Evaluation of Chlorine Dioxide as an Antimicrobial Against *Botrytis cinerea* in California Strawberries

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ABSTRACT

Strawberries are highly sensitive to deterioration by microbial decay after harvest and *Botrytis cinerea* is one of the most common diseases that infect them. Previous work has reported successful extension of strawberry shelf life through the use of chlorine dioxide (ClO₂) gas treatments. In this study, California strawberries purchased at retail stores were treated with continuously generated ClO₂ gas over concentrations ranging from 0.01 to 5.0 mg/L for durations ranging from 7 to 1000 min to determine how ClO₂ gas impacts fungal mortality and fruit quality as a function of treatment concentration, treatment duration, and ClO₂ absorption by the fruit. Repeated measures and Gompertz models were used to infer on the efficacy of the ClO₂ gas, and a novel method was used to quantify the ClO₂ absorption by fresh produce. It was found that ClO₂ gas treatments have minimal effect at delaying the onset or growth rate of *Botrytis cinerea* for treatments followed by incubation at 4 or 22 °C even when corrected for natural variability of *Botrytis cinerea* presence among batches of strawberries, indicating that treatments are not sufficient for shelf-life extension.

Key words: Strawberries, *Botrytis cinerea*, chlorine dioxide, Gompertz models, Repeated measures

1. INTRODUCTION

Food spoilage is both a sustainability and a commercial issue because visible mold and undesirable odors lead to consumer rejection, which in turn causes significant economic losses and food waste. According to Kantor et al. (1997), the annual edible food available to the U.S is reduced by about 27% due to food spoilage and waste at retail and consumer levels and fruits and vegetables are one of the main categories responsible for food losses and waste (Kantor et al 1997). This percentage is especially significant in the context of a life cycle assessment (LCA) study for food systems where input factors, including energy, water irrigation, packaging and transportation, are considered (Heller et al 2000, Peano et al 2012). It is such a relevant issue that the Environmental Protection Agency (EPA) and the United State Department of Agriculture (USDA) both raise awareness about food waste management and assist entities of the U.S food chain to meet their food-waste goals through initiatives such as “EPA's Food Recovery Challenge” and “U.S. Food Waste Challenge”.

Within the food production chain, fruits and vegetables may be contaminated at different stages since they are exposed to bacteria, parasites, viruses, and fungi/molds through multiple sources including insects, irrigation water or rain, soil, air, manure based fertilizers, manual handling by workers during the harvest and packaging process, food processing facilities and transportation (Brakett 1994, Wei et al 1995, Yuk et al 2006,). All these factors directly influence their mode of decay and the time to reach the end of shelf life. Specifically, mold spoilage of food results from a biological process that begins with contamination by fungal spores which germinate and extend and into hyphae to form a visible mycelium over time resulting in the end of the product's shelf life (Dantigny et al 2007). Mold growth is affected by environmental factors such as water activity (a_w), pH and temperature, and also by food constituents like proteins, carbohydrates, lipids and organic acids (Dagnas and Membre 2013, Garcia et al 2009). Optimum conditions for mold growth depend on the type of microorganism, but in general, food spoilage will occur more rapidly at temperatures above 25 °C and a_w above 0.85 as long as the food system is contaminated with spores and that those spores are able to germinate ((Dantigny et al 2005, Gougouli and Koutsoumanis 2012.)). Therefore, to improve the quality of fruits and vegetables, and to help prolong their shelf life, it is necessary to both understand the behavior of fungal growth and to identify methods that effectively reduce the population of microorganisms. Researchers have proposed models to describe microorganism population growth as a function of time, and to simulate and predict the behavior of different types of fungi growth

at different environmental conditions and at different growth stages (germination and hyphae extension) such as logistic, the Gompertz and the Baranyi models (Dantigny et al 2006, Dantigny et al 2007, Declerck et al 2001, Loez et al 2004).

Understanding how molds behave and what factors influence their growth could, among others, help identify suitable sanitation technologies that would effectively reduce the fungi population that cause food spoilage. Chlorine dioxide (ClO_2) gas for example, has been extensively studied for its proven efficacy as a potent sanitizer and disinfectant capable of reducing populations of pathogenic microorganisms that compromise food safety (Abdul-Raouf et al 1993, Benarde et al 1967, Costilow et al 1984, Du et al 2002, Han et al 2000^a, Han et al 2000^b, Netramai et al 2001, Reina et al 1995, Wang et al 2014). Some organizations have established limits for the exposure of food to ClO_2 such as the U.S Food and Drug Administration recommend a maximum of 3 mg/L in water for sanitizing fresh produce (FDA, 2012), the United States EPA allows levels of 0.8 mg/L and 1.0 mg/L for ClO_2 and chlorite in drinking water (EPA, 2006) while the Food and Agricultural Organization / World Health Organization (FAO/WHO) considers safe a daily consumption of body weight per day of 0.03 mg/kg of chlorite and 0.01 mg/kg of chlorate (Gómez-López et al., 2009).

