Quantification of the Toxicity of Aqueous Chlorine to Spores of *Penicillium digitatum* and *Geotrichum citri-aurantii*

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ABSTRACT

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Chlorine toxicity to *Penicillium digitatum* and *Geotrichum citri-aurantii*, causes of green mold and sour rot of citrus, respectively, was quantified. In 3% wt/vol NaHCO₃ containing 200 µg free chlorine per ml at pH 8.3, 95% of *P. digitatum* spores died (LT₉₅) by 180 s at 5°C, while only 32 s were required at 24°C. The LT₉₅ of *G. citri-aurantii* arthrospores was 108 and 31 s at 5 and 24°C, respectively. Mortality slowed 2- to 4-fold for each unit of increase from pH 7 to 10. The LT₉₅ of *P. digitatum* spores in 200 µg free chlorine per ml at 24°C at pH 7, 8, 9, and 10 was 13.2, 19.1, 29.4, and 88.4 s, respectively. The LT₉₅ of *G. citri-aurantii* at pH 7, 8, 9, and 10 was 3.0, 12.6, 56.6, and 114 s, respectively. Models were prepared describing mortality. Brief immersion in 200 µg free chlorine per ml reduced viable spores of *P. digitatum* and *G. citri-aurantii* from 10⁶ to 10³ spores per lemon, and naturally occurring yeast and molds from 10⁶ to 10⁴ CFU. In fruit wound-inoculated and immersed 24 h later in water, 4,000 µg free chlorine per ml, or 3% wt/vol NaHCO₃, green mold occurrence after storage was 98.5, 68.3 and 7.5%, respectively.

Additional keywords: *Geotrichum candidum*, green mold, sodium bicarbonate, sodium hypochlorite, sour rot

Chlorine is used commonly to sanitize fresh fruit and vegetables during processing after harvest. For citrus fruit, it is used in dump tanks, drenches, and high-pressure fruit washing systems. Recommendations state it should be used at 50 to 200 µg free chlorine per ml and the pH should ideally be controlled to approximately 7 and should not exceed 8 (3–5,9,21,23). Chlorine alone at these levels cannot prevent decay after wounds on fruit are inoculated; it kills spores in process water and on the surface of fruit, so that fruit with wounds made before processing are not inoculated when they contact process water (9). Chlorine at these relatively high rates is needed because the time fruit are in contact with the solution is brief, from 15 s to several minutes, and because fungal spores are comparatively resistant to chlorine and require relatively long periods to die compared to many other microbes (31). For example, Robbs et al. (24) reported cells of

Erwinia carotovora subsp. *carotovora* 50 times more sensitive to chlorine than conidia of *Geotrichum candidum*.

First used more than 75 years ago (2,10), sodium carbonate and sodium bicarbonate are in common commercial use to reduce losses by the postharvest citrus diseases green mold (16,28,29), blue mold (19), and sour rot (29), caused by *Penicillium digitatum* (Pers.:Fr.), *P. italicum* Wehmer, and *G. citri-aurantii* (Ferraris) R. Cif. & F. Cif. ("*G. candidum*" Link), respectively. These pathogens require wounds for infection to occur (9). The fruit must be immersed in tanks of the solutions for a minute or more (28) or washed with the solution applied at high pressure (30) to obtain the most effective control. Sodium carbonate and bicarbonate reduce the incidence of these diseases (reduction in occurrence of 50 to 95%), even when applied 24 h after inoculation of wounded fruit, they are equally efficacious, and sodium salts were superior to potassium or ammonium salts (29). A significant shortcoming of these solutions is that they are fungistats (14) and can become contaminated with spores of these pathogens and other microbes. Chlorine is often added at 200 µg free chlorine per ml to sodium bicarbonate solutions because it destroys inoculum and other microbes suspended in the solutions, and it slightly but significantly enhances the control of green mold (29).

Although the principles describing chlorine toxicity have long been known (31) and use recommendations developed, studies that quantified the sporocidal activity of chlorine against citrus pathogens are few (5,9,24) and none examined its toxicity when combined with bicarbonate salts. Two aspects of bicarbonate solution, high pH and ionic strength, can impact chlorine toxicity. As pH increases, the toxicity of chlorine decreases (27,31). The pH of sodium bicarbonate solution is high at rates (3% wt/vol) it is used on citrus fruit (29), about pH 8.3 when freshly prepared, and the pH increases when solutions open to the atmosphere lose carbon dioxide. The rate of change depends on the surface area of the tank, temperature, and amount of agitation; in some facilities, it can increase to over pH 9 in a day, in others, in about 4 or 5 days. The pH of sodium carbonate is very high, about 11.5 when freshly prepared, and the pH declines as the solution accumulates carbon dioxide from the atmosphere. Sodium carbonate is not typically chlorinated commercially because it is anticipated that its very high pH would render the chlorination ineffective. High ionic strength, a characteristic of these bicarbonate solutions, increases ionization of weak acids such as hypochlorous acid, indicated by a decrease in the dissociation constant (pK_a) of about one-half unit, and the reduction in hypochlorous acid content which presumably diminishes chlorine toxicity.

