 37 $^{\circ}C$ for 4 h in the dark. Then, 100 μL of Detergent Solution was added to each well of the 96-well plate and incubated for another 2 hours, protected from light. Then the bacterial liquid was vibrated in an incubator shaker for 10 minutes to dissolve the precipitate. Finally, the absorbance of each sample was measured at an OD of 540 nm with a microplate reader (SynergyH1, BioTek, Vermont, USA). Inhibition rate of *E. coli* proliferation was reported as OD values.

Bacterial membrane potential

A BacLight™ Bacterial Membrane Potential Kit (Thermofisher, B34950) was used to determine the cellular membrane potential during the disinfection process. This kit contains two stains: carbocyanine dye $DiOC_2(3)$ (3,3-diethyloxacarbocyanine iodide, Component A) and CCCP (carbonyl cyanide 3-chlorophenylhydrazone, Component B), both in DMSO. $DiOC_2(3)$ exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by larger membrane potentials. Proton ionophores such as CCCP destroy membrane potential by eliminating the proton gradient. The assay was used based on the manufacturer's protocol. Briefly, 1 mL of the treated bacteria (at each time point) was added to 9 mL of sterilized 0.01 M PBS solution containing 0.1% (wt./vol.) sodium thiosulfate and centrifuged at 5000 rpm for 5 min. Then the supernatant was removed and cells were resuspended in 1 mL sterile 0.01 M PBS solution. One milliliter aliquots of bacteria suspensions (n = 3) for each time point were added to flow cytometry tubes. FCM analysis needed three processed samples: stained, depolarized control and an unstained control. One of the tubes is unstained control and no stains will be added to it. To make the depolarized control, 10 μL of 500 μM CCCP (Component B) and 10 μL of 3 mM $DiOC_2(3)$ (Component A) were added to another tube and mixed. In the last tube, only 10 μL of 3

mM $DiOC_2(3)$ (Component A) was added and mixed. Samples were incubated at room temperature for 15–30 minutes. Stained bacteria was assayed in a flow cytometer (BD LSRFortessa™ X-20 Flow Cytometer, Amersham Biosciences Corp, NJ, USA) equipped with a laser emitting at 488 nm. Green fluorescence was collected in the FL1 channel (520 nm), whereas red fluorescence was collected in the FL3 channel (613 nm).

6.3 Results and discussion

6.3.1 Inactivation efficiency of chlorine and disinfection kinetics model

The change in free chlorine concentration as well as inactivation of *E. coli* under chlorination is presented in Fig. 6.3. Fig 6.3A, shows that there is a fast decay in FC concentration within a minute of chlorine addition followed by a slow decay. It is probably due to reaction with different organic compounds in the bacterial cell wall. Compounds that include nitrogen in their molecule react quickly with chlorine [60], while other organic compounds have smaller reaction rate with chlorine [60]. Also, the change in the FC concentration for experiments with FC of 0.25 and 0.5 $mg.L^{-1}$ are almost the same which shows that the chlorine demand is related to the bacterial load not the initial chlorine concentration.

The average removal rates of *E. coli* were 30, 90, and 99%, respectively, after exposure to 0.12, 0.25, and 0.5 $mg.L^{-1}$ free chlorine for 1 minute (Fig. 6.3B). For all three free chlorine concentrations tested, no culturable cells could be observed after 10 min. These results support our hypothesis that chlorination has a significant effect on *E. coli* inactivation. These results are also in agreement with our previous results presented in chapter V. Previously in section 5.3.1 we have shown that the average inactivation coefficient of *E. coli* by free chlorine (α_{max}) was 70.4 $\pm$ 3.2 $L.mg^{-1}.min^{-1}$ ($\approx$ 30.57 $L.mg^{-1}.min^{-1}/log_{10}^{e}$ $\approx$ 3.69 $\mu M^{-1}.min^{-1}$) and the CT values for 2- to 4-log inactivation of *E. coli* were in 0.065 – 0.131 $mg.min.L^{-1}$ range. Increasing the chlorine concentration lowered the time for removal of 99% bacterial load from 10 minutes for FC of 0.12 $mg.L^{-1}$ to 2 minutes for FC of 0.25 $mg.L^{-1}$ and 30 seconds for FC of 0.5 $mg.L^{-1}$ (Fig. 6.3B).

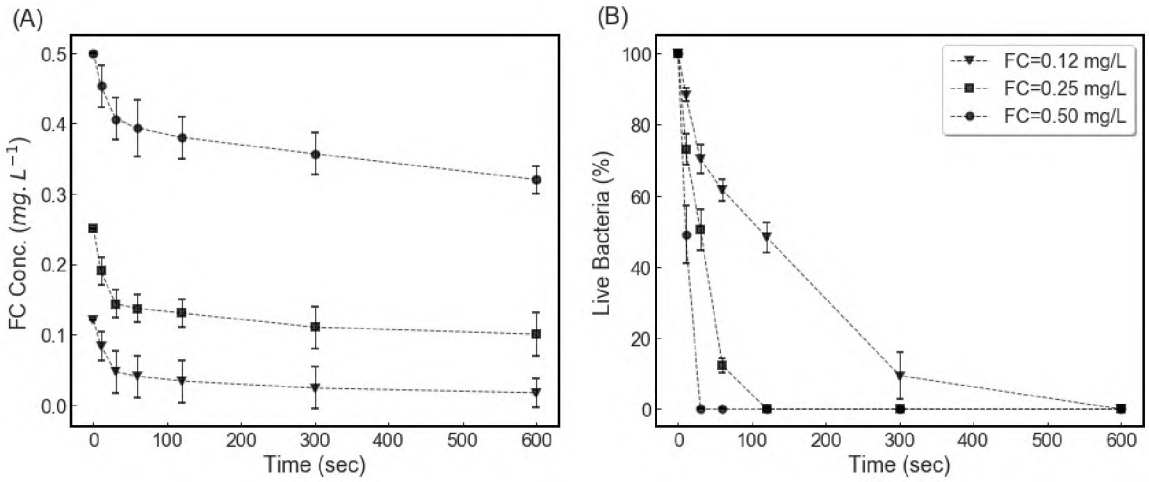

Figure 6.3: Free chlorine decay (A) and *E. coli* survival rate (B) in presence of three initial free chlorine concentrations: 0.12, 0.25 and 0.50 $mg.L^{-1}$. Reaction conditions: $pH = 7.1, T = 4\,°C$, initial concentration of bacteria: $\sim 10^6\ MPN.mL^{-1}$