While less research has focused on studying its effect on spoilage molds and bacteria, the few results found in the literature are promising and worth exploring. For example, Spotts et al. (1980) investigated the effect of ClO_2 on fungi for the control of d'Anjou pear decay and found that ClO_2 did not affect the germination of *Botrytis cinerea* when concentrations lower than 5 mg/L were used even when held for 10 min, but that ClO_2 was effective when pears were treated with 10 mg/L concentration for 10 min. Roberts et al. (1994) studied the percentage spore mortality of four fungal species after *in vitro* exposure to different ClO_2 concentrations and times and found that *Botrytis cinerea* was one of the most resistant species, sensitive only to high concentrations of 3 and 5 $\mu\text{g/mL}$ within 1 min of exposure. This information seems to suggest i) that contrary to what has been documented for pathogenic microorganisms, it has been challenging to reduce populations of *Botrytis cinerea* using treatments with low concentrations of ClO_2 and ii) that despite Spotts et al. and Roberts et al. agreement on the concentration of the effective treatment (5 mg/L), the difference in exposure times (1 versus 10 min.) implies that the biological subject where *Botrytis cinerea* is present has an effect on the efficacy of ClO_2 treatments. Strawberries are a unique fruit with irregular shape, seed-studded surfaces, and high organic acid content (Han et al 2004, Kallio et al 2000). Moreover, their shelf life is short due to susceptibility to rot-causing pathogens and fast ripening due to high

respiration rates (Kim et al 2010). Sy et al 2005 found that yeast and mold populations (not specified what type of population) on strawberries were significantly reduced when treated with gaseous ClO_2 at concentrations of 8 mg/L for 120 min. More recently, Aday et al. (2010) found that treatments with ClO_2 on strawberries are capable of extending the shelf life of the fruit; and Vardar et al. (2012) found that the incidence of decay of strawberries by *Botrytis cinerea* was significantly reduced by treatments with ClO_2 (1500 and 2000 $\mu\text{L L}^{-1}$) with little impact on sensorial characteristics. However, as a result of ClO_2 treatments, it and its by-products may persist in the fruit (Wise et al 2012) requiring knowledge of the quantities of these chemical compounds, particularly in the case of fruits and vegetables because they are often consumed raw. Therefore, a holistic approach to assess the feasibility of use of ClO_2 as a sanitizing technology for reducing *Botrytis cinerea* on strawberries should focus on its efficacy, impact on quality, and ClO_2 consumption by the strawberries. The main goal of this study was to determine the efficacy of ClO_2 gas in the delay of onset of *Botrytis cinerea*, the most common microorganism responsible for spoilage of strawberries, meanwhile quantifying the concentration range that does not produce visual damage to strawberries. The specific objectives of this work were to (i) expose strawberries to different ClO_2 treatments to determine its efficacy while maintaining the color and moisture content of the fruit, (ii) to quantify total ClO_2 absorption by the fruits and (iii) to correlate total ClO_2 absorption with concentration and exposure time.

2. MATERIALS AND METHODS

2.1 Selection of California strawberries. California strawberries were purchased from a local supermarket and were harvested approximately 1-week prior to experiments and shipped in refrigerated trucks to Michigan. There were differences between strawberry batches in terms of the suppliers and cultivars based on size and shape of the berries, but appropriate control populations from each batch are compared. All strawberries were thoroughly inspected before use. Only fruits similar in size and color that were free of bruises and other damage were chosen and were randomly assigned to treatments. Raw strawberries were weighed and conditioned at $22 \pm 2^\circ\text{C}$ for an hour prior to treatments in order to avoid condensation during treatments and to maintain a constant temperature inside the treatment chamber.

2.2 Design of experiments. A set of three completely randomized block designs (CRBD) (hereafter experimental designs) with appropriate controls for each design were conducted in order to determine appropriate ClO_2 sanitizing treatments on California strawberries for delaying the growth of *Botrytis cinerea* without affecting the visual quality of the fruit in terms of bleaching and desiccation of the tissue or calyx. Table 1 shows the different treatments in

pairs of concentration and time of exposure selected for each experimental design. The order of the treatments was randomized to account for variability as treatments were performed sequentially.

The first experimental design consisted of a 10-point response surface design covering low and high ClO₂ concentrations for short and long periods of time. Intentionally, combinations of high concentration with long exposure times, and low concentrations with short exposure times were excluded because it was anticipated that in the first case the quality of the produce would be significantly diminished (*e.g.*, excessive bleaching and desiccation), and that in the second case the sanitizing effect of the gas would be minimal. Exposures consisted of 12 strawberries per condition, with randomized replicates of some conditions, for a total of 132 berries treated and a control was removed at each time point for a total of 120 untreated berries. Mold growth conditions were 22 ± 2 °C and approximately 100% relative humidity.

The second experimental design consisted of four of the conditions from the above design plus an additional center point treatment of 0.41 mg ClO₂/L for 17 min. In this case, 4 replicates of each condition with 18 berries per replicate were used to increase sample size and improve power level of the experiment. A total of 360 strawberries were treated and 360 untreated berries were used as a control. Mold growth conditions were 22 ± 2 °C and approximately 100% relative humidity.