Our objectives were to determine the persistence of chlorine in bicarbonate salt solutions, quantify the impact of temperature and pH on the toxicity of chlorine to spores of *P. digitatum* and *G. citri-aurantii*, measure the influence of chlorine treatment on populations of pathogen spores and natural microbe populations on the surface of fruit, and to determine if chlorine at very high rates could control the decay of lemons or oranges after the inoculation of wounds with spores of *P. digitatum*.

MATERIAL AND METHODS

Culture of pathogens. *P. digitatum* (isolate M6R from J. W. Eckert, University of California, Riverside) was cultured 1 to 2 weeks on potato dextrose agar (PDA). Spores were harvested by adding 5 ml of water containing 0.05% Triton X-100 to the petri dish, rubbing the surface with a

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sterile glass rod, and passing the suspension through two layers of cheese cloth. The suspension was diluted with water to an absorbance of 0.1 at 425 nm determined with a spectrophotometer; this density contains about 10^6 spores per ml (8). *G. citri-aurantii* isolate 99-3 was similarly cultured and prepared, except the number of arthrospores was determined using a hemacytometer.

Free and total chlorine. The definition of free chlorine we use is the sum of available HOCl and OCl^- ; that of total chlorine is the sum of free HOCl and OCl^- plus reversibly combined chlorine that is liberated just before the analysis is conducted (1). To determine chlorine content, *N,N*-diethyl-*p*-phenylenediamine (DPD) was added in excess to the test solutions diluted to contain about 4 μg per ml chlorine and the development of red color measured at 520 nm with a colorimeter (Model DR 890 or Model Free and Total Colorimeter 46700-00, Hach, Inc. Loveland, CO). For free chlorine determination, in the absence of iodide ion, free chlorine reacted instantly with DPD to produce a red color, which was measured immediately (1). For total chlorine determination, an excess of iodide ion was added, which liberates chlorine from monochloramine, dichloramine, and other substances, and the absorbance was recorded after 2 min. Sodium bicarbonate was diluted sufficiently that it did not interfere with the accuracy of the analysis. The accuracy of the DPD analysis was checked by two methods: (i) by comparison of free chlorine content of a 20 $\mu\text{g}/\text{ml}$ chlorine standard by titration with sodium thiosulphate, and (ii) using a color standard that represented DPD analysis results with 0.2, 0.8, and 1.5 $\mu\text{g}/\text{ml}$ chlorine (SpecCheck secondary standards, Hach Co., Loveland, CO). Glassware used in contact with chlorine solutions was made "chlorine demand-free" by soaking it in a 5% (vol/vol) solution of laundry bleach (5.25% a.i.) in water for 1 h before use and rinsing it with deionized water (dH_2O).

Compatibility of chlorine with bicarbonate salt solutions. The chlorine content of sodium hypochlorite solution (Sigma Chemical Co., Chicago, IL) was determined using DPD colorimetric analysis. Chlorine solution was added to 25 ml of distilled water (dH_2O) or 0.5 M NaHCO_3 , KHCO_3 , or NH_4HCO_3 solutions to make a final concentration of 200 $\mu\text{g}/\text{ml}$ free chlorine. The pH of each solution was 8.3 and the temperature was 22 to 24°C. At intervals of 24 h for the next 240 h, the free hypochlorite content of each solution was determined by dilution in dH_2O of a single 0.4 ml aliquot of each followed by DPD analysis. The clear glass containers, which were exposed to ambient light in the laboratory, were opened briefly only to extract the aliquot and resealed. The experiment was conducted twice.

Impact of temperature and pH on the mortality of spores of *P. digitatum* and *G. citri-aurantii* in NaHCO_3 and chlorine. To determine the impact of temperature on the mortality of spores, a solution containing final concentrations of NaHCO_3 of 3% wt/vol and 200 μg free chlorine/ml was adjusted to pH 8.3 with 1 N HCl or KOH, sealed in an air-tight flask, and incubated at 5 and 24°C for 24 h. The spore mortality apparatus and all reagents used resided in a temperature-controlled room where the tests were conducted. To determine the impact of pH on the mortality of spores at 22 to 24°C, a solution containing NaHCO_3 (3% wt/vol) and 200 μg free chlorine/ml was adjusted to pH 7.0, 8.0, 8.3, 9.0, and 10.0 with 1 N HCl or KOH just before use in the procedure to quantify the mortality of spores in solutions. Preliminary tests showed that without chlorine the procedure did not reduce the germination of the spores. Each test combination of pH and temperature was conducted three times.