6.3.2 Morphology observation by SEM

SEM was used to observe the structure of *E. coli* and visible damage in the cellular surface during chlorination. The untreated *E. coli* cells exhibited an intact and rod-shaped morphology (Fig. 6.4). After 30 seconds of chlorination, little damage could be observed in the surface structure for all three levels of free chlorine concentration. These cells appeared slightly rough and wrinkled. SEM images revealed that with increasing time, cell surface turned rough and plicate at the beginning of chlorination, while holes and wrinkles appeared on the cell surface over time, which might have led to the release of intracellular compounds. Finally, continued chlorine exposure or higher concentrations at early exposure destroyed the entire cell wall and disintegrated the cell. For FC level of 0.12 $mg.L^{-1}$, visible changes on the cell surface were observed even after 120 seconds of exposure, while there was more than 99.9% inactivation of *E. coli*. When FC level was 0.25 $mg.L^{-1}$, the pace of structure deterioration was faster and some *E. coli* cells had visible compromise in the cell wall even at 30 seconds of exposure, leading to the formation of holes on the cell surface. After treatment with an initial FC concentration of 0.5 $mg.L^{-1}$ for 10 sec-

onds, holes and wrinkles were clearly observable on the cell surface (Fig. 6.4) indicating severe damage. Taken together, significant morphological changes were noted after 120 seconds of chlorination, irrespective of initial FC concentration, when most cell walls are irreversibly damaged. These results suggest that chlorination causes cell membrane damage in *E. coli*. Irreversible damage in the surface structure is a major step for microbial inactivation during chlorination. Pattison and Davies, (2001) [88] have reported that the N-terminal amino acids of peptidoglycan located on the cell wall could be oxidized during chlorination, which could lead to significant damage in the cellular surface, resulting in the release of vital intracellular compounds.

6.3.3 Live/Dead Assay for Cell Survival

Representative fluorescence images of *E. coli* during chlorination under three levels of initial FC was shown in Fig. 6.5. Representative images for suspensions of bacteria with known live/dead cell ratio are shown in Fig 6.5A. When the suspension is made of only live cell (100/0 or control-live) only green fluorescence was observed, while bacteria of the negative control (0/100 or Control-dead) present only red stain (dead cells), and they were obtained after 15 min of exposure to 0.25 $mg.L^{-1}$ chlorine. The fluorescence images of the control samples before chlorination (labeled "0 sec") revealed mostly green fluorescence and demonstrated that almost all the bacteria were alive. Comparison between three levels of FC indicates that the shift from green to red is faster as the chlorine concentration increases. For example, for FC concentration of 0.12 $mg.L^{-1}$, green stained cells are visible up to 2 minutes while for FC of 0.25 $mg.L^{-1}$ green stained cells are hardly observable after 1 minute exposure. When FC level was 0.5 $mg.L^{-1}$, green dots are not seen after the first 10 seconds. These results are in agreement with the culturable cell count data presented in Fig 6.3. Since the red dye can only penetrate damaged cells, these results could be interpreted in terms of the cell membrane damage. For instance, SEM images for inital FC of 0.5 $mg.L^{-1}$ after 10 sec of chlorination reveal damages to the cellular surface