The third experimental design was performed with a ClO₂ treatment condition of 0.63 mg/L for 84 min which was replicated 16 times. Here, the temperature of mold growth was assessed at 22 ± 2 °C and 4 ± 2 °C and approximately 100% relative humidity. This variable was explored to both avoid the growth of *rhizopus* in the strawberries and to delay the rate of fungal growth in both treated and control samples in an attempt to better distinguish differences between ClO₂-treated strawberries and the control for the onset of *Botrytis cinerea* growth. A total of 576 strawberries were selected and treated in batches of 18 strawberries per replication. Another set of 576 strawberries was selected as the control group.

2.3 Incubation tubs. After each ClO₂ treatment the fruits were assessed by incubation tests consisting of arranging the fruit on 0.5-cm mesh screens in aluminum pans (26 cm x 32 cm) ensuring the fruit was equidistantly separated from one another. The tubs were filled with approximately 100 mL of water below the screen level and covered with plastic film to maintain low air flow to minimize cross-contamination of fungal growth and a relative humidity equal or close to 100%. Incubations in these tubs were performed at 22 °C or refrigerated at 4 °C as prescribed by the experimental design. This approach is similar to that used by Schilder et al 2005 in evaluation of

fungicide efficacy in fruit rot control. Both mesh screens and aluminum tubs used were properly sterilized by autoclaving prior to each experiment. For all experiments, the incubation tubs were analyzed for fungal growth, which was rated daily (see rating of fungal growth section).

2.4 Evaluating efficacy of ClO₂ against the growth of *Botrytis cinerea*. California strawberries for all experiments were rated individually by establishing the percent area of each fruit that was infected on a daily basis. The continuous data collected for experimental designs 1 and 2 ranged from 0 to 1, where 0 represents no signs of mold and 1 represents 100% of the area covered with the fungus. Due to the size of experimental design 3, strawberries were rated every other day using a binary code where 0 represents no sign of mold, and 1 represents at least 10% of area covered with the fungus (the amount of fungal growth that would be visible to a consumer and might lead to rejection of the fruit – an economically important level of infection). In other words, the data collected in experimental designs 1 and 2 represents both the onset and the growth rate of fungus while data collected from experimental design 3 only captures the onset which can be translated into the probability of a strawberry to be infected at any given time post-treatment which is indicative of how a consumer would reject the product based on its quality. Rating was stopped when the majority of the strawberries reached approximately 90% infection.

2.5 Fruit quality assessment after treatment. Photos were taken before and after treatments in order to identify the location and assess the magnitude of the discoloration or desiccation that could result after exposure with ClO₂. Images were taken daily during rating to record the fungal growth process. A professional Canon EOS 40D camera equipped with a Canon EF-S 15-85mm f/3.5-5.6 IS USM Lens (Canon, Melville, NY, USA) was placed and fixed on top of the exposure chamber at a distance of approximately 70 cm from the platform on which the berries were placed. The glass chamber was fixed to the table with Velcro™ adhesive in order to avoid any movement during exposure. Camera settings, contrast background color, amount of light, amount of flash lights used and distances to the chamber were fixed and unchanged throughout treatments to ensure that any changes in the photos from before and after ClO₂ exposure were only due to the effect of treatments in strawberries. These images were analyzed using ImageJ 1.47v (National Institutes of Health, USA), to compare the respective color histograms of the various regions of the berries before and after exposure to determine the effect of ClO₂ treatment on the berry quality after exposure.

2.6 Continuous ClO₂ gas treatment. The system for treating strawberries with ClO₂ was previously described by Arango et al. (2013) (Arango et al 2013). **Figure 1** shows a schematic diagram of the system developed for (i) the continuous ClO₂ exposure and (ii) the quantification of ClO₂ absorption by fresh produce (e.g., strawberries). In summary, the equipment could be divided into three main components. Component 1 consists of a Minidox-M ClO₂

gas generator from ClorDiSys Solutions Inc (Lebanon, NY) equipped with a 98% N₂ and 2% ClO₂ tank, an airtube with filter and pressure regulator, and a set of three Aalborg GFC mass flow controllers (Orangeburg, NY). This component continuously generates and dilutes ClO₂ gas in air to reach gas concentrations below 1 mg/L (minimum ClO₂ concentration of the Minidox-M ClO₂ gas generator). The equipment is calibrated so that any ClO₂ gas concentration from 0.01 to 5 mg/L could be produced. Component 2 consists of an 11 L glass chamber with three inlet/outlet ports, a glass lid, a polytetrafluoroethylene O-ring, a metallic clamp to provide sealing and closure respectively and a stainless steel platform to hold the produce inside the chamber. The chamber is connected to the mass flow controller 3 (MFC3 from Figure 1) from Component 1, and its main function is to hold the strawberries and the gas during treatments. Component 3 consists of a Shimadzu UV 1800 spectrophotometer (Scientific Instruments, Columbia, MD) and a 100 mm cylindrical quartz flow cell with two windows (Quartz spectrophotometer cell, cylindrical, Starna Cells, Atascadero, CA). This component continuously detects the headspace concentration inside the chamber before and during treatments upon the gas exit from the chamber.

