SODIUM HYPOCHLORITE AND ALTERNATIVE SANITIZERS FOR SANITATION OF SIMULATED PACKINGHOUSE WATER SYSTEMS

By

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This document is dedicated to the memory of my grandfather, Albert George Fiśa.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

SODIUM HYPOCHLORITE AND ALTERNATIVE SANITIZERS FOR SANITATION OF SIMULATED PACKINGHOUSE WATER SYSTEMS

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Tomatoes are an important economic product of the United States, with Florida production accounting for 47% of the total fresh market winter crop. Microbial contamination can lead to decays or consumer health hazards. Combating either type of contamination in water handling systems currently relies on chlorination; experience has shown that potential hazards may not be controlled in this system. This research involves determining the capability of plant pathogens to survive biocidal treatments and screening of some potential alternative treatments to chlorination.

Determining a biocide's usefulness should be based on product availability, ease of use, known biocidal activity and safety. Five biocides were selected for testing here: chlorine (from sodium hypochlorite), acidified sodium chlorite (ASC); peroxyacetic acid (PAA); and chlorine dioxide (ClO_2), as an aqueous solution ($ClO_{2(aq)}$) or a vapor ($ClO_{2(g)}$). The biocides were tested for efficacy against three common postharvest pathogens of tomatoes: the soft rot bacterium, *Erwinia carotovora* subsp. *carotovora*; the

watery soft rot/nest inducing fungus, *Rhizopus stolonifer*; and the sour rotting yeast, *Geotrichum candidum* pv. *candidum*. Biocides were tested against each pathogen in vitro and in fruit, as either protective or curative agents.

ASC was shown to be more effective than chlorine in curing fruit of bacterial or yeast infections, while both were equivalent against fungal infections. ASC was slightly better than chlorine in protecting fruit from cross contamination. Concerns about the application and cost of ASC exist. As a protective agent, PAA had efficacy equal to chlorination against all organisms, but neither were sufficient curative agents. Aqueous ClO_2 showed some efficacy against bacteria and yeast, but its instability made it less desirable. Gaseous ClO_2 showed great efficacy against bacterial and yeast inoculations and some efficacy against fungal infections. Unlike the other biocides, the method of application of gaseous ClO_2 involves dry delivery after, or prior to, packinghouse water handling procedures.

Based on the results of this study, maintaining current recommendations of chlorination in tomato packinghouse flume systems is effective and economic for minimizing cross-contamination. However, as chlorination will not cure infested fruit, a ClO_2 vapor treatment may be an effective pre-dump treatment for reducing the initial pathogen load.

CHAPTER 1
INTRODUCTION

Tomato Production

The majority of tomatoes (*Lycopersicon esculentum* Mill.) grown for fresh market in Florida are harvested when mature green. Tomato fruit are hand harvested into containers, which are emptied into field bins on a flat-bed truck or a larger, modified truck trailer called a gondola. The fruit are then hauled to a packinghouse, potentially hundreds of miles away. At the packinghouse, fruit are transferred to a packing line, where they are washed, sorted, graded and packed into shipping containers, usually corrugated fiberboard boxes containing 11.34 kg (25 lbs.) of fruit each. During sorting, fruit that show any pink or red color are culled or, along with breakers or light pinks, may be transferred to a separate line and packed at a different grade. Green fruit are preferred for shipping because they are better capable of withstanding shear and compression forces of packing and transport. In contrast, pink or red fruit have begun to soften and are more susceptible to bruises or other surface injuries. Mature green tomatoes are treated with ethylene gas immediately after packing to assure that upon arrival at their destination, or shortly thereafter, fruit are uniformly red ripe for consumer use. This ethylene treatment may last for 7 days or more, but is recommended to be no longer than 3 to 5 days.

Although green tomato fruit are relatively resilient to bruising, rough handling during transfers such as from picking container to field bin or field bins to packing lines, can lead to bruises, cracks, abrasions, cuts or punctures. Such injuries may cause fruit to

be culled or rejected at the market. Due primarily to the weight involved, fruit are particularly prone to physical injury when transferred from field bins to the conveyor belt of the packinghouse. A water dump tank is used to cushion fruit during this transfer. Since green tomatoes float in water, unloading bulk loads from the field into a dump tank allows the fruit to spread out into a single layer, which can be flumed to, and picked up by, the belts of the packing line. Unfortunately, large volumes of water are required for these systems, which tend to require a closed circulation whereby the same water is used for multiple fruit unloads. The water becomes increasingly contaminated with microorganisms and other debris, which produces a cross-contamination hazard.