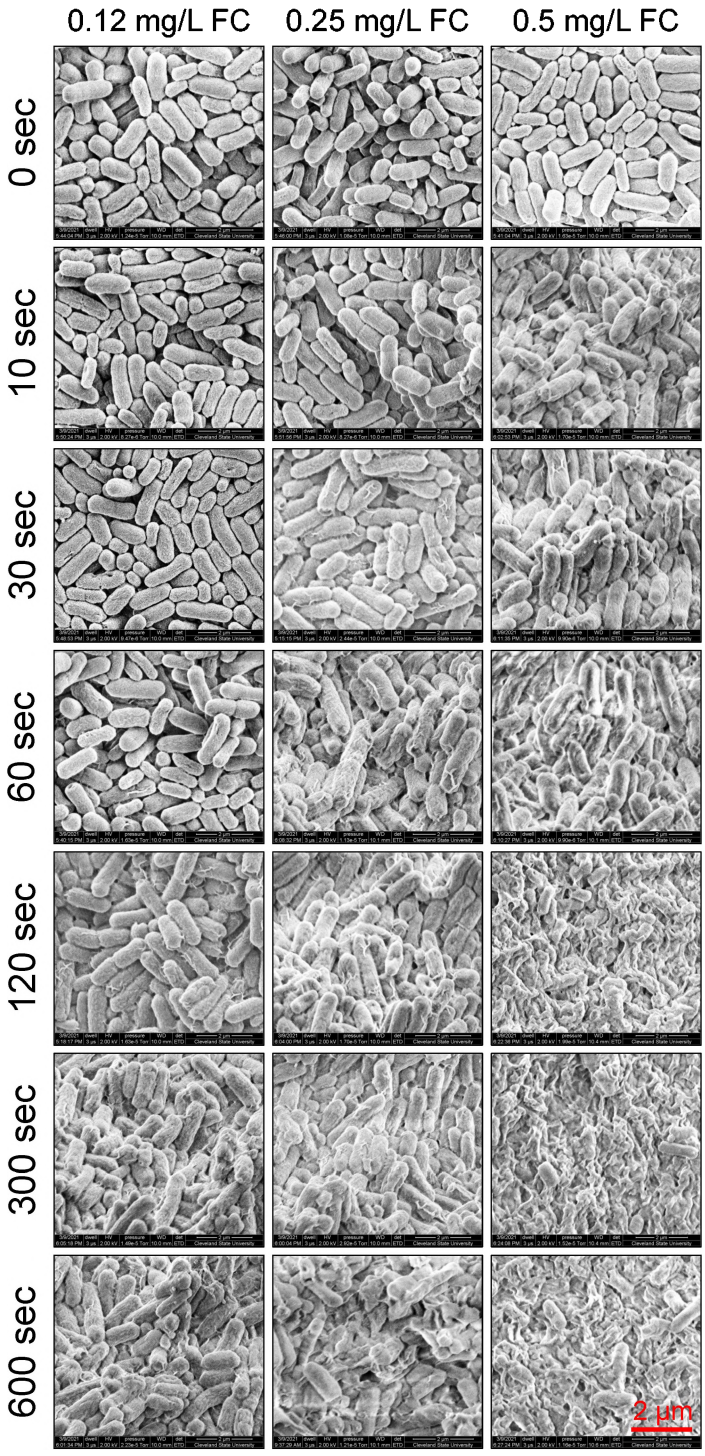

Figure 6.4: Representative SEM images of *E. coli* treated with various initial FC doses. Images corresponding to 0 sec refer to the control sample (i.e., before adding chlorine).

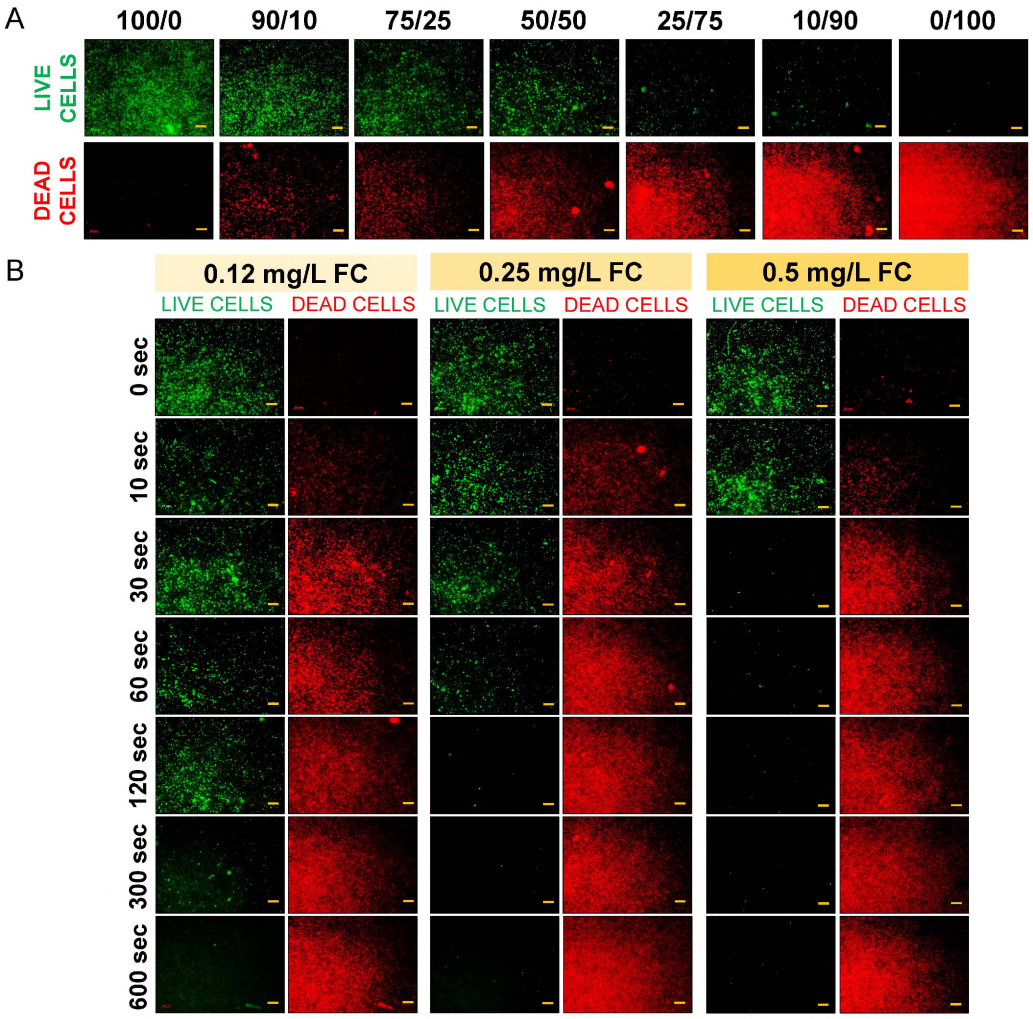

Figure 6.5: FM detection of *E. coli* during chlorination. (A) FM images for suspensions with known live/dead proportions. (B) FM images for treated solutions under different concentration of free chlorine. Reaction conditions: $pH = 7.1$, $T = 4\ ^{\circ}C$, initial concentration of bacteria: $\sim 10^6\ MPN.mL^{-1}$. The scale bar shown in the images is 20 μm.

while the fluorescence staining shows that most of the cells were dead after 10 seconds. Thus, the combination of fluorescence labeling and SEM images reveal corroborative and complimentary information about the inactivation process of *E. coli* by chlorine. It appears that the major mechanism of disinfection by *E. coli* is through the destruction of cellular membrane organization and membrane proteins, causing deformation in cell structure and functionality [65].