At the beginning of each treatment, strawberries are loaded into the 22 ± 2 °C chamber that has an air atmosphere part of which was displaced in order for the concentration of ClO₂ to build up. For this reason, a two-step process was designed to speed up the chamber filling. The first step consisted on injecting only ClO₂ gas at a higher concentration than desired until the concentration in the chamber headspace was past set point. In the second step, air started flowing and diluting the ClO₂ gas to achieve the desired concentration. At the end of the two-step process, the concentration entering the chamber and the concentration in the chamber headspace equilibrated to the desired concentration and the UV-vis detector recorded a steady value over time.

2.7 Quantification of ClO₂ absorption by fresh produce: Total ClO₂ absorption was calculated by equation (1), as discussed by Arango et al. (2013)(Arango et al 2013), after recording curves of the headspace concentration over time for both the empty and the full chamber (*e.g.*, with strawberries).

$$Total\ ClO_2\ Absorption\ [mg] = \left(\frac{A_e - A_f}{A_e} \right) * c * t * fr \quad (1)$$

where A_f is the area under the curve with chamber containing produce (or full), A_e is the area under the curve with the empty chamber, c is the ClO₂ concentration of exposure (mg/L), t is the time of exposure (min) and fr is the flow rate at which the gas enters the chamber (always set at 500 mL/min).

Under the same conditions of temperature, materials used, exposure time and concentration, the percentage difference between the curves was attributed to the ClO₂ absorption by fresh produce.

2.8 Statistical Analysis. To evaluate ClO₂ efficacy, statistical analyses were performed using the repeated measures methodology considering that the same strawberries were subjected to quality assessment as a function of time. With the repeated measures methodology, it was possible to compare all treatments with the control independently at each day of rating. Simultaneously, the non-linear Gompertz growth model was used to fit the data as described by equation (2):

$$y = \alpha e^{-e^{-\beta(t-\theta)}} \quad (2)$$

where y is the fraction of fungal growth at time t , and α , β , and θ are estimates of the asymptote, growth rate, and inflection point parameters, respectively (Dantigny et al 2007, Garcia et al 2009). To assess the effect of exposure time and concentration on ClO₂ absorption, a surface response analysis was conducted using JMP 10.0.0 (SAS Institute Inc., Cary, NC) and Matlab_R2013a (The MathWorks, Inc., Natick, MA). All other statistical analyses were done using SAS 9.3 (SAS Institute Inc., Cary, NC).

3. RESULTS AND DISCUSSION

3.1 Quality Assessment. Visual inspection of the exposed California strawberries showed that exposure to either prolonged treatments such as 0.22 mg ClO₂/L for 84 min, 0.01 mg ClO₂/L for 1000 min, 0.08 mg ClO₂/L for 1000 min and 1.26 mg ClO₂/L for 332 min, or exposure to high concentration such as 5 mg ClO₂/L for 7 and 37 min discolored strawberries by changing the red pigment of the fruit from red to pale yellow as well as it dried and bleached the calyx evidenced by a texture and color change from fresh green to an ashy yellow color. Furthermore, the degree and area of bleaching increased as the concentration and/or time increased. Figures 2a and 2b (online version) show an example of strawberries before and after exposure to treatment 5 mg ClO₂/L for 7 min, where the quality of the fruit was moderately affected as every single treated strawberry tissue and calyx had a shift in overall color, (as recognized by RGB and gray scale image analysis - data not shown). On the other hand, discoloration of strawberries exposed to treatments 0.63 mg ClO₂/L for 7 min, 0.04 mg ClO₂/L for 21 min and 0.01 mg ClO₂/L for 191 min was minimum and almost unnoticeable, which could be tentative treatment for California strawberries sold directly to consumers.

3.2 Quantification of ClO₂ absorption by fresh produce. Total ClO₂ absorption by strawberries was calculated for each treatment using equation 1 and the results are shown in Table 2. Combining this information with the results from the visual evaluation of the fruit, it can be demonstrated that the quality of the fruit after exposure (discoloration or bleaching) is in agreement and positively correlated to ClO₂ absorption by the fruit as presented on Figure 3. The data fits an exponential decay model with 2 parameters with a r²=0.89 shown in equation (3):

$$Q = 100e^{0.11A} \quad (3)$$

where Q is the percentage of exposed strawberries per treatment presenting signs of discoloration measured between 0 and 1 where 1 means all of the strawberries after treatment were discolored, and 0 means that none of them were discolored, and A is the total ClO₂ absorbed by the strawberries in each treatment. The steep behavior of the graph shows a fast quality deterioration of the strawberries with small changes in ClO₂ absorption. This information is of important concern because it leaves a small window for treatments that could potentially be used for sanitizing without affecting the fruit's quality. In fact, according to this set of experiments, significant bleaching fraction of strawberries occur at levels of absorption greater than at least 7.9 mg ClO₂/g of fruit, and an acceptable limit for ClO₂ absorption would be at least 4.5 mg ClO₂/g of fruit. This quantification further illustrates that the quality of berries is directly related to the quantity of ClO₂ consumed rather than the conditions at which it was exposed.