Currently, the only treatments in use by the tomato industry to combat contamination hazards are the chlorination of water systems, the use of prompt cooling, the culling of defective fruit and the minimization of mechanical injury. Few of the microbial problems associated with fresh market tomatoes, including those that lead to human disease, can be corrected after fruit have been contaminated. Therefore, preventing contamination is a key strategy. In this study, water chlorination and alternatives will be studied for their use in the production of wholesome fruit and in minimizing losses.

Justification

Profitability for fresh market tomato operations, as with all businesses, is based on income exceeding expense. Postharvest shrinkage, meaning a loss of product after harvest, represents the most expensive type of loss since all production and harvest inputs have been made. Decay is a major postharvest shrinkage factor. Sanitation programs directly impact on postharvest decays, ideally reducing shrinkage and improving profits. Poor sanitation, however, often leads to decay development.

Food safety is a growing concern for fresh market commodities, including tomatoes. Two outbreaks of salmonellosis that occurred in the early 1990's were traced back to round tomatoes and three outbreaks in 1998-9, 2002 and 2004 were traced back to Roma tomatoes. Each of these outbreaks had uncertain origins, but the list of suspected sources for each included the possibility of coming from Florida fields. Such outbreaks have made the use of sanitary measures increasingly important for growers.

Objectives

The objectives of the research reported herein were designed to provide Florida growers and packers information to assist in the development of effective packing line sanitation procedures, as are directly relevant to water handling systems. Control of pathogen infestation was specifically modeled through the use of three common postharvest decay pathogens. Bacterial decay pathogens used in these tests were considered as model systems for similar control of some bacterial human pathogens that also may be present as contaminants.

Objective 1

The first objective was to assess the efficacy of potential alternative sanitizers. Water chlorination as currently practiced was compared with three possible alternative water treatments: peroxyacetic acid; aqueous chlorine dioxide; and acidified sodium chlorite. Considerations included the efficacy for controlling three test microbes, ease of use and hazards to workers. Use of each biocide as either a curative agent and as a protective agent was also explored.

Objective 2

The second objective was to explore fumigation with vaporous ClO_2 as a replacement for or in addition to flume and dump tank biocides. Methods of delivery

were investigated and efficacy rates monitored. The value of multiple treatments in succession, as compared with a single treatment, for further reduction in microbial hazards in fresh market tomatoes was also investigated.

CHAPTER 2
LITERATURE REVIEW

Terms Used and Definitions

For comparing the efficacy of biocides against particular organisms, a useful value to use is the concentration of biocide applied, C, multiplied by the time of the treatment, T, or the "C x T" value. Comparing these values shows that as the concentration or the time of application of a biocide is increased, the other can be decreased and still have the same value.

Reference to the chemical "chlorine" refers to aqueous solution including the free and total chlorine in solution, including all free and un-reacted species of chlorine that are present. This includes HOCl, OCl^-, NaHOCl, Cl^- and any others that may be present.

"Room temperature" refers to the ambient temperature of the laboratory, which ranged from 19°C to 24°C and averaged 22°C.

"Multiple hurdles" refers to the tactic of introducing multiple applications of one or more interventions to a single product. The application of several products is designed to create several 'hurdles' that a pathogen must surmount in order to survive to cause decay. Each application of a biocide or other treatment is considered a 'hurdle' to pathogen survival and parasitism of a product.

The oxidation-reduction potential (ORP) of a chemical is a measure if its ability to oxidize or reduce another compound. It is determined by sampling the electrical potential of a solution and is a quick and simple way of referencing a biocide's relative capacity to react with microbes and thus, presumably, its capacity to kill them.

Tomato Production

The United States of America produces approximately 10% of the world's fresh market tomatoes (*Lycopersicon esculentum* Mill.), totaling on average 11.3 million metric tons per year over the 10 year period from 1993 to 2003, which was second in production only to China (81). The two largest production areas are California and Florida, with Florida being the sole source of domestically produced tomatoes in the winter months (128). Florida produced 47% of the total U.S. fresh-market tomatoes from 1992 to 2002. Florida production trended upward until the early 90's and then either remained the same or decreased. Imports, possibly as influenced by the North American Free Trade Agreement (130), reduced Florida's share of the market (Figure 2-1) (146). Over 40,000 acres of tomatoes are grown annually in Florida, which represents nearly 20% of the state's total vegetable acreage.

Fresh tomatoes are a popular addition to many food dishes in the United States, second only to potatoes in both farm value and volume consumed (82). Americans consumed 5.72 kg of fresh tomatoes per capita in 1960, increasing to 8.07 kg per person in 2000 (Table 2-1) (82). Whole cherry or round tomatoes accompany many salads, sliced round tomatoes top sandwiches or are consumed directly.