6.3.4 Live/Dead cell count by fluorescence microplate reader

Previously, in section 6.3.1, the cell count results for inactivation of *E. coli* by chlorination were presented in terms of "MPN" - a culture-based method to enumerate the number of live cells. As we mentioned in the introduction section of this chapter, the disinfection performance can not be obtained by conventional culture-based methods alone. The reason is that during disinfection some cells might lose their ability to proliferate while they are still viable. Thus, to measure the number of viable cells, we used the live/dead fluorescence spectroscopy. After generating the standard curve (Fig. 6.1) for suspensions with known *E. coli* Live/Dead ratio ($Ratio_{G/R}$), the viable cell percentage was obtained for disinfection samples. Results are shown in Fig. 6.6. A quick comparison of the results from culture-based method (6.3) and staining method (6.6) shows that they both have strikingly similar trends, with almost no difference between them. Further analysis revealed that on average, the viable cell percentage from staining protocol is $3.45\% \pm 0.62$ higher than culture-based method. This proves that chlorination induces loss of culturability although bacteria are still deemed viable [103].

6.3.5 Chlorination effect on *E. coli* proliferation and culturability

To assess the effect of chlorine exposure on *E. coli* proliferation, a MTT colorimetric method was used. The OD_{540} absorbance values for cellular activity after treatment with chlorine at three different concentrations were shown in Fig. 6.7A. Similar to plate count and fluorescence spectroscopy, the proliferation inhibition is higher when the FC concentration is higher. The growth inhibition after 30 seconds of exposure to 0.12 $mg.L^{-1}$ FC is $\sim$20%, and $\sim$40% when the initial FC levels was 0.25 $mg.L^{-1}$, and $\sim$60% when initial FC was 0.5 $mg.L^{-1}$ (Fig. 6.7B). These results also suggest that cells can remain viable but non-culturable (VBNC) state during the disinfection process. Such cells continue to consume nutrients and might participate in transcription process [68].

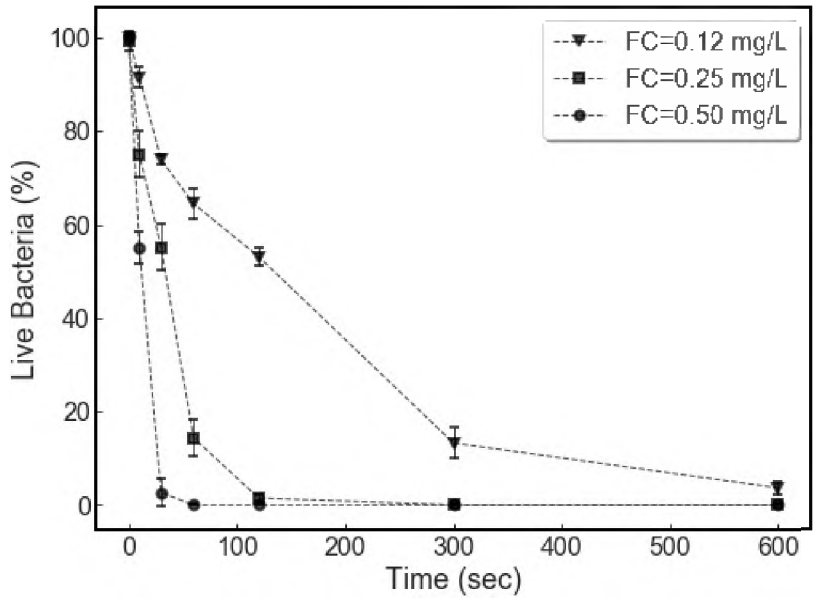

Figure 6.6: Analysis of relative viability of *E. coli* suspensions treated with free chlorine using live/dead fluorescence spectroscopy (fluorescence microplate reader). Reaction conditions: $pH = 7.1, T = 4\ ^{\circ}C$, initial concentration of bacteria: $\sim 10^6\ MPN.mL^{-1}$

6.3.6 The changes in total and intracellular ATP

Since ATP is the chemical energy that cells use for their metabolic activities, its levels could be an indicator for microbial activity and viability. The changes in total and intracellular ATP levels during the disinfection process of *E. coli* by free chlorine (Fig. 6.8) were obtained using an ATP Determination Kit and the standard curve plotted for known ATP concentrations (Fig. 6.2). During chlorination, ATP is released from the cells due to the damaged membrane. However the mechanism and the concentration of released ATP depends on the free chlorine concentration (Fig. 6.8). Before starting the disinfection experiments the intact cell concentration was $\sim$6-$log\ MPN.mL^{-1}$ with 91.8 $\pm$ 1.4% intracellular ATP (7.35 $\pm$ 0.17 nM) and 8.2 $\pm$ 1.4% extracellular ATP (0.66 $\pm$ 0.11 nM). The total and intracellular ATP remained unchanged for untreated control cell suspension over the ten minutes duration of the experiment.

Exposure to chlorine increased the total ATP (Fig. 6.8). Higher concentrations of chlorine resulted in more ATP production by the cells, possibly due to the damaged metabolic