Mortality of spores in chlorinated solutions. *P. digitatum* and *G. citri-aurantii* were cultured as previously described and suspensions in water containing approximately 10^6 spores per ml were prepared. A 0.5 ml volume of the spore suspension was dispensed on the surface of a 25 mm diameter, 3- μm pore size polycarbonate filter clamped in a 25-ml volume glass filter support, which had been fitted into a 1-liter flask with a side arm attached to a vacuum pump. The vacuum was adjusted so the flow of solution through the filter was 1.5 ml per min. To expose the spores to the test solution, the vacuum was applied briefly to withdraw free water from the spores residing on the filter surface, a 5-ml volume of sterile dH_2O was added and withdrawn, and the chlorine solution was added and the exposure time initiated. To prevent chlorine reaction products from forming around the spores and modifying the toxicity of the solution, the solution flowed constantly at 1.5 ml per min during exposure of the spores. At the end of the exposure period, 1 ml of 1,000 $\mu\text{g}/\text{ml}$ calcium thiosulfate was dispensed to the surface of the filter to reduce the residual chlorine to chloride, followed by two 5-ml volumes of sterile dH_2O , each added when liquid residing on the filter surface had passed through the filter. Preliminary tests showed that calcium thiosulfate did not reduce the germinability of the spores. The filter was removed, added to 10 ml of sterile dH_2O , and agitated at high speed with a reciprocating vortex device. Serial dilutions were prepared and 0.2-ml volumes each were dispensed and spread on duplicate 100-mm-diameter petri dishes containing dichloran rose bengal chloramphenicol agar (DRBC, Difco, Chicago, IL). The dishes were incubated at 20°C for 5 days, and the number of colonies enumerated. The minimum number of viable spores that

could be detected, approximately 5 colonies per plate, was 250 per ml.

Quantification of the mortality of spores and natural microbe populations on the surface of lemon fruit. Four hundred freshly harvested 'Eureka' lemons were randomized, 100 were not cleaned and 300 were rigorously washed with fresh tap water over rotating brushes on a packing line and dried in air. The fruit without prior cleaning were treated with the test solutions, while spores of *P. digitatum* or *G. citri-aurantii* were applied to the cleaned fruit before treatment. *P. digitatum* and *G. citri-aurantii* were cultured as previously described, suspensions in water containing approximately 10^7 spores per ml were prepared, and approximately 1 ml of the spore suspension per fruit was applied with a low-pressure air-brush sprayer. The fruit were dried in air and the treatments were applied 24 h later. Fruit were dipped for 2 min at 25°C ($\pm 0.5^\circ\text{C}$) into one of the following; (i) buffered dH_2O alone, (ii) NaHCO_3 (3% wt/vol) alone, (iii) 50 μg free chlorine/ml alone, (iv) 200 μg free chlorine/ml alone, (v) NaHCO_3 (3% wt/vol) with 50 μg free chlorine/ml, and (vi) NaHCO_3 (3% wt/vol) with 200 μg free chlorine/ml. All solutions contained 25 mM KH_2PO_4 and were adjusted to pH 7.7 before use and the pH did not change during the test. The treatments were applied to two replicates of 10 fruit each. The treatment solutions, each 10 liter in volume, were contained in a stainless-steel, computer-controlled heated tank system with 12 22-liter-capacity tanks. The solutions were stirred constantly with a 3-cm-diameter propeller revolving at 500 rpm. After treatment, the fruit were immediately rinsed for 5 s with 250 ml of dH_2O in a low pressure spray and then placed at 1°C for no longer than 48 h before further analysis. To determine the populations of spores or natural microbes, each replicate was sealed inside 4-liter-capacity sterile plastic bags containing 500 ml of 0.1% peptone and 0.01% Triton X-100 (each wt/vol), and placed in a reciprocating shaker (displacement 100 cm, 100 cycles per minute). After 20 min, the samples were inverted and shaken for an additional 20 min. Serial dilutions were prepared and 0.2-ml-volumes each were dispensed to duplicate 100-mm-diameter petri dishes containing DRBC or plate count agar (PCA). DRBC dishes were incubated at 20°C for 5 days, and colonies of *P. digitatum*, *G. citri-aurantii*, or "total yeast and molds" (20) enumerated. PCA was incubated at 37°C for 2 days and colonies of aerobic mesophilic bacteria (20) enumerated. The experiment was conducted three times.

