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Comparison of inactivation of *Listeria monocytogenes* within a biofilm matrix using chlorine dioxide gas, aqueous chlorine dioxide and sodium hypochlorite treatments

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ABSTRACT

The present research compared the effect of chlorine dioxide (CD) gas, aqueous CD and aqueous sodium hypochlorite (SHC) treatments on the inactivation of a five strain mixture of *Listeria monocytogenes* – containing biofilms. Four day old biofilms were developed on a stainless steel (SS 304) coupon by using a mixture of five cultures of *L. monocytogenes* (Scott A, N1-227, 103M, 82 and 311) using a 100% relative humidity (RH) dessicator for incubation at room temperature (22 ± 2 °C). After biofilm development, coupons were rinsed and dried for 2 h and treated with 0.3 mg/l CD gas at 75% RH, 7 mg/l of aqueous CD and 50 mg/l SHC. Initial $\log_{10}$ population of biofilm cells before CD gas, aqueous CD and SHC treatment was 4.80, 5.09 and 4.95 $\log_{10}$ CFU/cm^2. The Weibull model was used to fit non-linear survivor curves. Treatments and time points of 0.3 mg/l CD gas and 7 mg/l aq. CD solution were significantly different ($p < 0.05$). A 10 min treatment of 0.3 mg/l CD gas, 7 mg/l of aq. CD, and 50 mg/l SHC resulted in reductions of 3.21, 3.74 and 3.09 $\log_{10}$ CFU/cm^2, respectively. At 10 min, all treatments were not statistically different ($p > 0.05$). Low levels of CD (0.3 mg/l CD gas and 7 mg/l aq. CD solution) for 10 min resulted in similar log reductions compared to 50 mg/l SHC.

1. Introduction

Listeria monocytogenes is a food-borne pathogen of major concern. It is a psychrotrophic, gram-positive, non-spore-forming, facultative anaerobe bacterium, commonly found in soil, water, sewage, vegetation, fecal matter and animal feed (Moltz and Martin, 2005). It is ubiquitous in nature and capable of growth at temperatures between 1 and 45 °C, pH levels of 4–9 and salt level of up to 10% (Yousef and Carlstrom, 2003). It mainly afflicts immunocompromised individuals, pregnant women and cancer patients. According to a collaborative study conducted by the Centers of Disease Control and Prevention (CDC), *L. monocytogenes* causes approximately 2500 cases of illnesses and 500 deaths per year (Mead et al., 2000). The data indicate that *L. monocytogenes* has the highest mortality rate among all food-borne pathogens. Recent data from 2008 shows the number of reported cases in the United States of Listeriosis was 719 (CDC, 2009).

A biofilm is defined by as "a microbially-derived sessile community which is characterized cells that are irreversibly attached to a substratum or interface, or to each other, are embedded in a matrix of extracellular polymeric substance that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002). *L. monocytogenes* has the ability to adhere to and grow on a variety of surfaces found in food processing plants including stainless steel, rubber, glass and polypropylene (Lunden et al., 2000). The cells in a biofilm have the ability to survive cleaning and sanitization, and may detach and contaminate the processed food products. Due to its psychrotrophic nature, the organism can grow under refrigerated conditions of processed food storage. Post-process contamination is also a critical issue in the ready-to-eat (RTE) meat and poultry industries (Tompkin, 2002).

Bacteria in biofilms are generally more resistant to antimicrobial substances than planktonic bacteria (Frank and Koffi, 1990; Krysinski et al., 1992; Mustapha and Liewen, 1989). The exopolysaccharide (EPS) matrix restricts the access of antimicrobial substances to cells in the biofilm by reducing diffusion and maintains cells with slow growth rate leading to physiological changes responsible for antimicrobial resistance of biofilm cells (Kumar and Anand, 1998). The resistance to sanitizers increases with maturity of the biofilm, as reported by Lee and Frank (1991), who found that 8 day old *L. monocytogenes* biofilms grown on stainless steel were twice as resistant to chlorine as compared to 4 day old biofilms. There is a need to explore the potential of novel

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antimicrobial/sanitizing agents to control the formation and growth of biofilms in food processing environments to eliminate post-process contamination issues.