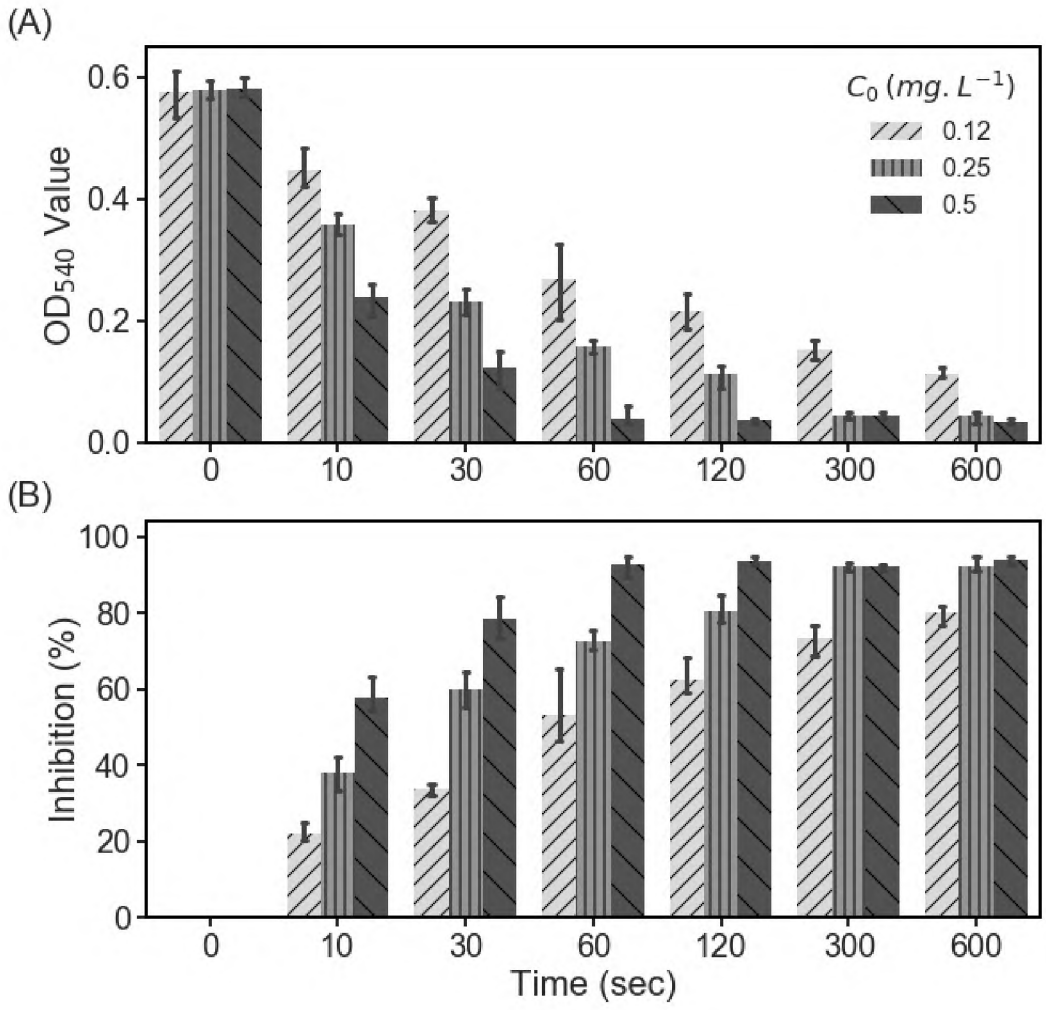

Figure 6.7: Inhibition of cell proliferation induced by free chlorine. (A) OD absorbance values from MTT proliferation assay. (B) Cell proliferation inhibition (%) after treatment with chlorine. Reaction conditions: $pH = 7.1, T = 4\ ^{\circ}C$, initial concentration of bacteria: $\sim 10^6\ MPN.mL^{-1}$.

system, and the imbalance in the normal equilibrium between synthesis and utilization of ATP generated [112]. However, the increase in total ATP happened for only a brief period of time (up to a minute) after the addition of chlorine, after which the total ATP level remained unchanged. Also, the intracellular ATP content decreased during the disinfection process and the most likely reason being that ATP is leaked from the cells due to damaged cellular membrane. So the loss in intracellular ATP can be an indicator of cellular membrane damage as well. As shown in Fig. 6.8, with the increase of FC concentration, the intracellular ATP levels declined proportionately faster, likely due to more damage to

cellular membrane at higher FC concentration. This establishes that chlorination has a significant impact on membrane integrity of the bacterial cells. Similar results were reported by Xu *et al.,* (2018) [102]. They have concluded that low doses of chlorine ($< 5\ mg.L^1$) affect membrane permeability and consequently increase extra cellular ATP levels indicating the leakage of intracellular ATP.

6.3.7 Flow Cytometry analysis for membrane potential changes

Flow Cytometry analysis was done to detect the changes in membrane potential of chlorinated cells. This was achieved using staining with $DiOC_2(3)$ and generating scatter plots of green versus red fluorescence. CCCP was used to generate depolarized control cell populations. Subsequently reasonable gates were drawn around the depolarized control group. The population-level changes in cellular membrane potential during the chlorination process of *E. coli* were expressed in Fig. 6.9. The plots for untreated samples presented as "0 sec" in Fig. 6.9 show that untreated viable cells are completely out of fixed depolarized gate with a shift toward red fluorescence. This is because $DiOC_2(3)$ stains all bacterial cells green fluorescence, but the fluorescence shifts toward red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by larger membrane potentials [65]. With increase of contact time in chlorine disinfection, the red fluorescence diminished gradually, resulting in the shift of the clusters towards the fixed depolarized-gate, resulting in almost complete migration to the depolarized zone. This indicates that the cell membrane potential of *E. coli* is declining during the chlorine disinfection process, mostly due to changes in structural membrane integrity of *E. coli* by chlorination.

The percentage of depolarized cells were quantified from these images using FlowJ software (`https://www.flowjo.com/`) and shown in Figure 6.10. Results show that the rate of membrane potential loss or depolarization is faster for higher FC concentrations. This is because the cellular membrane damage increased with increasing FC concentration, as noted in previous sections. Cheswick *et al.,* (2020) [103] also reported that treatment