A mathematical model that predicts ClO₂ absorption by fresh produce in terms of concentration of ClO₂ and exposure time was developed using a response surface method (RSM). For the response variable, the analysis of variance indicated that the model was significant (p<0.01) with significant linear and quadratic effects at a significance level lower than 0.01. The prediction model is described by equation 4, and the surface plot was bounded by the limits of concentrations and exposure times considered in the set of experiments as shown in Figure 4.

$$Abs_{ClO_2} = 16.62 - 41.27X_1 - 44.24X_2 + 45.34X_1X_2 + 17.32X_1^2 + 26.06X_2^2 \quad (4)$$

where Abs_{ClO_2} is the response variable absorption of ClO₂ in mg ClO₂, X_1 is the logarithm of concentration in mg/L, and X_2 is the logarithm of time in min.

3.3 Fungal growth. To simulate commercial conditions and fungal loads present on strawberries in marketing channels as they reach retail stores and consumers, strawberries used in these experiments were treated as they came

from the store, and no inoculation, pre-wash or calyx removal was performed. Preliminary experiments had also indicated that inoculation of strawberries with *Botrytis cinerea* made no difference in the amount of mold growth that developed as most strawberries tend to be already infected in the field prior to harvest (Maas 1995).

For these reasons, the variability in the degree of infection between strawberries was initially high, yet representative of real world systems. Therefore, there were cases when treated or untreated strawberries would remain intact after several days of incubation in the aluminum tubs while other strawberries in the same tray would be completely covered with fungal growth. Furthermore, even though strawberries within experiments were all from the same lot and same producer, strawberries between experiments were not, and so the variation in the rate of decay of strawberries between experimental designs 1, 2 and 3 was also wide. Evidence of this is that rating in experimental design 1 lasted only for 4 days, after which the majority of strawberries were rotted, while experimental design 2 was suspended after 7 days of incubation, and rating of strawberries from experimental design 3 incubated at 22 °C was suspended after 13 days. This observation did not indicate unprecedented variability as differences in cultivar susceptibility, harvest timing, and fungicide programs can influence the incidence and rate of decay of strawberries by *Botrytis cinerea* (Maas 1995, Wise et al 2012).

3.4 Assessment of ClO₂ efficacy and strawberry quality. To model repeated measures over time, the fungal growth data collected from experimental designs 1, 2, and 3 was fit to a first-order autoregressive model because, in this case, observations more adjacent to each other in time might be more highly correlated with each other than those observations farther apart in time within each strawberry. In experimental design 1, it was found that both the effect of the treatments ($p < 0.0150$) and the interaction of treatment with rating day ($p < 0.0217$) were significant. From Table 3 and Figure 5a, it can be observed that the efficacy of the ClO₂ gas against *Botrytis cinerea* was proven on treatments 5 mg ClO₂/L for 7 min, 0.22 mg ClO₂/L for 84 min, 1.26 mg ClO₂/L for 332 min, and 0.08 mg ClO₂/L for 1000 min, all which as of the second day post treatment showed significantly lower fungal growth than the control. Other treatments such as 0.63 mg ClO₂/L for 7 min and 5 mg ClO₂/L for 37 min showed significant differences from the control but only in some selective rating days which rendered them identical to the control for practical purposes.

Because the objective of these series of experiments was to find treatments where both efficacy and quality were met, treatments 5 mg ClO₂/L for 7 min and 0.22 mg ClO₂/L for 84 min (both with moderate quality damage

after exposure), and treatments 0.04 mg ClO₂/L for 21 min, and 0.63 mg ClO₂/L for 7 min were repeated in experimental design 2. Although the last two treatments were not statistically efficacious at reducing mold growth on this smaller population, they were selected because they did not affect the visual appearance of the strawberries after exposure and replicating them with increased sample size to increase confidence in the efficacy response was warranted (Figure 5a). Additionally, as explained in the design of experiments section, treatment 0.41 mg ClO₂/L for 17 min was selected because it was calculated with equation (3) that it would not damage the fruit after exposure (percentage of exposed strawberries with discoloration $Q \leq 16.36$).

In experimental design 2, it was found that the effect of the treatments was significant at $p < 0.0585$, while the interaction of treatment with rating day was significant at $p < 0.0101$. From Table 4 and Figure 5b, it can be observed that treatments 0.63 mg ClO₂/L for 7 min, 0.22 mg ClO₂/L for 84 min and 5 mg ClO₂/L for 7 min lower the amount of mold grown versus the control after rating day 4 at 95% confidence level, which translates to about a half day additional shelf life before 10% of the strawberries are infected. These results showed that both criteria ClO₂ gas efficacy and quality of the strawberries after exposure were met with the treatment 0.63 mg ClO₂/L for 7 min, which was then run alone versus the control in experimental design 3. Further analysis assuming a power of 0.8 and the observed variation within strawberries to test for significant differences at $p < 0.05$, resulted in a required minimum sample size of 144 strawberries per treatment.