Green mold on inoculated lemons and oranges after immersion in chlorine or NaHCO_3 solutions. Standard methods recommended to evaluate citrus postharvest fungicides were used (8). Lemons and

oranges used in all experiments were selected by hand from field bins soon after harvest, washed with water, and randomized. A suspension of 10^6 spores per ml of *P. digitatum* was prepared as previously described, and each fruit was inoculated by briefly immersing a steel probe with a 1 mm wide by 2 mm long tip into the spore suspension and immediately making a single wound on the equator of each fruit. After inoculation, the fruit were incubated for 24 h at 16 to 20°C, and then four replicates of 25 fruit each were immersed in 22 liters of the treatment solutions for 2 min at 24°C ($\pm 0.5^\circ\text{C}$). The following treatments were applied: (i) inoculated, untreated control, (ii) NaHCO_3 (3% wt/vol, pH 8.3), (iii) 50, 1,000, 2,000, or 4,000 $\mu\text{g/ml}$ free chlorine at pH 7.5, and (iv) 50, 1,000, 2,000, or 4,000 $\mu\text{g/ml}$ free chlorine at pH 9.0. Each solution contained 20 mM Na_2HPO_4 and the pH was adjusted with 1.0 N HCl and 1.0 KOH just before use. NaOCl concentrations were measured by DPD colorimetry and pH was measured with an electrode and neither changed during the experiment. Immediately after treatment, the fruit were rinsed briefly in fresh water and stored 1 week at 20°C before the occurrence of green mold was recorded. The experiment was conducted

Fig. 1. The persistence of free chlorine in 0.5 mM bicarbonate salt solutions.

Fig. 2. Impact of temperature on the viability of spores of *Penicillium digitatum* and *Geotrichum citri-aurantii* in 200 μg free chlorine/ml in 3% (wt/vol) sodium bicarbonate at pH 8.3 at 5 and 24°C.

twice, once with oranges and once with lemons.

Statistical analysis. The influence of pH on the mortality of spores in 3% wt/vol NaHCO_3 and 200 μg free chlorine/ml was analyzed using Finney's probit analysis (26) to estimate the LT_{95} and LT_{99} values in seconds. The LT_{95} and LT_{99} values are the time in seconds when 95 or 99%, respectively, of the spores were killed.

LT_{95} values and 95% confidence intervals were used to compare the rates of mortality of the fungi among pH levels. Concentration and time products were calculated by multiplying the LT_{95} and the HOCl concentration in 200 g chlorine/ml solutions. HOCl concentration was calculated using the actual pH of the solution, pK_a of hypochlorous acid (7.5), and the Henderson-Hasselbach equation (31).

LT_{99} values were regressed against pH using the following model:

$$\text{LT}_{99} = ab^{\text{pH}}$$

The plots of the LT_{99} prediction curves included 95% confidence bands on the predictions. A model prediction of LT_{99} rather than LT_{95} , was selected because this decrease in spore germinability, a 2-log reduction, would be more useable in practical packinghouse applications.

In tests to quantify the reduction in natural populations of yeasts and molds or spores of *P. digitatum* or *G. citri-aurantii* or in tests to control green mold, a one- to three-way analysis of variance (ANOVA) was applied followed by Fisher's Protected LSD ($P = 0.05$) to separate means (Super-ANOVA, Abacus Concepts, Berkeley, CA). When the percentage of infected fruit was analyzed, ANOVA was applied to the square root of the arcsine of the proportion of infected fruit, and actual values are shown.

RESULTS

Compatibility of chlorine with bicarbonate salt solutions. Free chlorine persisted with little decline for 7 days in 0.5 M NaHCO_3 or KHCO_3 , but declined rapidly in 0.5 M NH_4HCO_3 (Fig. 1).

Impact of temperature on chlorine toxicity. Spores suspended in chlorine and NaHCO_3 died much faster at 24 than 5°C

(Fig. 2), and the impact of temperature was greater on *P. digitatum* than *G. citri-aurantii* (Table 1). The LT_{95} of spores of *P. digitatum* in 200 μg free chlorine/ml and 3% wt/vol NaHCO_3 , pH 8.3, at 24°C was 28.3 s, while at 5°C in this solution the LT_{95} was 180 s, a 6.4-fold increase in LT_{95} . Under the same conditions, the LT_{95} for arthrospores of *G. citri-aurantii* at 24°C was 31.0 s, while at 5°C it was 108 s, a 3.5-fold increase in LT_{95} .

Impact of pH on chlorine toxicity. The pH of the NaHCO_3 (3% wt/vol) and chlorine (200 $\mu\text{g/ml}$) solution greatly influenced its activity towards spores suspended in the solution (Fig. 3). Spores died more slowly when the pH of the solution increased from 7 to 10, and the impact of pH was greater on *G. citri-aurantii* than *P. digitatum* (Table 1). The concentration $\times$ time product associated with the LT_{95} at each solution pH, the product of the calculated HOCl concentration multiplied by the LT_{95} ($\mu\text{g/ml} \times \text{s}$), was not constant and declined as pH increased above 8 (Table 2).