Chlorine dioxide (CD) disinfects by oxidation and exhibits a wide range of bactericidal and sporicidal activity (Foegeding et al., 1986; White, 1972; Jeng and Woodworth, 1990). Advantages of using CD vs. use of chlorine, for disinfection includes formation of less toxic disinfection by-products (DBPs), effectiveness at low concentrations, low reaction time, ease of generation and effectiveness over a broad range of pH (Chang et al., 2000). When chlorine/CD is dissolved in water containing organic matter, formation of DBP's such as total trihalomethanes (TTHM) and haloacetic acids (HAA) occur (Gates, 1998). These compounds are potential carcinogens and have been associated with various health problems (Rand et al., 2007). Use of CD instead of chlorine, for water disinfection, result in formation of significantly lower levels of TTHM and HAA (Chang et al., 2000; Gates, 1998). CD also forms chlorite, chlorate and chloride ions. All of these compounds are non-mutagenic (Meier et al., 1985) and chlorite is non-carcinogenic (Kurokawa et al., 1986; Yokose et al., 1987). The mechanism of disinfection by CD still needs to be explored (Simpson, 2005). Earlier researchers have proposed mechanisms which include reaction of CD with amino acids (Noss et al., 1983) and free fatty acids (Ghandbari et al., 1983). It blocks protein synthesis (Bernarde, 1967) and increases permeability of the outer cell membrane (Aieta and Berg, 1986). Therefore, it reacts with membrane proteins and lipids resulting in increased membrane permeability (Noss et al., 1983; Ghandbari et al., 1983). Previous studies indicate higher bactericidal activity of aqueous chlorine dioxide as compared to chlorine solution. 5 mg/l CD had similar effect as 34 mg/l chlorine when used in poultry processing plant chiller water (Lillard, 1979). Higher dose of liquid chlorine (2.6 mg/l) in water at pH 7 was required to attain 99.9% inactivation of *Sarcina* as compared to CD (1.5 mg/l) (Huang et al., 1997).

Efficacy of chlorine dioxide gas to disinfect biofilms has not been studied in detail. Jang et al. (2006), reported a decreasing concentration level of CD from the biofilm surface towards the coupon. They suggested that inactivation of bacteria embedded deep in the biofilm can be achieved either by treatment with high concentrations of CD or with increased contact times (Jang et al., 2006).

The main objective of this study was to compare the $\log_{10}$ reductions for the inactivation of four day old, five strain cocktail of *L. monocytogenes* biofilms after treatment with chlorine dioxide gas and aqueous chlorine dioxide and compare the effectiveness of both treatments with conventional sodium hypochlorite treatment at 10 min. Different concentrations were selected to correspond to equilibrium conditions between liquid and gas phases of chlorine dioxide. Under ambient temperature and equilibrium conditions, CD is 23 times more concentrated in the aqueous phase as compared to the gas phase (Taube and Dodgen, 1949). Therefore, an aq. CD solution concentration of 7 mg/l (pH 5.7) was used to compare with CD gas treatment at 0.3 mg/l concentration at 22 ± 2 °C. Treatment of biofilms with sodium hypochlorite solution of 50 mg/l (pH 9) at 22 ± 2 °C was used to compare results with earlier research work reported by Robbins et al. (2005) and evaluate the effectiveness of both treatments with conventional sodium hypochlorite treatment.

2. Materials and methods

2.1. Bacterial cultures

A mixture of five strains of *L. monocytogenes* was used. The selection of cultures was based on various criteria that included association with outbreaks, food source and ability to form biofilms. *L. monocytogenes Scott A*, serotype 4b ribotype DUP 1042 (source: USFDA, Human, Mass outbreak), *L. monocytogenes 103 M*, serotype ½ a (source: Sausage) and *L. monocytogenes FSL N1-227*, serotype 4b and ribotype DUP 1044A (Source: Bilmar lunch meat, US food epidemic, 1998–1999 CDC (M. Weidman, Cornell)) were obtained as frozen cultures from Dr. Arun Bhunia's lab, Purdue University (West Lafayette, IN). *L. monocytogenes 311 and 82* (source: Environmental isolates from commercial chicken further processing plant) (strong biofilm formers) were obtained from Dr. Mark. E. Berrang, USDA ARS, Russell Research Center (Athens, GA) as stab cultures on brain heart infusion (BHI) agar. The cultures were activated by transferring a bead of frozen culture or loop of individual cultures into separate tubes containing 10 ml tryptic soy broth (Dot Scientific Inc., MI) with 0.6% yeast extract (Bacto, MD) (TSBYE). These tubes were then incubated for 24 h at 37 °C with continuous shaking at 100 rpm on a platform shaker (Max Q 2000, Barnstead Lab-line, Melrose, IL, USA). Three consecutive serial transfers (100 μl) of each culture were done prior to use. Cultures were maintained by serial transfers in TSBYE throughout the course of experiments. One hundred μl of activated cultures were transferred to separate10 ml TSBYE tubes and incubated at 37 °C for 18 h for use in biofilm formation.