In experimental design 3, 576 strawberries were divided into 4 groups: control at 4 °C incubation temperature, control at 22 °C, treated at 4 °, and treated at 22 °C. The rating of strawberries stored at 4 °C was suspended after 24 days, while the rating of those stored at 22 °C was suspended after 13 days. Fungal growth in these strawberries took almost twice as long as in experimental design 2, and 3 times as much than experimental design 1, which could be interpreted as resulting from an inherently more disease-resistant batch of strawberries or a significantly less infected batch or another of the considerations discussed above. Figure 5c shows the results of experimental design 3 for both incubation temperatures. It was found that the simple effects of rating day and temperature were significant while the effect of the treatment 0.63 mg ClO₂/L for 7 min was not ($p < 0.3458$). However, the interaction of the treatment across rating days was significant at $p < 0.0529$ and for strawberries stored at 4 °C, it was found that the treatment was significantly better than the control between days 17 through 20 with $p < 0.0586$. To further understand how ClO₂ was being effective at reducing the growth of *Botrytis cinerea* in strawberries, the Gompertz model as described in equation (2) for mold growth was fitted to the data.

Figure 6 shows the plots for experimental designs 1, 2 and 3 fitted to the Gompertz model, and Table 5 summarizes the results for the three parameters α , β , and θ for all treatments. One of the advantages of using a parametric model such as the Gompertz model to understand the effect of the treatments is that the parameters provide physical meaning to the behavior of the fungal growth, and therefore they allow for better understanding of the ClO₂ gas efficacy. For example, Table 5 shows that treatments 0.63 mg ClO₂/L for 7 min, 5 mg ClO₂/L for 7 min, 1.26 mg ClO₂/L for 332 min, and 0.08 mg ClO₂/L for 1000 min from experimental design 1, as well as treatments 0.63 mg ClO₂/L for 7 min, 5 mg ClO₂/L for 7 min, and 0.22 mgClO₂/L for 84 min from experimental design 2, show the lowest β and highest θ values. This indicates that the infection growth rate is slower for these treatments and that as a result, the onset of *Botrytis cinerea* is delayed. Therefore, in cases where ClO₂ gas was found to be effective, it acted as both an inhibitor of initial growth (delay of onset) and as a regulator along time since the speed at which the disease grew was significantly slower. These results are in good agreement with the findings from the repeated measures modeling since the treatments that were selected for their better efficacy are the same across experiments. Equally, the results from the Gompertz modeling show that the treatment selected for experimental design 3 behaves similarly to the control especially at incubation temperature 22 °C, which means that ClO₂ was not effective at delaying the onset and growth of *Botrytis cinerea* on the strawberries.

One of the main differences in studies of effects of ClO₂ on human-pathogenic microbes on fruit compared to fungal decay of field-grown strawberries naturally infected with *Botrytis cinerea* may be that the former are usually surface contaminants or applied to the fruit surface by researchers, whereas *Botrytis cinerea* is a fungal plant pathogen that infects fruit in the field prior to harvest, such that the fungus may already be present within plant tissues by the time the ClO₂ treatment is applied. This reduces the capacity of ClO₂ to reach and interfere with fungal growth. Furthermore, ClO₂ treatment on the berries after shipping across the United States for least 5-7 days, even though under refrigerated conditions, may be too late to achieve strong and consistent efficacy of ClO₂ as *Botrytis cinerea* may continue to grow, albeit slowly, during the shipping process. In addition, regardless of our careful selection process, bruising of berries during shipping was inevitable, thus increasing the susceptibility of fruit tissue to fungal decay.

3.5 Conclusion. Absorption of ClO₂ by fresh produce as a function of concentration and exposure time and its relationship with strawberry quality and fungal growth were presented including several statistical approaches for evaluating the response surface methodology designs. For the first time, data conclusively indicate that the quantity

of ClO₂ absorbed, rather than the time and concentration profile at which the gas is administered, is highly related to fruit appearance post treatment. Moreover, experimental designs 1 and 2 showed a subset of ClO₂ gas treatment conditions that effectively delayed the onset of *Botrytis cinerea* versus control on small population strawberries. However, the practical shelf life extension was only about a half of a day in the best cases and high natural variability existed even when statistical improvements were observed. When the treatment that met both quality standards and efficacy against the mold was further explored on a larger sample size in the final experimental design, this study shows that ClO₂ gas was not an effective treatment versus control to extend the shelf life of strawberries due to infection and presence of *Botrytis cinerea*. Therefore, while ClO₂ may indeed be an effective antimicrobial at some conditions it is likely not practical for a meaningful extension of the shelf-life of strawberries as it does not delay the onset of *Botrytis cinerea* growth versus control samples which contain realistic field concentration of spores.