Chlorine toxicity models. A model that estimated a 2- $\log_{10}$ (LT_{99}) mortality in seconds at 24°C from pH 7 to 10 for *P. digitatum* spores was: $\text{LT}_{99} = 0.212(1.88)^{\text{pH}}$ (Fig. 4). Solutions at pH 7.0, 8.0, 8.3, 9.0, and 10.0 for the LT_{99} of spores of *P. digitatum* were 18.3, 32.8, 64.3, and 120 s, respectively. A model that estimated a 2- $\log$ (LT_{99}) mortality in seconds at 24°C from pH 7 to 10 for *G. citri-aurantii* arthrospores was: $\text{LT}_{99} = 0.000004.98(6.18)^{\text{pH}}$ (Fig. 4). Solutions at pH 7.0, 8.0, 8.3, 9.0, and 10.0 for the LT_{99} of arthrospores of *G. citri-aurantii* were 3.4, 15.6, 63.3, and 402 s, respectively.

Impact of chlorine treatment on the viability of spores and natural microbe populations. Immersion of lemons for 1 min at 24°C in 200 μg free chlorine/ml, alone or with 3% wt/vol NaHCO_3 , both at pH 7.7, reduced the number of viable spores of *P. digitatum* and *G. citri-aurantii* from approximately 10^6 to 10^3 spores per fruit (Fig. 5). Natural yeast and mold populations were significantly ($P > 0.001$) more resistant to 50 and 200 μg chlorine/ml than *P. digitatum* or *G. citri-aurantii* and reduced from approximately 10^6 to 10^4

Table 1. The time in seconds when 95% of the spores (LT_{95}) of *Penicillium digitatum* and arthrospores of *Geotrichum citri-aurantii* died in 200 μg free chlorine per ml and NaHCO_3 (3% wt/vol) at 5 and 24°C^z

pH	Temp ($^\circ\text{C}$)	<i>P. digitatum</i>			<i>G. citri-aurantii</i>		
		95% CI			95% CI		
		LT_{95}	Lower	Upper	LT_{95}	Lower	Upper
7.0	24.0	13.2	11.7	16.8	3.0	2.5	4.1
8.0	24.0	19.1	16.3	25.1	12.6	10.1	19.1
9.0	24.0	29.4	24.3	42.7	56.6	51.9	68.8
10.0	24.0	88.4	77.8	108.3	114.0	100.0	154.0
8.3	24.0	28.3	24.7	33.9	31.0	25.0	42.8
8.3	5.0	180.2	149.1	250.5	108.4	91.0	136.9

^z The LT_{95} and 95% confidence intervals (CI) were determined by probit analysis.

CFU/fruit by this treatment. Free chlorine at 50 µg/ml was significantly (paired *t* test; *P* = 0.0495) less effective than at 200 µg/ml. Treatment with water or NaHCO₃ alone did not reduce spore or microbe populations, and the presence of NaHCO₃ had no significant influence on chlorine efficacy.

Green mold on inoculated lemons and oranges after immersion in chlorine or NaHCO₃ solutions. Green mold occurrence was reduced more than 90% on lemons and oranges by immersion in NaHCO₃ (3% wt/vol), while immersion in chlorine alone at concentrations as high as 4,000 µg/ml reduced green mold no more than 40% (Table 3). Chlorine efficacy was generally similar at 1,000 to 4,000 µg per ml and significantly superior to 50 µg per ml. Efficacy was similar at pH 7 and 9. The fruit were not visibly harmed by these treatments.

DISCUSSION

The toxicity of aqueous chlorine was substantially influenced by temperature and the magnitude of the increase in toxicity was similar to that reported for other microbes. A temperature increase from 5 to 24°C of the chlorinated bicarbonate solution increased the rate of mortality of *P. digitatum* and *G. citri-aurantii* by factors of 6.4 and 3.5, respectively. Phillips and Grendahl (22) reported that the percentage of spores of *Monilinia fructicola* that survived 5 min exposure in 3 µg/ml chlorine

at 0, 5, 10, 15, 20, and 25°C was 95, 90, 80, 60, 32, and 2, respectively. Kostenbauder (17) reported that a temperature coefficient, termed *Q*₁₀, described the increase in the rate of mortality of many microorganisms in many toxicants for each 10°C rise in temperature. It is analogous to the impact of temperature on the first order rate constant of simple chemical reactions. In reviews by Dychdala (7) and Hoff (13), the *Q*₁₀ for many microorganisms in chlorine was 2 to 3 until the rise was sufficient that thermal destruction began to occur. The *Q*₁₀ for *P. digitatum* and *G. citri-aurantii* we observed was 3.4 and 1.8, respectively.