The U.S. demand for fresh-market tomatoes usually exceeds its production capacity, particularly during winter months. Imports, primarily from Mexico, make up most of the difference between domestic production and demand (80). Often, foreign production exceeds demand, leading to depressed prices. Maintaining or growing U.S. tomato production for domestic markets or export requires efficient and reliable field, packing and shipping practices, which support a wholesome, sanitary and high-quality product. From the USDA/ERS web site (80):

The U.S., a net importer of fresh tomatoes, had a tomato trade deficit in 1999 of $567 million. Fresh tomato imports mostly arrive from Mexico (about 83 percent of imports in 1999), with an increasing volume of greenhouse and hydroponic products from Canada (9 percent of imports), the Netherlands (3 percent), and others. Fresh tomato exports primarily go to Canada (91 percent of exports in 1999) with exports to Mexico (4 percent) a distant second. An expanding volume (now about 1 percent) is also exported to Japan—a market previously closed by phytosanitary restrictions to U.S. shippers until 1997.

Tomato Morphology

A tomato fruit is covered in a waxy cuticle that is nearly impervious to gas and water exchange. Any gas or liquid movement from within a fruit to the outside environment, or from the environment internal to a fruit, requires a break in this cuticle to take place. All tomato fruit have two natural openings: the stem scar and the blossom scar. These openings are necessary for a fruit as gas exchange is required for cellular respiration and to avoid internal tissues from becoming anaerobic. In order for gas to exchange with the surface, internal channels must exist. These channels consist of intercellular space.

Another means by which a tomato's internal contents and the external environment can be exchanged is through damage to the fruit cuticle. At wound sites, a tomato fruit is temporarily susceptible to liquid and pathogen infiltration. Upon internalization, microorganisms may begin to parasitize fruit tissues. Even if treated with a sanitizer 7 s after contact with a fresh wounded surface, microorganisms, as modeled in a dye/chlorination system (15), would be protected from inactivation by the sanitizer.

Physical and Biological Injury

Efficiency in production requires that shrinkage be minimized. Postharvest losses are particularly expensive in that they represent a loss of all input values, from field inputs such as fertilizers and labor, to production, harvest, packing, storage and

transportation inputs. Losses to microbial disease or from physical injury after harvest has been estimated to be around 6.7% of bulk pack tomatoes, in a survey from the Greater New York and Chicago areas in the early 1980's (30). If fresh market production loses in are similar in Florida, about 355,000 metric tons of fresh market tomatoes are lost based on total farm value in 2002 (146); this amounts to about 34 million dollars annually.

Postharvest shrinkage is largely due to abiotic or biotic damage (3). Abiotic damage consists of physical or metabolic injuries, such as from physical impacts, temperature extremes, pH fluctuations of water or soil, weather events, availability of free water (from excess to insufficient), or other environmental extremes that may occur anywhere along the production line from field to shipping. Biotic, or infectious, damage arises from primary or secondary effects of other living organisms on the commodity, such as disease, waste, feeding or parasitism and/or physiological responses.

Abiotic Losses

Many agents of abiotic damage occur in the field, including: too high or too low temperatures; lack or excess of soil moisture or light; lack of oxygen in the root zone due to flooding; air pollution; nutrient deficiencies; mineral toxicities; soil pH extremes; pesticide toxicity and improper cultural practices (3). After harvest, mechanical injuries or environmental stresses are the primary causes of abiotic losses. Directly or indirectly, abiotic injuries lead to grade reductions, value losses and increased postharvest decays (4).

Mechanical injuries

Injuries from mechanical sources may be caused by impacts or by compression (92). Impact injuries result from a single collision or a series of collisions that produce

cellular damage at the point of impact. In contrast, compression injuries result from a static pressure that changes the shape of a fruit irreversibly. Both impacts and compression can lead to tissue bruising. Internal bruising of tomato fruit may initially appear as water soaking due to cell rupture, especially in the locule tissue surrounding the seeds. Internal bruising can be either transient or persistent, depending on the severity. Such damage may not be apparent when fruit are packed, but as these fruit ripen, portions of the damaged locule may dry or breakdown. External bruising, conversely, is visible, but does not occur as readily (111;112). Sargent et al. (112) reported that 5 to 45% of the interior of green tomato fruit was bruised as a result of a 20 cm drop onto a firm, flat surface. Increased internal bruising resulted from increasing the drop height to 30 cm, whereas external bruising was not observed. Bruising injury increases with drop height, number of impacts and fruit ripeness, and varies with area of fruit involved. Impacts over locules lead to greater damage compared to those that occur over a septum. Internal bruising does not generally promote decay.