2.2. Preparation of stainless steel coupons

Stainless steel (SS) coupons (type 304, 2B finish) with dimensions 1.4 cm × 5 cm (area 7 cm^2) were used. The coupons were prepared by washing them using a procedure described by Frank (2003) with some modifications as follows. The coupons were placed as a single layer on a wire basket kept inside an ultrasonic bath (model 3510R-MT, Branson, CT) with 100 ml/l solution of Micro-90 soap (International Products Corporation, NJ) and sonicated at 60 ± 5 °C for 1 h. This was followed by rinsing the coupons in deionized water and sonicating again in 1.5% phosphoric acid solution at 60 ± 5 °C for 20 min. The cleaned coupons were rinsed with deionized water again and air-dried. These were then placed in plastic autoclavable containers and sterilized by autoclaving.

2.3. Attachment and biofilm formation

The method used for biofilm formation was similar to that as described by Robbins et al. (2005) with a few modifications. The five activated strains of *L. monocytogenes* were mixed in equal quantities (1 ml each, population levels of 9.5–9.9 $\log_{10}$ CFU/ml, absorbance at 600 nm between 0.9 and 1.0) in a sterile test tube. The mixture was vortexed and 100 μl of the mixed culture was placed on each sterile SS coupon kept in a sterile disposable Petri dish. A sterile disposable inoculation loop was used to spread the culture on the top surface of each coupon. The Petri dish was covered and kept in a dessicator with 100% relative humidity (RH) at room temperature (RT, 22 ± 2 °C) for 3 h for initial attachment. After 3 h, the coupons were washed with 20 ml of sterile potassium phosphate buffer (PPB; 50 mM, pH 7) to remove unattached cells. One hundred μl of sterile tryptic soy broth (TSB) was added on top of each coupon and the covered Petri dishes were held at 100% RH for an additional 24 h. After each 24 h period, coupons were washed using PPB and sterile TSB was added in a similar manner as mentioned above for 4 days. Prior to each treatment, the coupons with 4 day old biofilm were rinsed with 20 ml PPB, and air-dried at room temperature for 2 h in a laminar air flow chamber. Usually disinfection with liquids is not carried out on dry equipment. However, when using gas-phase disinfection, a wet surface cannot be guaranteed in all areas. Therefore, coupons were air-dried prior to all treatments to obtain the worst case scenario for gas-phase disinfection.

2.4. Treatment with sodium hypochlorite solution

Fifty mg/l of sodium hypochlorite solution was prepared using sterile deionized water and bleach (5.25% sodium hypochlorite). The final concentration was measured after dilution using DPD method no. 8021 (using Hach DR/2500, Method 8021-USEPA accepted for reporting for drinking water analysis and procedure equivalent to Standard Method 4500-CL G (Greenburg, 1992)) and reported as mg/l free chlorine. Twenty ml of this solution was transferred to a glass tube (50 ml capacity). The dried coupons were then immersed in the tubes containing sodium hypochlorite solution for 1, 5 and 10 min at 22 ± 2 °C with the caps on to keep them airtight during the treatment. The coupons were then immediately transferred to 50 ml sterile centrifuge tubes containing sterile 20 ml solution of 10% sodium thiosulfate (Sigma, St. Louis, MO) to neutralize the effect of residual chlorine.

2.5. Chlorine dioxide gas treatment system

CD gas (109 ppm or 0.3 mg/l) was generated based on a method described by Simpson (Simpson, 2005) as shown in Fig. 1. Briefly, the gas was generated by passing 0.5% chlorine gas (Matheson Tri-Gas, Joliet, IL) through three sodium chlorite cartridges in series (Clordisys Solutions, Inc. Lebanon, NJ) producing 100 mg/l of CD gas in nitrogen at 20 ml/min flow rate. The level of gas in the treatment chamber was verified and maintained using a CD sensor (Model – AF26, optek-Danulat, Germantown, WI) and a programmable logic controller (DL-06, Automation Direct, Cumming, GA). The gas was circulated within the treatment chamber using a fan (4" i.d. fan, air velocity 9–20 ft/min). Relative Humidity during the treatment was maintained using a humidifier connected to a single-loop, feedback controller (Taylor Micro-scan 500) with a humidity sensor (Model – C1210032, Vaisala, Helsinki, Finland).