The toxicity of aqueous chlorine was also substantially influenced by pH and the magnitude of effect on toxicity was similar to that reported for other microbes. In our work, a 1.5- to 4-fold decrease in chlorine potency occurred for every unit of pH increase from 7 to 10. Brown and Wardowski (5) reported that chlorine at 100 µg/ml at pH 7 killed *P. digitatum* and *G. citri-aurantii* spores within 10 s, while at pH 9, the chlorine concentration had to be doubled to kill all the spores within 10 s. In their work, they observed the germination of spores on agar media 24 h after treatment, while we counted colonies of these fungi that developed 5 days after treatment. In preliminary tests, we found observation of spore's germination on media overestimated chlorine toxicity, because the germination of many spores was delayed rather than stopped. This difference in the determination of spore germinability might explain the greater chlorine potency they observed than we did in this study. Robbs et al. (24) reported that a 2-log reduction in the viability of arthrospores of *G. candidum* in 2 min required 11, 14, and 25 µg free chlorine/ml at pH 6, 7, and 8, respectively. Segall (27) found that the rate of mortality of spores of *Alternaria tenuis* in 3 µg/ml chlorine was similar at pH 6 to 7.5 and only slightly reduced at pH 8. Rudolph and Levine (25) reported that for *Bacillus metiens* in 25 µg/ml chlorine, they observed an average decrease of toxicity of 3.4-fold for each unit of increase in pH between pH 7 and 13. Granum and Magnussen (11) evaluated chlorine toxicity to *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and spores of *B. subtilis* from pH 5 to 9 and concluded that hypochlorite

potency decreased by a factor of 2.5 for each unit of pH increase from 5 to 8, and by a factor of 6 from 8 to 9.

While the rate of spore mortality decreased as pH increased, it occurred much faster at high pH than would be expected if chlorine toxicity were entirely due to calculated hypochlorous acid content present in the solution. If the sole toxic component were hypochlorous acid, a 10-fold decrease in chlorine toxicity would be expected with each unit of pH increase above the dissociation constant (about pH 7.5) for chlorine. This interpretation overestimates the influence of pH on chlorine toxicity observed in our work and other empirical studies. Chlorine toxicity would be proportional to the product of exposure period and hypochlorous acid content. In our work, the product of the HOCl concentration and LT₉₅ (µg/ml * s) was not constant and declined as pH increased above pH 8 (Table 2). This result suggests that other factors such as the hypochlorite ion, are contributing to chlorine toxicity. Hadfield (12) stated that as hypochlorous acid is consumed during disinfection, protonation of the hypochlorite ion will occur and hypochlorous acid will be continuously produced, a phenomenon termed the "reservoir effect". Potency of the chlorine solution depends on the amount of HOCl absorbed by the microbe. At lower pH, a large portion of chlorine is in this form and mortality is more rapid, while at higher pH the HOCl is elaborated more slowly by its equilibrium with OCl⁻ ion and mortality occurs more slowly. Another interpretation is that hypochlorite ion itself has toxicity, from 20 to 300 times less than hypochlorous acid, depending on the microorganism (31). In our work, *P. digitatum* died much more rapidly in high pH chlorine solutions than *G. citri-aurantii*, so *P. digitatum* was more sensitive to hypochlorite ion. The contribution of hypochlorite ion to toxicity increases when contact times are long (12) or when the chlorine solution is cool (31).

In this work, we assumed the dissociation constant (*pK*_a) of chlorine was 7.5. However, the *pK*_a of chlorine is moderately influenced by temperature and ionic strength of the chlorine solution (31). The *pH* of the dissociation constant (*pK*_a) of chlorine shifts downward as temperature increases, which reduces the hypochlorous acid content of the solution. By the method of Morris (18), at 5°C the *pK*_a of hypochlo-

Fig. 3. Viability of spores of *Penicillium digitatum* A, and *Geotrichum citri-aurantii* B, in 200 µg free chlorine/ml and 3% (wt/vol) NaHCO₃ at pH 7, 8, 9, or 10.