Sharp impacts or impacts on sharp surfaces may produce breaks in the fruit's cuticle and epidermis. Large splits tend to occur through collisions with edges of equipment or forceful drops. Such fruit are culled out during grading and are considered an immediate loss. Small breaks or punctures in the fruit surface arising from fruit impacts against sand grains, rough wood surfaces, or similar damage may not be detected on the packing line. These small wounds may allow infection leading to subsequent decay, or can harbor other hazardous microorganisms (92;137).

Temperature extremes

Prolonged exposure to temperature extremes predispose fruit to bruising and early decay if too cool, or cell wall breakdown if too warm (92). Mature green and breaker

tomatoes exposed to temperatures less than 13^{o}C for prolonged periods experience changes in lipid metabolism and membrane lipid catabolism after re-warming to 20^{o}C (107;142). As the length of time at chilling temperatures increases, the type and extent of damage also increases. Tomato chilling injury symptoms include incomplete ripening, uneven coloration, water soaking, surface pitting, or shriveling (92). Often, chilling injury is first apparent after fruit have re-warmed. Fruit cultivar, ripeness and chilling severity factor in to the extent of damage. Chill damaged tomato fruit are less flavorful (88), sometimes visually unappealing, and more susceptible to bruising or other physical damage (73). Microorganisms, ranging from plant pathogens to certain human pathogens, such as *Geotrichum candidum* or *Salmonella*, respectively, are more likely to grow in chilling injured tissues (132). H of exposure to low temperature are cumulatively damaging (92;107). Fruit exposed to low temperatures in the field are more sensitive to chilling temperatures in storage (92).

Moisture extremes

Variation in the moisture available to tomato plants in the field can lead to surface injuries in the fruit, particularly as fruit approach maturity. A rapid increase in soil moisture can lead to concentric or radial fruit cracking. Concentric type growth cracks form rings that sequentially emanate from (usually) stem scars and progress perpendicular to the axis of the fruit. Radial growth cracks also generally originate from a stem scar, but instead are oriented parallel to the axis of the fruit. All growth cracks begin at the cuticle, but may or may not progress deeper into fruit tissue (148). While unsightly and a cause for fruit to be culled, growth cracks are more important as potential infection courts for invading decay organisms, particularly if such fruit are not culled. Weather conditions that favor cracking are generally unavoidable. Crack resistant cultivars are

available, but the resistance is not absolute. Surface cracks may also occur as a result of the absorption of water by fruit during water handling procedures (20).

Biotic Losses

Often called ‘decays,’ infectious diseases that develop and progress in harvested fruit are caused by pathogenic bacteria, fungi and/or yeast (other organisms that cause biotic disease include: protozoa; nematodes; viruses and parasitic higher plants, these are not generally considered problematic postharvest) (3). Most of these pathogens are considered wound invaders, as they cannot directly penetrate the cuticle of a fruit. Additionally, most of the postharvest pathogens of tomatoes are relatively non-specialized; they are widely dispersed in the environment and have wide host ranges. In Florida, some of the most significant postharvest decay pathogens include *Erwinia carotovora* subsp. *carotovora* (*E.c.c.*) (71), *Rhizopus stolonifer* (*R.s.*), *Geotrichum candidum* pathovar. *candidum* (*G.c.c.*) and the black molds.

Erwinia carotovora (L. R. Jones) (26) subsp. *carotovora* is an ubiquitous bacterial pathogen of many plants and a major cause of postharvest disease in many commodities. It is the primary postharvest pathogen of tomatoes gown in Florida (102) where control of this pathogen is of primary concern. This and related bacteria are ‘soft-rotters’ because they produce pectolytic enzymes that destroy the structure of host tissues, leading to a liquid mass. The soft rot erwinias have been isolated from many different environments and host tissues, including most fresh fruits and vegetables, the soil in growing fields, fresh water lakes throughout the United States, raindrops and even mist from ocean spray along the California coastline (60;89).

Geotrichum candidum (32) is a yeast and the cause of sour rot (also known as watery rot) of fruits and vegetables, including a tomato strain, *G. candidum* pathovar.

candidum (*G.c.c.*) (28). Typical sour rot lesions often include fungal signs that resemble 'cottage cheese' and are characterized by a sour, citrus-like aroma. *G.c.c.* is found world-wide in varied habitats (32) and is a facultative plant parasite under specific conditions (28;29). Arthrospores of *G.c.c.* are generally dispersed by rain splash or mechanical contact, but appear to be too heavy for direct wind dispersal. *G.c.c.* growth is affected by fruit ripeness. Both tomato and (to a lesser extent) citrus pathogenic species of *G. candidum* are capable of causing decay in red ripe tomatoes, whereas green tomato fruit are susceptible only if fruit are predisposed to infection, such as by chilling injury (29).