2.6. Treatment with chlorine dioxide gas

The dried stainless steel coupons were placed in individual sterile glass tubes with caps. After placing the glass tubes with coupons on a tray inside the chlorine dioxide treatment chamber, the caps of the tubes were removed and kept aside on the tray. The chlorine dioxide gas chamber was then closed and the coupons were exposed to 75% RH for 10 min (pre-conditioning). After pre-conditioning with 75% RH, 2 coupons were removed from the chamber through an airlock before injecting CD gas (negative control-with untreated biofilm cells) and used to record the initial count of biofilm cells prior to treatment. The chlorine dioxide gas was then injected into the chamber and coupons were treated for 2, 4, 6, 8, and 10 min at 0.3 mg/l (109 ppm) chlorine dioxide gas at 75% RH and 22 ± 2 °C. After completion of each treatment time, coupons were removed through an airlock and transferred to sterile centrifuge tubes containing 20 ml neutralizing buffer (DIFCO, Maryland). Duplicate coupons were removed at every time point mentioned above.

2.7. Treatment with aqueous chlorine dioxide solution

A stock solution of aqueous chlorine dioxide was made by using a Minidox-M chlorine dioxide generator (ChlorDisys Solutions, Inc., Lebanon, NJ) before the start of each experiment. Chlorine dioxide gas generated by the system was bubbled through sterile deionized water in a glass bottle for 1 min. The bottle was then covered with metal foil and kept in the dark at ambient temperature. The concentration of chlorine dioxide was measured using the DPD (N, N-diethyl-p-phenylenediamine) method (using Hach DR/2500, Method 10 126 procedure equivalent to *Standard Methods*, 18 ed., 4500 CDD). The stock solution was diluted using sterile deionized water to a final concentration of 7 mg/l. Twenty ml of this solution was transferred to an amber colored glass tube (tube capacity 50 ml). The coupons with biofilms were immersed in the tubes containing aqueous CD solution for 2, 4, 6, 8, and 10 min at 22 ± 2 °C with the caps on to keep them airtight during the treatment. The coupons were immediately transferred to sterile centrifuge tubes containing 20 ml Neutralizing buffer (Difco™, BD, Franklin lakes, NJ) to neutralize the effect of residual CD.

2.8. Enumeration of cells in the biofilm

The method used was similar to that reported by Moltz and Martin (2005) with some modifications. The centrifuge tubes with 20 ml neutralizing agent and coupon with biofilm were vortexed (VWR Mini-vortexer at speed 10) for 1 min and serial dilutions were subsequently performed using 0.1% peptone water (DIFCO, Sparks, MD). Aliquots of 1 and 5 ml for each dilution were then pour plated with tryptic soy agar (DOT Scientific Inc., Burton, MI) + 0.6% yeast extract + 1% sodium pyruvate (TSAYEP) (Sigma, St. Louis, MO) at 50 °C to aid the recovery of injured cells. After vortexing, the coupons were also placed in a sterile Petri dish and pour plated with TSAYEP agar to enumerate cells remaining on the coupon. The Petri plates were then incubated at 37 °C for 48 h. Results were reported as $\log_{10}$ CFU/cm^2 of coupon surface area.