Table 2. Calculated concentrations (µg/ml) of HOCl and OCl⁻ at pH 7, 8, 9, and 10 in NaHCO₃ (3% wt/vol) and 200 µg/ml free chlorine (HOCl + OCl⁻) at 24°C, LT₉₅ in seconds of spores of *Penicillium digitatum* and arthrospores of *Geotrichum citri-aurantii*, and the product of HOCl concentration and LT₉₅ (µg/ml * s)

pH	HOCl	OCl ⁻	<i>P. digitatum</i>		<i>G. citri-aurantii</i>	
			LT ₉₅	LT ₉₅ × HOCl	LT ₉₅	LT ₉₅ × HOCl
7.0	152.0	48.0	13.2	33.4	3.0	7.6
8.0	48.1	151.9	19.1	15.3	12.6	10.1
9.0	6.1	193.9	29.4	12.9	56.6	5.8
10.0	0.6	199.4	88.4	0.9	114.0	1.1

rite in water is 7.76, while at 24°C it is 7.55. Increasing ionic strength reduces the pK_a of chlorine, and sodium bicarbonate at 3% wt/vol would reduce the pK_a moderately. White (31) addressed both the impact of temperature and ionic strength on the pK_a of chlorine. At 25°C, the pK_a of chlo-

Fig. 4. Models predicting seconds to 99% mortality (LT_{99} , $\pm 95\%$ confidence interval) of spores of *Geotrichum citri-aurantii* and *Penicillium digitatum* in 200 μ g free chlorine/ml and 3% (wt/vol) $NaHCO_3$ at pH 7, 8, 9, or 10.

Fig. 5. Populations of natural yeasts and molds, or viable spores of *Penicillium digitatum* or *Geotrichum citri-aurantii* on lemon fruit after immersion 1 min in water, 50 or 200 μ g free chlorine/ml, $NaHCO_3$ (SBC), or 50 or 200 μ g free chlorine/ml with 3% wt/vol $NaHCO_3$. Columns within each class of organism with unlike letters differ significantly by Fisher's LSD ($P = 0.05$).

rine declined from 7.5 to 7.1 in an idealized 4% wt/vol salt solution, while at 5°C, the pK_a of chlorine declined from 7.8 to 7.3 in this solution. Therefore, at pH 8.3, in this salt solution, hypochlorous acid content at 25°C is 6.0%, while at 5°C it is 2.0%. Therefore, the increase in chlorine toxicity expected by temperature impact on reaction rate is moderated by the decrease in pK_a caused by temperature, which reduces hypochlorous acid content. A further complexity is the influence of temperature on the pK_a and subsequent ionic strength of the carbonate and bicarbonate ions. As temperature increases, the pK_a of the bicarbonate-carbonate equilibrium will decrease from 10.2 and carbonate, a bivalent anion that contributes twice the ionic strength of the monovalent bicarbonate anion, will increase. Carbonate ion concentration increases at higher pH, and this increase in ionic strength reduces the pK_a of chlorine, which reduces the proportion of hypochlorous acid. The influence of these factors on chlorine toxicity was probably too modest to be detected in our study. For example, the magnitude of the reductions in populations of spores and microbes on lemon fruit surfaces was similar whether sodium bicarbonate was present or not (Fig. 5).

In citrus packinglines, the residence time of fruit in chlorinated dump tanks or bicarbonate solutions should be of sufficient length to kill spores in the water and on the fruit. Prolonging the contact period also improves control of green mold by sodium bicarbonate (29). If spores that emerge at the end of the packingline tanks, belts, or brush beds are alive, where subsequent rinse water could rescue them from additional chlorine contact, they could contaminate subsequent processing steps and increase decay incidence. A residence time of 2 min in water or sodium bicarbonate containing 200 μ g free chlorine per ml is sufficient to kill 99% or more of the spores of *P. digitatum* and *G. citri-aurantii*, even if the pH of the solution is as high as 9. Surface populations of spores of these pathogens and natural yeasts and molds should also be reduced similarly.

We observed natural yeasts and molds on fruit surfaces were reduced about 2 logs by immersion in 50 or 200 μ g chlorine/ml, while spores of *P. digitatum* and *G. citri-aurantii* were reduced about 3 logs by this treatment. The reductions we observed when the fruit were immersed in the chlorine solutions were greater than reported in prior reports when these solutions were sprayed on fruit. We have repeatedly observed that the immersion of fruit into fungicides or sanitizers is superior to spraying the solutions over rotating brushes (28). Brown and Wardowski (5) reported that while a 15 s spray on oranges with up to 1,000 μ g chlorine/ml killed all of the spores of *P. digitatum*, it reduced those of *G. citri-aurantii* only by approximately 50%. Pao and Brown (20) reported commercial washing with either water alone or 200 μ g chlorine/ml sprayed over rotating brushes reduced yeast and mold counts similarly and only about 1 log.