'Lactic acid bacteria' are a collective group of Gram-positive bacteria including certain *Lactobacillus* spp. and *Leuconostoc* spp., etc., which are causal agents of a decay visually similar to early sour rot lesions that is often accompanied by a 'pickled' (i.e. vinegar) aroma (16;35). Decay caused by the lactic acid bacteria is visually water soaked, pale and firm. Similar to *G.c.c.*, tissue exudates in lesions caused by lactic acid bacteria tend to be acidic, pH 4.0-4.9, whereas decays caused by pectolytic bacteria tend to be in the pH range of 5.6-8.4 (6). Apoplastic fluid from healthy pink to red tomato fruit are approximately pH 4.4-4.8. The same fluid in green fruit may have a pH as high as pH 6.7. By contrast, when tissues lose their structure and their vacuolar membranes break, addition of symplastic liquid to the internal fluid pH may fall to pH 4.1 to pH 4.5 (120). Lactic acid decays have been linked to possible synergistic effects with sour rot (Bartz and Concelmo, *unpublished*).

Rhizopus stolonifer (*R.s.*) is an aggressive fungal pathogen that spreads as a creeping mycelial mat, or through the air as copious numbers of lightweight, hyaline spores (11). Hyphae of *R.s.* grow quickly and may cause 'nesting' in boxes of fruits or

vegetables. Young lesions feature water soaking, but the mycelium of *R.s.* provides structure to such lesions and the tissue liquefaction characteristic of bacterial soft rot does not occur. Free liquid is usually released from the decaying tissues as lesions enlarge. At this stage, spores may be produced and enable aerial dispersal. *R.s.* spores are very tolerant to both adverse environments and many biocides.

Black mold rot, caused by any one of the black mold pathogens (*Alternaria alternata, Stemphyllium botryosom, S. consortiale* and/or *Pleospora lycopersici*), appears capable of infecting fruit in the field, then becoming quiescent until the fruit ripens or is stored (9). Growth and sporulation resume after harvest, as fruit ripen or become weakened by chilling, cracking, senescence, or any other types of tissue breakdown. Black mold spores may infest the corky ring tissue, breaks in the fruit's cuticle, or the stigma or flower prior to pollination (135). Lesions do not generally develop unless fruit are stored for excessive periods of time and/or are chill damaged (90). Fruit in contact with the ground while maturing may develop Alternaria rot quicker than other fruit.

The decays that affect tomato fruit tend to be affected by the climate in which the fruit are grown. While *Erwinia* losses are reported throughout the U.S., they are less of a problem in drier production areas, such as California, where gray mold rot, caused by *Botrytis cinerea*, is the most important postharvest disease. Comparatively, tomatoes grown in the eastern United States have higher levels of Erwinia rot and black mold rot than do those produced in the western states (126). Several other decay organisms exist, but are usually less problematic in Florida produced fruit.

Combined Effects

Biotic agents and abiotic injuries may act additively or synergistically to cause postharvest losses in fruit (92). For example, breaks in a fruit's cuticle may lead to biotic

loss by creating an infection court for decay pathogens, potentially producing survival sites for other hazardous microbes, including certain human pathogens (117). Also, chilling damage can predispose fruit to increased susceptibility to many diseases, including Rhizopus rot, Alternaria rot, bacterial soft rot (*E.c.c.*), sour rot (*G.c.c.*), or several others (12;124;142). Some diseases may not even occur unless a fruit is first chill damaged, such as is the case with green tomatoes and *G.c.c* (29). Although chilling injury tends to be less severe as fruit ripen (142), if tomato fruit of any ripeness stage are exposed to continuous temperatures of 7^{o}C (45^{o}F) or less for 5 days prior to inoculation, either in the field, during storage, or as a combination of both, they will show a greatly increased susceptibility to soft rot (12). Similarly, if green fruit are exposed to near freezing temperatures for 7 to 10 days, they become highly susceptible to sour rot (28).

Both physical injury and decays are much more common in red fruit than in green. Green fruit are firmer and better able to withstand impacts and compression associated with harvesting and handling (110-112). Harvesting pink or red fruit increases the risk of physical damage as well as the incidence of bacterial soft rot (14) or sour rot, during or after shipping.

Unwholesomeness

Tomato fruit may also carry human pathogens (22;27;56;146;150). Contamination may occur through contact with water, fecal matter, domestic or wild animals, or employees that are ill or have recently been ill and thus shedding propagules (52). Bacteria, viruses or parasites responsible for human disease usually do not affect the fruit or its quality and, as a result, are considered harmless commensals as far as the fruit is concerned. This type of contamination represents an invisible hazard that can, however, produce major impacts on fresh tomato markets (95).