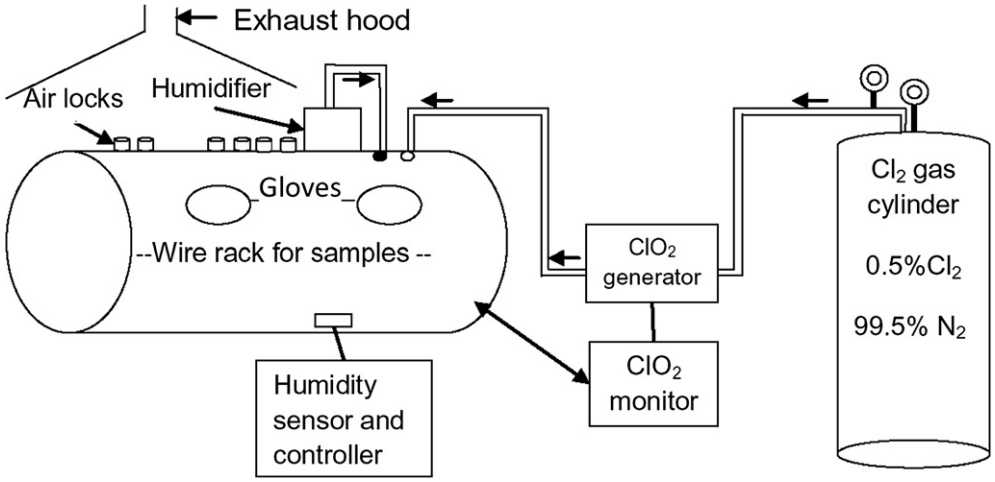

Fig. 1. Schematic diagram of chlorine dioxide (ClO_2) treatment system.

2.9. Non-linear model – Weibull

The Weibull model was fitted to log surviving fractions using the equation (Peleg, 1999; Peleg and Cole, 1998):

$$\log_{10} S(t) = -bt^n \quad (1)$$

where, $S(t)$ is the surviving fraction and n is the shape parameter. The value of n relates to the concavity of the survival curve. b is a coefficient in the Weibull distribution, called scale factor (Avsaroglu et al., 2006; Peleg, 1999). The Weibull model was used for non-linear curves obtained from the data. Excel™ was used for calculations and survival curves. Graph Pad Prism 5 software (Graph Pad Software, LaJolla, CA) was used to calculate Weibull model parameters. t_{lethal} was calculated using the relation, $t_{lethal} = (d/b)^{1/n}$, where, d is the desired number of log cycle reductions and t_{lethal} is the time to reach d log reductions (Peleg, 1999).

2.10. Statistical analysis

Each experiment represented three replicates consisting of two coupons per treatment time resulting in six values per treatment time and three coupons for the initial biofilm cell count per treatment. Log reduction values, mean and standard deviation were determined using the compiled data. Statistical analysis was performed using SAS 9.1.3 software (SAS Institute, Inc. Cary, NC). The "Proc mixed" procedure and differences of least square means were used to find significant differences between the treatments and treatment time points. Weibull model parameters and R^2 values were calculated using GraphPad Prism 5 software.

3. Results and discussion

3.1. Treatment with sodium hypochlorite

The results of inactivation of four day old, five strain cocktail biofilm by 50 mg/l sodium hypochlorite solution are shown in Fig. 2c. Weibull model was used to fit the curve obtained from log reduction vs. treatment time. The b and n values for sodium hypochlorite treatment calculated from the model are shown in Table 1.

Treatment of four day old *L. monocytogenes* five strain cocktail biofilm with 50 mg/l sodium hypochlorite (pH 9) for 1, 5 and 10 min resulted in log reductions± standard deviation of 0.78 ± 0.27, 1.6 ± 0.39 and 3.09 ± 0.49 $\log_{10}$ CFU/cm^2 respectively. In a similar study reported earlier, four day old L. monocytogenes, strain 10403S biofilm was treated with 50 mg/l calcium hypochlorite and found that treatment for 1, 5 and 10 min resulted in $\log_{10}$ reduction of approximately 2, 2.4 and 5.1 log CFU/cm^2 respectively (Robbins et al., 2005). Our study provides results similar to Robbins et al. except for the difference that the biofilm developed from five strain mixture was more resistant to sodium hypochlorite treatment.

3.2. Treatment with chlorine dioxide gas and aqueous chlorine dioxide

Initial recoverable populations of biofilm cells before CD gas and aqueous CD treatment were 4.80 ± 0.07 and 5.09 ± 0.2 $\log_{10}$ CFU/cm^2 respectively. The non-linear curves obtained by plotting log reduction vs. treatment time were analyzed using the Weibull model. The b and n values for both treatments are shown in Table 1. The fitted curves are shown in Fig. 2a and b. Statistical analysis of the data indicates that both treatments (0.3 mg/l CD gas and 7 mg/l aqueous CD) were significantly different ($p < 0.05$).