The cycling period of chlorinated water or sodium bicarbonate through pressure washing systems, dump tanks, or drenches should be of sufficient duration that spores are killed during this cycle before they can contact fruit. For example, if a solution of 3% $NaHCO_3$ and 200 μ g free chlorine/ml at pH 9 was in use in a drencher and if each cycle of passage of the solution through the system was 15 s, only about 50% of the spores of *P. digitatum* would have been killed in this brief cycle and many viable spores could be applied to the fruit. If a retention time of 2 min was employed, perhaps by the addition of an accumulation tank to prolong the contact period of the spores to chlorine before the water is reapplied, 99% or more of the spores of *P. digitatum* and *G. citri-aurantii* would be killed, even if the solution pH was as high as 9.

In this work, only freshly prepared chlorine solutions in water or sodium bicarbonate were used, while solutions used in citrus packinghouses can become turbid and contaminated with soil and fruit constituents. The impact of these changes on the toxicity of chlorinated solutions in packinghouses deserves additional study be-

Table 3. Green mold between lemons and oranges wound-inoculated with spores of *Penicillium digitatum* 24 h before treatment by immersion in the solutions for 2 min followed by storage at 20°C for 1 week

Treatment	Green mold occurrence (%) ^y			
	Oranges		Lemons	
	pH 7.0	pH 9.0	pH 7.0	pH 9.0
Untreated control	100.0 a	95.0 a	100.0 a	100.0 a
Water alone	97.0 a	88.0 b	100.0 a	100.0 a
50 μ g/ml chlorine	98.0 a	92.0 ab	93.3 a	92.8 ab
1,000 μ g/ml chlorine	75.0 b	74.0 c	64.0 b	62.8 c
2,000 μ g/ml chlorine	82.0 b	59.0 d	66.3 b	76.8 b
4,000 μ g/ml chlorine	79.0 b	59.0 d	65.0 b	70.0 b
$NaHCO_3$ (3% wt vol) ^z	7.0 c	7.0 e	8.0 c	8.0 d

^y Values followed by unlike letters differ significantly ($P \leq 0.05$) according to Fisher's Protected LSD.

^z The pH of $NaHCO_3$ solution was 8.3.

cause it could be significant. The efficacy of chlorine used for water disinfection declines as turbidity increases (31). Free chlorine declines and is usually replenished automatically or manually as process water is used in citrus packinghouses. Free chlorine becomes bound or is reduced to chloride. Amines, from ammonia, proteins, and other nitrogen-containing organic substances, will react with chlorine to form the "nuisance disinfectants" of nitrogen trichloride and less toxic or nontoxic chloramines (7). Our result that free chlorine did not persist in an ammonium bicarbonate solution was probably a result of the formation of these compounds. Nitrogen trichloride is very toxic to fungi and this gas was once employed to control postharvest decay of citrus fruit (15). Among the nuisance disinfectants, only nitrogen trichloride gas causes irritation to workers' eyes (31), a common complaint among packinghouse personnel near chlorine solutions. To determine if organic nitrogen significantly interferes with chlorination efficiency as the solutions age, the amount of nitrogen available to react with chlorine could be determined (31). If the ammonia to chlorine ratio exceeds 1:5 and chlorine continues to accumulate, the chloramines are oxidized and destroyed by additional chlorine, and free chlorine can now increase readily (7). Other constituents released from the fruit, such as starches, lipids, alcohols, and sugars, should have little effect on the germicidal activity of chlorine. If the ionic strength of the solutions increases as they age, this would impact the pK_a of chlorine as previously discussed and decrease chlorine toxicity (7). However, simple observations in packinghouses that we have done, suggest that chlorination under commercial conditions is at least approximately as effective as we describe it here. For example, we found that viable spores of *P. digitatum* were present in packinghouse tanks containing 200 µg free chlorine per ml and 3% NaHCO_3 while large numbers of fruit were passing through the tanks, but none could be found several minutes after they had passed out of the tank.

Immersion of inoculated fruit in chlorine solutions alone, even at concentrations as high as 4,000 µg free chlorine per ml, did not cause a useful reduction in the subsequent green mold and was inferior to immersion in NaHCO_3 for this purpose. D'Aquino et al. (6) similarly reported that immersion of minneola tangelos in 500 µg free chlorine per ml for 2 min did not reduce subsequent green mold, significantly increased ethylene production and respiration rate, and that the treated fruit aged more rapidly. We found that adjusting the chlorine solution pH had no impact on its

ability to control green mold. Granum and Magnussen (11) stated that since chlorine stability increases at higher pH, optimal disinfection of the surfaces of fresh products might occur at higher pH than is optimal for chlorine toxicity. We did not see evidence of improved penetration at higher pH; chlorine was presumably too reactive to penetrate into the wounds where the spores resided. Although incapable of controlling inoculum in wounds, immersion of fruit in high concentrations of chlorine dramatically reduced surface populations of *P. digitatum* and *G. citri-aurantii*, and substantially reduced natural yeast and mold populations.

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