Five outbreaks of food poisoning have been traced back to raw tomatoes in the U.S. since 1990. Three different serovars of *Salmonella enteritidis*: Javiana; Montevideo; and Baildon, were implicated in these events (there may have been more), which occurred in 1990, 1993, 1998-99, 2002 and 2004 (37;61;127). The first two incidences were traced back to a single packinghouse in South Carolina where insufficient sanitation was cited as the most likely cause (61). The 1998-99 outbreak was traced back to either a tomato processing plant in California, or two possible packing facilities in Florida; the exact source could not be identified (37). The 2002 case involved consumption of diced Roma tomatoes at the "2002 Transplant Games" in Orlando, Florida (127). The source of contamination has not been specifically identified (123). Roma tomatoes were again implicated in a 2004 outbreak of salmonellosis in six states, involving at least 470 individuals and as many as five or more serovars of the bacteria; anecdotal evidence suggests the source was again undetermined, but Florida, Georgia and South Carolina were suspect (104). In each of these cases, more effective sanitation practices would have likely averted contamination and the subsequent outbreaks. It is impossible to determine how many potential outbreaks are averted annually as a result of adequate handling and sanitation.

Mammalian and plant pathogens historically have been considered to survive in a delicate balance with their hosts and to subsequently not survive well under alternate conditions (122). This directly suggested that human pathogens should not survive well in vegetable material and that human and plant pathogens should not be of concern for cross infection. While cross contamination of organisms capable of crossing major taxonomic (Kingdom) lines to cause disease is still not considered an issue, recent studies

have found certain animal pathogens, while not causing disease in plants, could survive, possibly even multiply, in or on various plant products long enough to be of concern for individuals that consume those products (46;140). Guo et al. (55) reported that tomato plants grown in a greenhouse and inoculated with *Salmonella* either in the flowers or injected directly into vascular tissues of their stems produced fruit that were infested with the bacterium up to 25% of the time.

Several human pathogens have been isolated from, or should be considered as potential contaminants of, fresh market fruits and vegetables, including *Salmonella*, *Escherichia coli*, *Shigella* spp. and *Listeria monocytogenes* (63;125;140). Viruses have also been known to be transmitted from person to person through raw fruits or vegetables as vehicles, although no reports specific to tomato have been identified as of this writing.

Salmonella

Salmonella enterica subsp. *enterica* is one of the most commonly reported food-borne bacterial pathogens causing gastroenteritis (52). It has multiple serovars (68), each commonly named by the geographic location in which it was first isolated. Similar to bacterial soft rot of tomatoes, rainfall events are linked to population peaks of coliforms, like *Salmonella,* in the environment (78). *Salmonella* survives under varied conditions and, while it may not replicate at optimal rates in stored tomatoes, it has been shown to survive on diced Roma cultivar tomatoes stored at 4^{o}C for up to 5 days (138). A number of salmonellosis incidents have been attributed to vegetables that were properly refrigerated, including two that were traced back to raw tomatoes (62;147). *Salmonella* cells have also shown a wide range of pH tolerance. Proteins called acid-shock proteins are synthesized by the bacterial cells during exposure to acidic conditions and allow for

survival during subsequent exposure to very acidic environments, as low as pH 3.3 (50). These same proteins may also contribute to survival under other extreme conditions.

Shigella spp.

The genus *Shigella* has several species that are a common cause of gastrointestinal hemorrhaging through the production of shiga-toxins (49). Similar toxins are produced by enterohemorrhagic *E. coli* strains, such as *E. coli* O157:H7 (91), which are collectively referred to as capable of producing 'shiga-like toxins' to cause disease.

Escherichia coli

Escherichia coli is a common non-parasitic inhabitant of most mammalian intestines (31;49). Certain *E. coli* strains, however, are capable of causing disease. Common strains of *E. coli* (EC) that cause food borne illness include: enteroaggregative (EAEC);enteropathogenic (EPEC); enteroinvasive (EIEC); enterotoxigenic (ETEC) and enterohemorrhagenic (EHEC) (49;99). The EAEC, EPEC, EIEC and ETEC are causes of diarrhea, most often associated with children, the elderly or immunocompromised individuals (49). ETEC, for example, is the cause of 'traveler's diarrhea.' ETEC also causes a cytotoxin mediated diarrhea, but it is more severe and fatalities are more common. The EHEC strains include *E. coli* O157:H7, a cause of severe illness and death. The toxin produced by O157:H7 is a shiga-like toxin that may lead to intestinal perforation and hemolytic uremic syndrome (HUS) which may progress to kidney damage and renal failure, or even brain seizure, coma and death (49;74). Children tend to be more likely to develop HUS than adults. The largest outbreak to date occurred in Japan, where over 9,000 individuals became ill and 11 died. The outbreak was due to infested radish sprouts in salads served to school children (136).