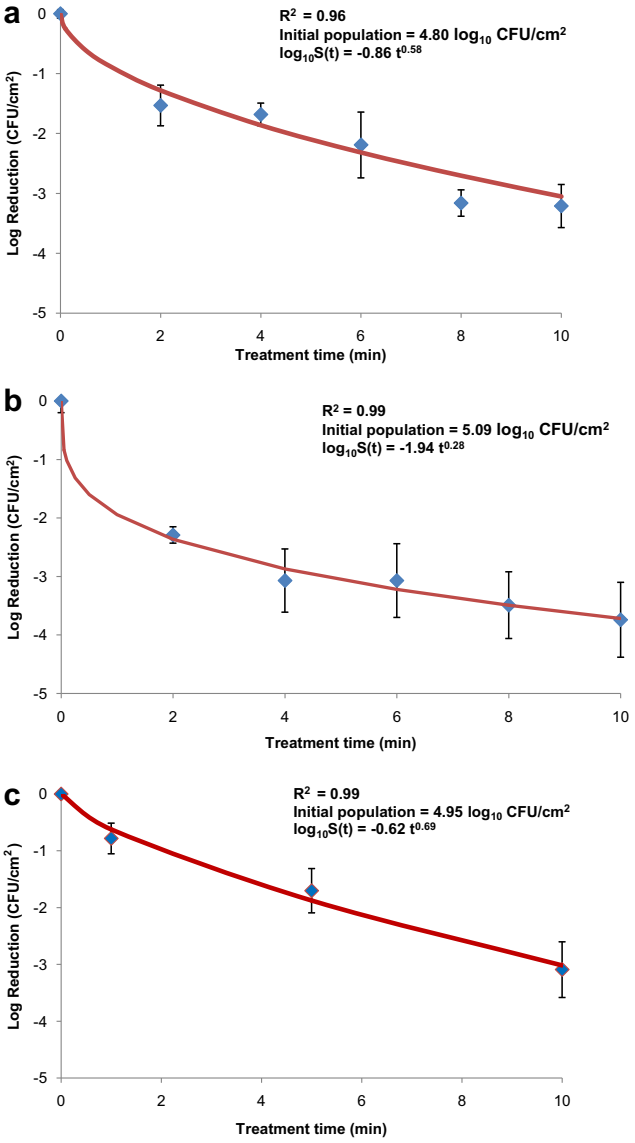

Fig. 2. Effect of (a) chlorine dioxide gas (0.3 mg/l @ 75% RH), (b) aqueous chlorine dioxide treatment (7 mg/l) (middle) and (c) sodium hypochlorite (50 mg/l) on four day old five strain *Listeria monocytogenes* biofilm (points on the graph indicate the mean log reduction ± std. dev. ($\log_{10}$ CFU/cm^2), $n = 3$ at each time point). The Weibull model was used for curve fitting.

Aqueous CD resulted in a significantly greater log reduction of biofilm cells for shorter treatment times (2, 4 and 6 min) as compared to CD gas treatment. This may be a result of slow diffusion of CD gas into glass sample tubes and/or the time required for the gas to dissolve in water present on the surface of the biofilm. The difference in log reductions between treatments was smaller and not significant for the longer treatment times (8 and 10 min)

Table 1
Weibull model parameters (mean ± std. dev.) and R^2 values for three treatment conditions.

Treatment	b	n	R^2
Sodium Hypochlorite (50 ppm)	0.62 ± 0.29	0.69 ± 0.22	0.99
Chlorine dioxide gas (0.3 mg/l @ 75% RH)	0.86 ± 0.26	0.58 ± 0.15	0.96
Aqueous chlorine dioxide solution (7 mg/l @ pH 5.70)	1.94 ± 0.18	0.28 ± 0.05	0.99

(Table 2). Once the CD gas is dissolved in water, it is similar in effectiveness to aqueous CD. Therefore, it takes a longer time to show a comparable bactericidal effect using CD gas as compared to aqueous CD when the two concentrations (gas and aqueous) are kept at their equilibrium values.

Earlier research work reported the inactivation effect of 0.3 mg/l CD gas treatment for 31 min and 15 °C of *Escherichia coli* 0157: H7 cells on green peppers increases with an increase in % RH from 55 to 95% (Han et al., 2001a). In our study, seventy-five percent RH was selected for the treatment to simulate the % RH conditions which would be easier to maintain in a food processing plant. A higher humidity during treatment may increase the gas effectiveness at the shorter treatment times.

Values of t_{lethal} for both the treatments of CD gas and aq. CD (Table 3) indicate that the difference in t_{lethal} values is greater at lower *d* (desired log reduction) values mainly 1 and 3 log CFU/cm^2. This is consistent with the differences mentioned at shorter treatment times. However, when the desired log reduction is extrapolated to 5 log CFU/cm^2, the t_{lethal} values for both the treatments are very close i.e. 20.8 for 0.3 mg/l CD gas treatment and 29.4 for 7 mg/l aqueous CD treatment.