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AUTHOR CONTRIBUTION

Juliana Arango: Participated in the design of the study, collected the data, analyzed data, drafted the manuscript

Maria Rubino: Participated in the design of the study, analyzed the data and drafted the article

Rafael Auras: Participated in the design of the study, analyzed the data and drafted the article

Jerri Gillett: Collected the data and analyzed data

Annemiek Schilder, Participated in the design the study, analyzed the data, and reviewed the final copy of the article

Adam Grzesiak: Participated in the design of the study, analyzed data and drafted the manuscript

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Figure Legends

FIGURE 1. Schematic diagram of the three component system for continuous chlorine dioxide exposure and quantification of chlorine dioxide absorption by fresh produce (Adapted from Arango 2013 et al (3))

FIGURE 2: **a)** strawberries before exposure to ClO_2 and **b)** after exposure to treatment 5 mg ClO_2/L for 7 min where the calyx showed evident of color changes and desiccation and some parts of the strawberries tissue were discolored

FIGURE 3. Relation between percent of strawberries without any discoloration versus total chlorine dioxide absorption in mgClO_2/kg fruit

FIGURE 4. Response Surface plot for ClO_2 absorption by fresh produce in terms of concentration and time.

FIGURE 5. Repeated measures model over time for all treatments in experiment: **a)** 1 at 22 °C **b)** 2 at 22 °C and **c)** 3 at 4 and 22 °C

FIGURE 6. Gompertz model fit for treatments in experiment: **a)** 1 at 22 °C **b)** 2 at 22 °C and **c)** 3 at 4 and 22 °C

TABLE 1. Summary of treatments 1, 2, and 3

Experiment 1				
Concentration [mg/L]	Exposure Time [min]	Replicates	No. strawberries per treatment	
0.01	191	1	12	
0.01	1000	1	12	
0.04	21	1	12	
0.08	1000	1	12	
0.22	84	2	12	
0.63	7	1	12	
1.26	332	2	12	
5	37	1	12	
5	7	1	12	
Control		10	12	
Experiment 2				
Concentration [mg/L]	Exposure Time [min]	Replicates	No. strawberries per treatment	
0.04	21	4	18	
0.22	84	4	18	
0.41	17	4	18	
0.63	7	4	18	
5	7	4	18	
Control		20	360	
Experiment 3				
Concentration [mg/L]	Exposure Time [min]	Replicates	No. strawberries per treatment	Incubation temperature [°C]
0.63	7	16	9	22 ± 2
Control		16	9	22 ± 2
0.63	7	16	9	4 ± 2
Control		16	9	4 ± 2

459 Note: There is a set of control for each replicate of each treatment, therefore there are 4 replicates*5 treatments*18
 460 strawberries per treatment.

461 Note: Incubation temperature for experiments 1 and 2 was 22 ± 2°C. Temperature numbers are reported as mean ±
 462 standard deviation during the treatment

463

TABLE 2. Summary of total chlorine dioxide absorption by California strawberries after exposure to the different treatments considered in experiments 1, 2, and 3.

Concentration [mg/L]	Exposure time [min]	Total ClO ₂ Generated [mg] ^B	ClO ₂ absorption [mgClO ₂ /kg fruit]	Percent Strawberries without discoloration [%]
0.01	191	0.96	1.8E-03 ^A	100
0.01	1000	5.00	10.9 ^A	0
0.04	21	0.42	0.5 ^A	95
0.08	1000	40.00	73.7 ^A	0
0.22	84	9.24	10.2 ± 0.8	51.5
0.63	7	2.21	1.4 ± 0.2	95
1.26	332	209.16	234.8 ± 1.3	0
5	7	17.50	7.9 ± 0.3	35
0.41	17	3.49	4.5 ± 0.2	89

Note. Data in column 4 is given as mean ± standard error. ^A Treatments not replicated, therefore only means are presented. ^B Flow rate was 500 mL/min

TABLE 3. Repeated measures results for treatments in experiment 1 giving the mean percent of strawberries infected for any given ClO₂ condition over time.

Treatment	Rating Day			
	1	2	3	4
Control	9.78 ^A	34.77 ^A	58.45 ^A	78.79 ^A
0.63 mg/L for 7 minutes	1.33 ^A	13.83 ^A	35.17 ^B	67.33 ^A
5 mg/L for 7 minutes	1.00 ^A	4.83 ^B	19.92 ^B	43.42 ^B
0.04 mg/L for 21 minutes	5.83 ^A	24.25 ^A	51.00 ^A	65.83 ^A
5 mg/L for 37 minutes	2.83 ^A	12.50 ^A	35.25 ^B	55.08 ^B
0.22 mg/L for 84 minutes	2.17 ^A	10.39 ^B	29.83 ^B	49.50 ^B
0.01 mg/L for 191 minutes	0.92 ^A	19.25 ^A	43.33 ^A	68.92 ^A
1.26 mg/L for 332 minutes	2.92 ^A	11.58 ^B	27.42 ^B	54.08 ^B
0.08 mg/L for 1000 minutes	0.08 ^A	10.83 ^B	21.75 ^B	41.42 ^B
0.01 mg/L for 1000 minutes	3.08 ^A	24.33 ^A	48.50 ^A	78.00 ^A

Note. Data in columns are given as mean. ^{A,B} Within columns, means followed by the same letter are not significantly different ($\alpha=0.05$).