The transmission of human pathogens to food sources, including fresh fruits and vegetables, is most likely through contact with contaminated water or direct mammalian contact (via the fecal-oral pathway) (21). Cattle have been suspected of being the primary reservoir of EHEC (139), suggesting that beef products or bovine waste material is the source of primary contamination. However, the common housefly has been shown capable of spreading bacterial cells and could carry *E. coli* from feces to fruits or vegetables (98). Once introduced to plant surfaces, such as lettuce, cucumbers and carrots, it may be able to multiply (1). Additionally, wound sites may afford this bacterium shelter from chlorination treatments or desiccation (116). One outbreak of *E. coli* O157:H7 in 2001 was traced back to lettuce served in a restaurant (91). An outbreak in the United States and Canada in 1996 was traced back to consumption of fresh, unpasteurized apple cider, prepared from fruit that included several drops that may have been retrieved from the orchard floor after contamination by feces from wild animals. (5).

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive bacteria of the *Listeriaceae* family (96). It is a human pathogen that has become an emerging disease in regards to foodborne illness over the past two decades (79). It is a psychrotroph which is able to survive and multiply in unusually cold environments, including those as low as -0.4^{o}C (52). Dairy and other animal products, in particular soft cheeses, have been most often the source of the disease. However, listeriosis has been traced to fresh fruits and vegetables, including cherry tomatoes (63;79;132). *L. monocytogenes* generally has a low mortality rate, except in immunocompromised individuals, the elderly, neonatal patients (<5 days old), or when healthy individuals are exposed to extreme populations. In these cases, it is capable of causing meningitis or septicemia, with a mortality rate as high as 30% (149). Another

major concern with *L. monocytogenes* is with pregnant women, where infection often affects the fetus and can lead to miscarriage (116).

Viruses

A virus is an obligate parasite and cannot replicate outside of its host, so food can only act as a vehicle and not as a multiplication site. Contamination is usually through contact with contaminated water sources or a shedding individual (49).

Interactions of Tomato Fruit and Human Pathogens

Wells and Butterfield (140) reported a significant correlation between isolation of enteric pathogens, specifically those of the genus *Salmonella*, and bacterial soft rot in fruit and vegetables sampled from a grocer's market. They also showed that when *Salmonella* was co-inoculated with the bacterial soft rot pathogen *E.c.c.*, the enteric pathogens could not only survive in the tissues of various plants, including tomato fruit slices (Mahovic and Schneider, *unpublished*), but were able to multiply to 10-fold greater numbers (52). Co-inoculation of fruit with *Salmonella* and either *B. cinerea* or *R. stolonifer* increased the incidence of *Salmonella* contaminated fruit after 48 h (141). *Salmonella* populations increased after co-inoculation with *G. candidum* in tomato fruit stored at 15 or 25^{o}C for up to 10 days, in particular if fruit had previous chilling injury (133).

Identifying and Controlling Microbial Hazards during Tomato Handling

In the handling of fresh market commodities or ready-to-eat fruits or vegetables, those that have been in storage longer, were more perishable, or were handled more often tended to have more microbial contamination (21). Microbial contamination can lead to direct losses if it causes postharvest decay or indirect losses if an outbreak of human disease is traced back to a particular commodity. Often, more than just single, implicated

operations are closed; an entire market can collapses because consumers lose confidence in the safety of the product and refuse to buy it regardless of the source. Analysis of potential critical points of contamination and the development of methods to deal with these points is an important practice for minimizing possible occurrence of infestation and/or infection of either type of microbe. Keeping records documenting the steps taken to prevent contamination helps to build consumer confidence and protect a producer from tort law claims.

Identifying Hazards

For any agricultural operation involving fresh produce, including tomatoes (108), there are at least five general areas to consider as potential sources or vectors of human or decay pathogen infestation:

1. Employee or animal contact with the product
2. Water that comes in contact with the product during any stage: growing, harvesting or processing
3. Field sanitation
4. All aspects of the packing line
5. Containers and other equipment used to store and transport the product

Attention must be paid to each of these steps to ensure that potential sources of hazardous microorganisms are controlled or isolated from the product. Employees and animal manures are the most likely source of human pathogens (21). Water and containers or other handling equipment have the greatest potential to disperse hazardous microorganisms.

Employees

Direct or indirect unsanitary human contact with fresh fruits and vegetables is the primary source of contamination of food (95). Direct contact involves the touching of produce by hands or other parts of an individual that is shedding pathogen structures.