To find the number of cells remaining on the coupon after vortexing, direct pour plating of the coupon with TSAYEP agar and incubation at 37 °C for 48 h was done, to obtain the cell counts. It was observed that, a cell population of 0.3–1.2 $\log_{10}$ CFU/cm^2 was left on the surface of the coupons (data not shown). These methods are not capable of recovering all the biofilm cells from the coupons and used with an assumption that pre- and post-treatment cell recovery rates are similar. In this research the biofilm was directly exposed to sanitizing agents without any pre-cleaning treatment to represent the worst case scenario and verify the effect of sanitizing agent on the biofilm cells directly. It was also verified from initial experiments that higher inactivation in case of aqueous CD or sodium hypochlorite treatments was not due to washing off of the biofilm cells into the solution (data not shown).

Statistical analysis of the log reduction data obtained from all three treatments at 10 min treatment time, show that all the treatments were not statistically different ($p > 0.05$). This data indicate that even low levels (7 mg/l aqueous CD and 0.3 mg/l CD gas @ 75% RH) of CD treatment for 10 min resulted in similar log reduction compared to 50 mg/l sodium hypochlorite for biofilm cells. Earlier research work indicates similar decontamination effect of low levels of 3 mg/l CD solution as compared to 200 mg/l of chlorine (sodium hypochlorite solution) on *L. monocytogenes* cells present on fruit surfaces (Rodgers et al., 2004).

Aqueous and gaseous CD efficacy was studied earlier to reduce *L. monocytogenes* on green peppers with injured and uninjured surfaces (Han et al., 2001b). It was reported that both gas and aqueous treatments were compared at similar concentrations (0.3 and 3 mg/l) and gaseous CD was more effective in reducing *L. monocytogenes* Scott A on green peppers. In this study, equilibrium concentrations of CD gas and aqueous solution based on Henry's Law were used, as it was hypothesized that CD gas dissolves in water and reaches the cells in order to inactivate them. Therefore, a CD gas treatment will effectively concentrate in any water on the cell surfaces to the equilibrium concentration at that temperature if given enough time. Since the longer treatment times had statistically similar effects on the biofilms, this helps support the hypothesis that CD gas and aqueous CD are similar in effectiveness when compared at their equilibrium concentrations (i.e. ~23:1 ratio at 22 °C).

This study has also demonstrated that low levels of CD gas (0.3 mg/l @ 75% RH) and aqueous CD (7 mg/l) have equivalent inactivation of *L. monocytogenes* cells in a biofilm matrix as compared to conventional 50 mg/l sodium hypochlorite treatment. Further research needs to be done to establish levels of CD and treatment times required for complete inactivation of biofilm cells. The potential for CD use should be explored further for the purpose of equipment sterilization and inactivation of biofilms from food processing equipment surfaces.

Table 2
Mean log reduction of *Listeria monocytogenes* biofilm cells on stainless steel (AISI 304) coupon surfaces after chlorine dioxide gas and aqueous chlorine dioxide treatments.

Time of treatment (minutes)	Mean log reduction (CFU/cm^2)	
	Treatment with chlorine dioxide gas (0.3 mg/l @ 75% RH)	Treatment with aq. chlorine dioxide solution (7 mg/l @ pH 5.70)
2	1.53 ± 0.34a	2.29 ± 0.14b
4	1.68 ± 0.19a	3.07 ± 0.54b
6	2.19 ± 0.55a	3.07 ± 0.63b
8	3.16 ± 0.22a	3.49 ± 0.57a
10	3.21 ± 0.36a	3.74 ± 0.64a

*$n = 3$ and values reported are mean ± std. dev.
Means with the same letter (a or b) in same row are not significantly different ($p > 0.05$).

Table 3
Calculated values of t_{lethal} (similar to traditional *D*-value) for two different treatments of *Listeria monocytogenes* biofilm cells on stainless steel (AISI 304) coupon surfaces calculated from Weibull models fit to the data.

d (desired log reduction, log CFU/cm^2)	t_{lethal} (min.)	
	Chlorine dioxide gas treatment (0.3 mg/l @ 75% RH)	Aq. chlorine dioxide Treatment (7 mg/l @ pH 5.70)
1	1.3	0.1
3	8.6	4.7
5[a]	20.8	29.4

[a] Extrapolated value.

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