475 TABLE 4. Repeated measures results for treatments in experiment 2 1 giving the mean percent of strawberries infected for any given ClO2 condition over time..

Treatment	Rating Day						
	1	2	3	4	5	6	7
Control	-2.84 ^A	0.76 ^A	5.90 ^A	27.71 ^A	52.99 ^A	66.82 ^A	77.28 ^A
0.63 mg/L for 7 minutes	-2.84 ^A	0.46 ^A	2.36 ^A	15.78 ^B	29.85 ^B	52.08 ^B	64.00 ^B
0.41 mg/L for 17 minutes	-9.24 ^A	0.49 ^A	3.99 ^A	27.50 ^A	51.08 ^A	68.42 ^A	86.61 ^A
0.04 mg/L for 21 minutes	-4.26 ^A	0.15 ^A	6.28 ^A	29.93 ^A	49.89 ^A	66.49 ^A	81.39 ^A
0.22 mg/L for 84 minutes	-5.68 ^A	1.08 ^A	4.85 ^A	15.49 ^B	38.68 ^B	58.33 ^A	73.17 ^C
5 mg/L for 7 minutes	-1.38 ^A	0.49 ^A	1.74 ^A	11.14 ^B	38.49 ^B	60.13 ^A	73.28 ^C

476 Note. Data in columns are given as mean. ^{A,B} Within columns, means followed by the same letter are not significantly different ($\alpha=0.05$). Negative values for
477 column 1 are due to the repeated measurement statistical method of analysis.
478

479 TABLE 5. Gompertz model parameters for treatments in experiments 1, 2, and 3.

Experiment 1			
Treatment	Asymptote (α)	Growth Rate (β)	Inflection Point (θ)
Control	1.05 ± 0.15	$0.7 \pm 15\text{E-}04$	$2.2 \pm 25\text{E-}04$
0.63 mgClO ₂ /L for 7 minutes	1.73 ± 3.41	$0.5 \pm 68\text{E-}04$	3.9 ± 0.04
5 mgClO ₂ /L for 7 minutes	1.11 ± 2.89	$0.6 \pm 11\text{E-}03$	3.9 ± 0.04
0.04 mgClO ₂ /L for 21 minutes	0.81 ± 0.34	$0.9 \pm 70\text{E-}04$	$2.2 \pm 64\text{E-}04$
5 mgClO ₂ /L for 37 minutes	0.88 ± 0.90	$0.7 \pm 81\text{E-}04$	2.9 ± 0.02
0.22 mgClO ₂ /L for 84 minutes	0.81 ± 0.52	$0.7 \pm 49\text{E-}04$	3.0 ± 0.01
0.01 mgClO ₂ /L for 191 minutes	1.05 ± 0.87	$0.7 \pm 68\text{E-}04$	2.8 ± 0.01
1.26 mgClO ₂ /L for 332 minutes	1.39 ± 2.08	$0.5 \pm 52\text{E-}04$	3.9 ± 0.03
0.08 mgClO ₂ /L for 1000 minutes	1.02 ± 2.73	$0.5 \pm 96\text{E-}04$	3.8 ± 0.05
0.01 mgClO ₂ /L for 1000 minutes	1.35 ± 1.21	$0.6 \pm 54\text{E-}04$	3.0 ± 0.02
Experiment 2			
Treatment	Asymptote (α)	Growth Rate (β)	Inflection Point (θ)
Control	0.82 ± 0.06	$0.9 \pm 17\text{E-}04$	$4.1 \pm 14\text{E-}04$
0.04 mgClO ₂ /L for 21 minutes	0.93 ± 0.10	$0.7 \pm 14\text{E-}04$	$4.3 \pm 21\text{E-}04$
0.22 mgClO ₂ /L for 84 minutes	0.97 ± 0.17	$0.6 \pm 14\text{E-}04$	$4.9 \pm 36\text{E-}04$
0.41 mgClO ₂ /L for 21 minutes	1.02 ± 0.11	$0.7 \pm 14\text{E-}04$	$4.5 \pm 20\text{E-}04$
0.63 mgClO ₂ /L for 7 minutes	1.13 ± 0.34	$0.5 \pm 16\text{E-}04$	$5.5 \pm 69\text{E-}04$
5 mgClO ₂ /L for 7 minutes	0.83 ± 0.08	$0.9 \pm 19\text{E-}04$	$4.7 \pm 15\text{E-}04$
Experiment 3 - Temp 4 °C			
Treatment	Asymptote (α)	Growth Rate (β)	Inflection Point (θ)
Control	0.8 ± 0.04	0.4 ± 0.05	17.2 ± 0.20
0.63 mgClO ₂ /L for 7 minutes	0.9 ± 0.08	0.3 ± 0.05	18.3 ± 0.41
Experiment 3 - Temp 22 °C			
Treatment	Asymptote (α)	Growth Rate (β)	Inflection Point (θ)
Control	0.87 ± 0.03	0.6 ± 0.07	5.3 ± 0.14
0.63 mgClO ₂ /L for 7 minutes	0.9 ± 0.04	0.5 ± 0.08	5.6 ± 0.20

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