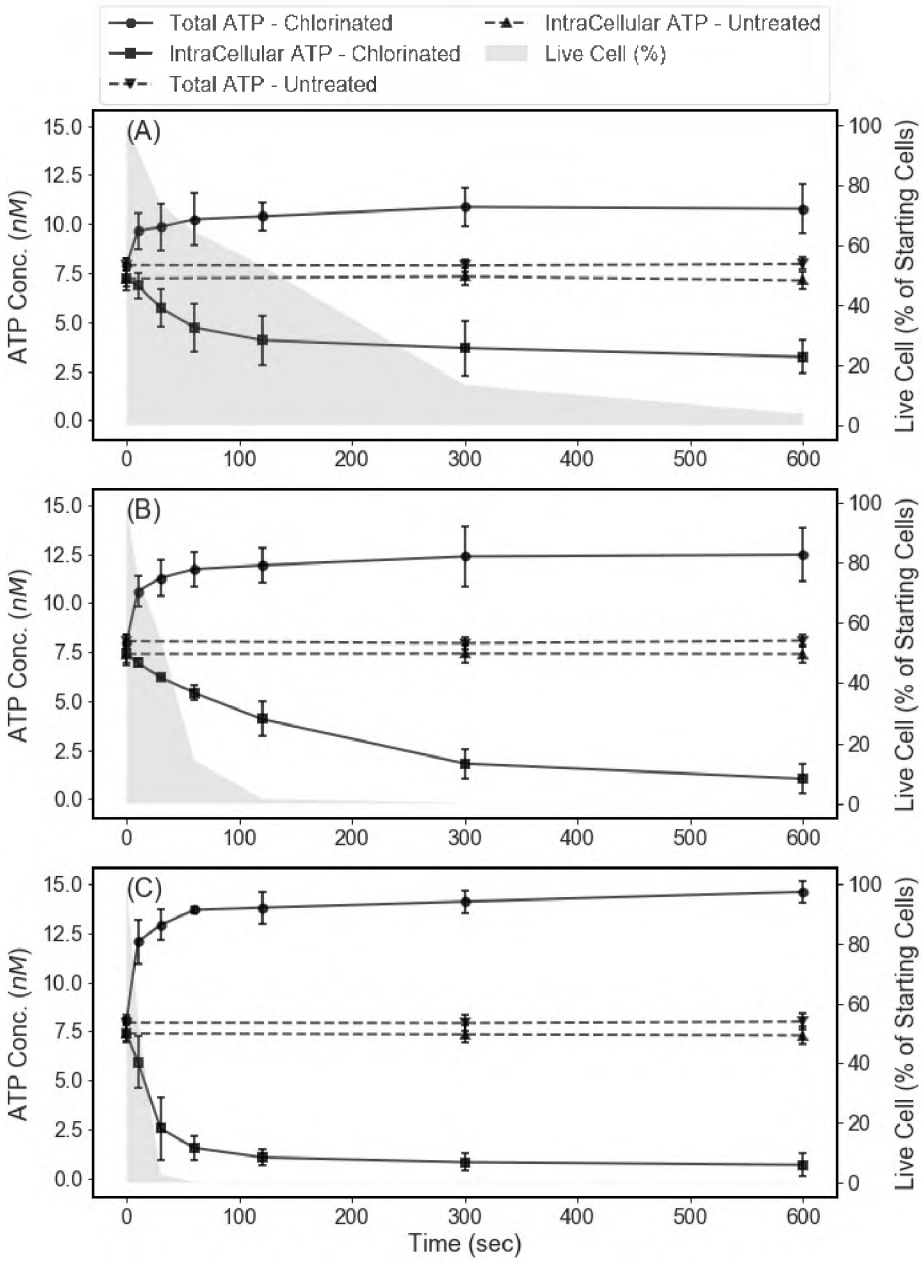

Figure 6.8: Effects of chlorine on the adenosine triphosphate (ATP) levels of *E. coli*. (A) FC = 0.12 $mg.L^{-1}$; (B) FC = 0.25 $mg.L^{-1}$; (C) FC = 0.50 $mg.L^{-1}$. The shaded area shows the percentage of live bacteria (N/N_0) from fluorescence spectroscopy data.

with chlorine led to a reduction in cellular membrane potential, and with prolonged exposure caused both a reduction in the intact cell count and fluorescence intensity indicating breaking down of the cell membrane.

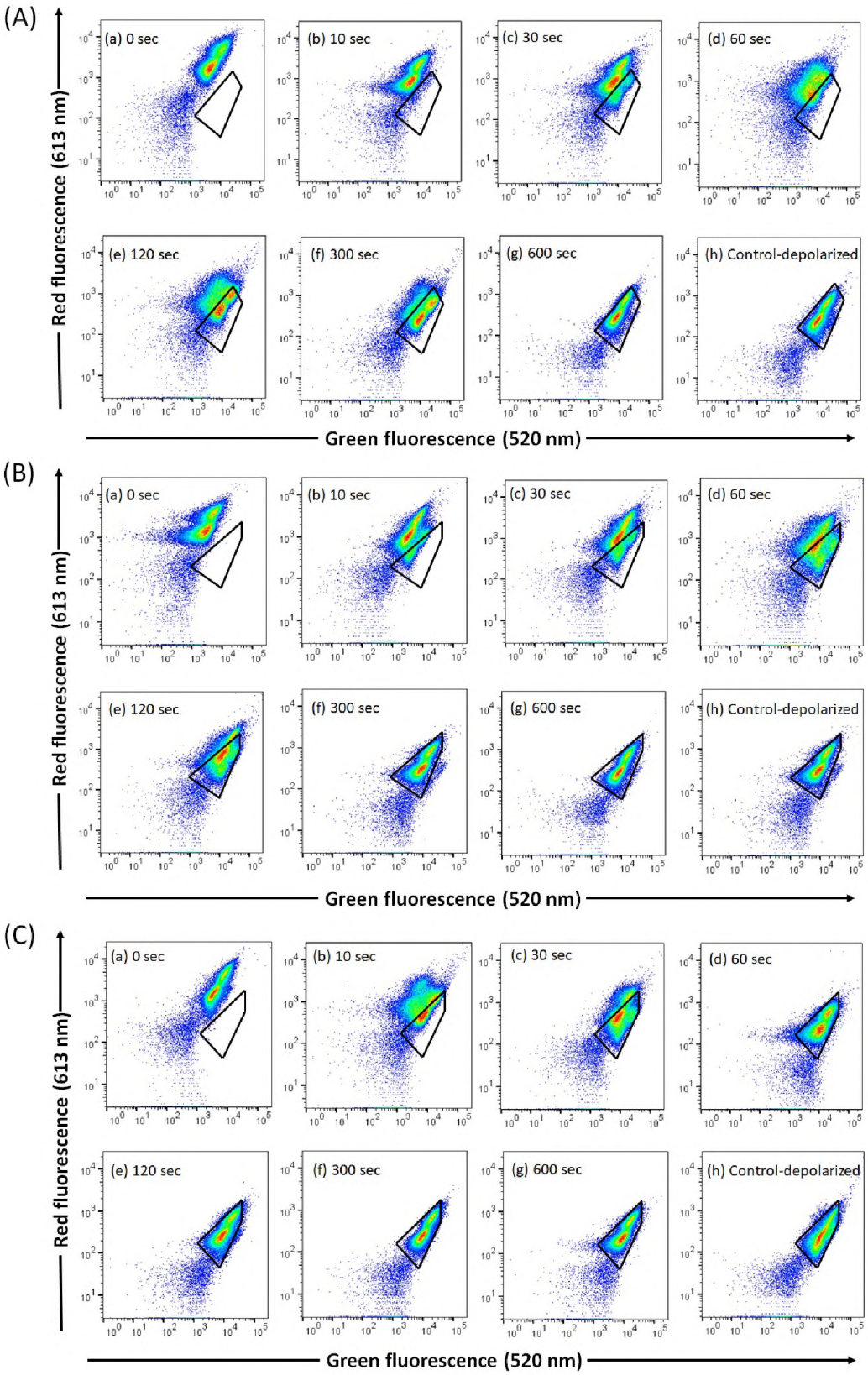

Figure 6.9: Flow cytometry analysis and membrane potential results for *E. coli* after exposure to (A) 0.12 $mg.L^{-1}$, (B) 0.25 $mg.L^{-1}$ and (C) 0.50 $mg.L^{-1}$.

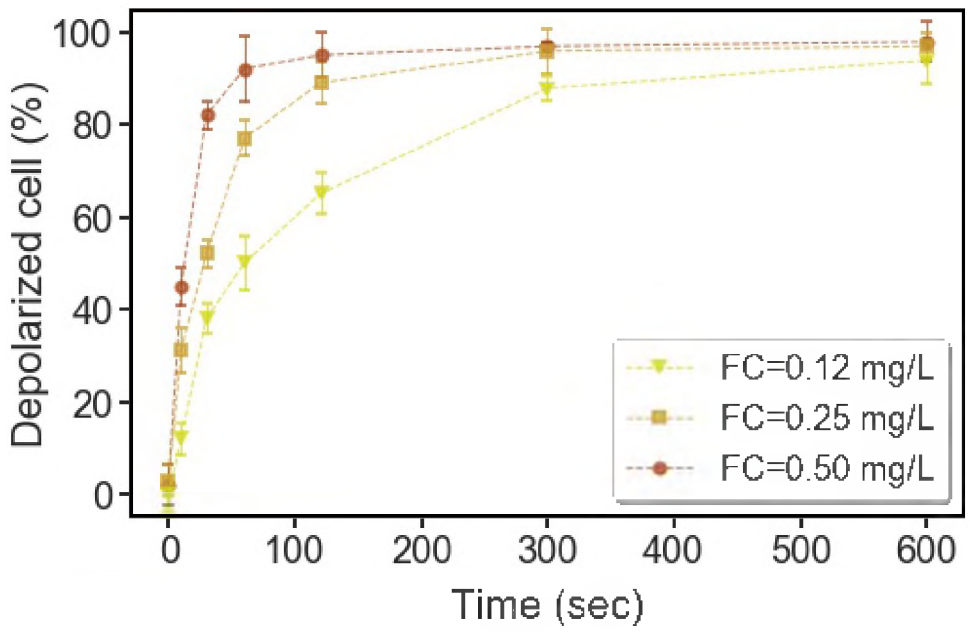

Figure 6.10: Depolarization of *E. coli* under chlorination with three FC levels.

6.4 Conclusions

This study demonstrated that chlorination has huge impact on inactivation of *E. coli*. At low levels of FC and shorter exposure times, cell surface became rough and plicate; however, holes and wrinkles formed on the cell surface at higher FC concentrations or at longer exposure times, causing significant damage to the cell membrane. The cellular permeability changed due to chlorination, resulting in a significant decrease in the number of viable cells. Besides, around $3.45\% \pm 0.62$ of cells lost their culturability and transform to viable but not culturable (VBCN) state during the disinfection process in low CT values (>0.2 $mg.min.L^{-1}$ - before complete inactivation). The rapid decrease in the intracellular ATP levels was also another indicator of cellular membrane damage. Finally, the cell membrane potential declined with increasing chlorination time, and the *E. coli* lost their membrane potential indicating extensive cell membrane damage might be the leading cause of mechanism by which chlorination disrupts *E. coli*.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

In this study, the dynamic changes of water quality during the washing process of different produce including lettuce (romaine, iceberg, green leaf, red leaf), carrots and green cabbage were studied. Expectedly, results showed that COD levels increased over time as more produce was washed and, in particular, the lettuce type impacted the rate of increase in organic load. As the produce was introduced to the washing solution, FC concentration decreased mainly due to consumption of chlorine by organic content. FC concentration is of high importance in washing of fresh-cut produce as it inactivates bacteria, and such depletion of FC could lead to cross-contamination in the washing water. Using results from a bench-top experimental setup, a mathematical model was developed for FC dynamics in the washing solution, to describe the mechanisms by which FC changes in the washing of fresh-cut produce.

The wash model developed in this study applies to a variety of produce types in the context of predicting FC dynamics (decay and re-dosing) across various scales. The fact that its predictions of FC levels hold at multiple experimental scales and across different produce types strongly suggests that the model illustrates fundamental chlorine dynamics that occur during fresh cut carrot/cabbage and iceberg lettuce washing. In particular, these findings give validity to performing future lab scale experiments to quantify FC kinetics

associated with different produce/cut types as well as experiments aimed at understanding the impact of continuous FC dosing on FC dynamics during produce washing. Specifically, the effect of pH dynamics should be explored in this context as the results from this work were obtained via wash water pH levels near 6.5.

In addition, it was shown that turbidity and TDS measurements may not be reliable in predicting FC levels (as opposed to chlorine demand) as there is no consistent, observable relationship linking the increase in organic load (in terms of change in COD) from cut carrots/cabbage/lettuce entering the wash tank and the corresponding increase in turbidity or TDS. The model's predictive success across scales and produce types provides a strong case that COD information is much more dependable than that of turbidity or TDS. In particular, the fraction of the COD increase due to washing produce was shown to be a consistent predictor of the associated produce/cut type organic load in wash water, and therefore a reliable predictor (via the model) of FC decay rates.

Further, replacing COD with amino acid (AA) concentration levels turned out to be a successful predictor of FC levels in produce wash water. The proposed mathematical model, based on the measurement of AA concentration in the wash water, accurately predicts FC levels. It is also very robust, and largely independent of the produce type. We propose that our results, when properly calibrated, could be of significant use in the produce industry to predict FC levels at regular intervals during the wash process, with implications not only for auto-regulation of appropriate FC levels in the wash water, but also for compliance purposes. Our model could also be potentially used to ensure that sufficient, but not excess FC concentrations are used, thus providing higher levels of safety during the washing process by the prevention of formation of higher concentrations of undesirable disinfectant byproducts – an unfortunate outcome from adding excessive amounts of chlorine-based sanitizers.

In addition to water quality dynamics, we used our complete model to not only confirm the utility of lab scale experiments in quantifying water-mediated cross-contamination, but

also illustrate how our model can provide guidelines for future experiments to establish quantifiable guidelines for cross-contamination control. Further studies under a wider range of operational conditions need to be conducted to corroborate these results, so that they may be used to understand the dynamics of FC and cross-contamination during the wash process, and to potentially improve existing industrial practices.

The current study showed that the organic load has a negative effect on the sanitizing efficacy of free chlorine during produce wash, and the chlorine inactivation coefficient for *E. coli* inversely varies with the organic load. Organic load not only consumes free chlorine in produce wash but also decreases chlorine disinfection efficacy, possibly due to competing consumption of FC by the organic compounds released from produce cut surface, although more studies are needed to prove these hypotheses. The disinfection model we developed in this study showed promise in predicting *E. coli* inactivation in the presence of organic load in produce wash processes. Our approach in predicting free chlorine concentration using only a proportion of COD level as an indicator for organic load in continuous wash of iceberg lettuce showed remarkable improvement in the prevailing knowledge of this process. Although the proposed mathematical model was trained on single-wash (batch) experiments, it predicted the free chlorine decay in continuous wash quite accurately. Our proposed model could have significant industrial applications in predicting free chlorine decay during continuous produce wash, usage of minimally appropriate chlorine concentrations to achieve sanitization goals and preventing formation of undesirable disinfection products. Our model, when appropriately calibrated, can predict bacterial survival in wash water as well as help prevent cross-contamination in washing flumes.

Finally, the chlorine inactivation mechanism of *E. coli* was investigated by analyzing the results from different methods such as culture-based cell count (plate-counting), SEM, fluorescence microscopy, live/dead assay, MTT proliferation assay, ATP levels detection assay, and membrane potential assay. Cell surface became rough and plicate at lower levels of initial FC and shorter exposure times; however, holes and wrinkles formed on the cell

surface at higher FC concentrations or at longer exposure times. Fluorescence microscopy confirmed that the cell membrane is severely damaged due to chlorination. A significant decrease in the number of viable cells was also noted as well as curtailment of their proliferation ability. Live/dead assay proved that $3.45\% \pm 0.62$ of cells lose their culturability and transform to viable but not culturable (VBCN) state during the disinfection process in low CT values $>0.2\ mg.min.L^{-1}$ (before complete inactivation). The intracellular ATP levels as well as membrane potential rapidly decreased with chlorination and exposure duration, indicating cellular membrane damage.

7.2 Future Directions

1. The developed model can be employed for other sanitizers such as peracetic acid and ozone.

2. Other fruits and vegetables can be studied and the parameters of the model should be tuned for them.

3. Other than fresh produce, chicken, pork and beef are also typically washed using similar process. So, the washing process of each of these food can be investigated.

4. The effect of pH dynamics should be explored in this context as the results from this study were obtained via wash water pH levels near 6.5.

5. In this study, the wash water from one batch was used for another batch to simulate the recycling in wash industries. However, future studies could study the effect of replacing some portion of wash water with fresh water.

6. The mechanisms of bacteria binding to fresh produce could be explored.

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APPENDIX A

PUBLICATIONS

1. **MD. Abnavi**, A. Alradaan, D. Munther, C. R. Kothapalli, P. Srinivasan,"Modeling of Free Chlorine Consumption and *Escherichia coli* O157:H7 Cross-Contamination During Fresh-Cut Produce Wash Cycles" in *Journal of food science*, vol. 84, 2019.

2. P Srinivasan, **MD Abnavi**, A Sulak, CR Kothapalli, D Munther, "Towards enhanced chlorine control: Mathematical modeling for free chlorine kinetics during fresh-cut carrot, cabbage and lettuce washing" in *Postharvest Biology and Technology*, vol. 161, 2020.

3. **MD Abnavi**, CR Kothapalli, P Srinivasan, "Total amino acids concentration as a reliable predictor of free chlorine levels in dynamic fresh produce washing process," in *Food Chemistry* , vol. 335, 2021.

4. **MD Abnavi**, C. Kothapalli, D. Munther, P. Srinivasan, "Chlorine inactivation of Escherichia coli O157: H7 in fresh produce wash process: effectiveness and modeling," in *International Journal of Food Microbiology*, 2021.