Thus, a harvest manager or packing facility must require that employees practice good hygiene at all times and that workers who are ill or have recently been ill be disallowed from working directly with the fresh product. Indirectly, employees can contaminate water, equipment, or other employees that subsequently contact the fruit or vegetable. Preventing the initial contamination or sanitizing any water or equipment that is potentially contaminated prior to its use in harvest or handling can reduce these hazards.

Water

Water is the most dynamic and difficult pathogen vector to control. Maintaining safe water is important as microbes disperse readily in water and all require at least some moisture for survival (49;95). Free water may also compromise plant defenses, enabling decay to begin. All water sources need to be monitored for contamination and sanitation, including irrigation water, water used in flumes, wet dumps or washes and the water that employees use for hygienic purposes.

Water that comes in contact with fruit in the field includes that used in irrigation, in the application of pesticides or fertilizers and rain splash or rainfall. Surface water is likely to contain human pathogens that have been shed by domestic and wild animals. Therefore, ponds, lakes or streams should never be used for overhead irrigation or for mixing pesticide sprays, unless the water is first sanitized (49;95).

Water used for handling, such as in wet dumps and circulating water tanks, provides a natural environment for the accumulation of microbial populations (100). The microbial load in a tank is directly related to water system sanitation, system cleanliness at startup and populations coming in on harvested fruit (13). Microbial populations on crops prior to harvest are usually highly variable with populations increasing during warm moist weather and decreasing during cool dry weather (118).

Field sanitation

Animals and workers are the primary sources of various human pathogens that may contaminate crops in the field. Mammals, birds, amphibians or reptiles may shed human pathogens (= zoonotic). These sources may directly contaminate crops. Alternatively, vectors such as flies, other insects, rain splash, etc. may move pathogens from fecal deposits to fruit surfaces (49).

The packinghouse

Florida grown tomatoes are generally hand-harvested into buckets, which are emptied into field bins or gondolas. These bins or gondolas are then trucked to a packing facility, potentially over 300 miles distant. At the packinghouse, the fruit-transport containers are emptied into a wet-dump or water flume system. This water handling system spreads the bulk load of fruit out into a single layer and then flumes them to a roller conveyor that moves the fruit through a washer, dewatering rollers, and a waxer. Workers stand along side the belts and sort the fruit for color and grade as they are transferred along the line. Finally, the fruit are automatically sized and dropped into corrugated fiberboard boxes.

The wet dump, water flume and washer steps remove debris, soil, chemical residue and microorganisms from fruit surfaces. Since flume water is recirculated during the workday, microbes can accumulate in the water leading to cross contamination, which is the potential contamination of all fruit entering the water system. High levels of contamination can occur, unless the water is continuously sanitized to reduce these populations (86).

Containers, equipment and transport

Harvesting, storage and shipping containers and equipment can be a type of vector that moves both decay pathogens as well as human pathogens to fruit surfaces (25). Transport vehicles also have been known to contaminate fresh fruits and vegetables with human pathogens (147).

Controlling Biotic Hazards

To minimize food safety hazards and decay potential and to meet the requirements of buyers, managers of fresh fruit and vegetable packinghouses are suggested to adopt a Hazard Analysis and Critical Control Point plan (HACCP), or a Good Agricultural Practices (GAPs) or Good Manufacturing Practices plan (GMPs). A HACCP or GAP/GMP program designed for a fresh-market produce handling operation consists of formalized criteria for observing and reacting to handling steps that may introduce biological, chemical or physical contamination of the final product (21). Both programs are designed to minimize threats of microbial contamination, but each takes into account different levels of control over critical points in the handling process.

HACCP plans require record keeping of any and all steps taken toward maintaining a safe environment and most importantly requires regular microbiological tests of the commodity as well as any equipment that contacts the commodity, including water. Microbial populations detected must remain below a maximum allowable limit for each organism of concern. The product tested must be tracked such that it can be retrieved if the allowable limit is exceeded. A GAP/GMPs program requires record keeping and procedural handling specifications that theoretically minimize the potential introduction and/or spread of disease causing microbes, but does not necessitate a microbiological testing schedule. GAPs and GMPs each are more easily applied to raw product

packinghouses where microbiological tests could be difficult to administer and the subsequent tracking of the sampled product nearly impossible (21).

Some purchasers are beginning to require evidence of such a plan being in place. Retail market chains are increasingly demanding that their suppliers adhere to a HACCP or GMP system, as verified by a third-party auditor. Federal agencies in the U.S., such as the FDA, do not yet require the use of a HACCP system. However, some 'at risk' crops may be the first to incur such a policy (21). A similar policy is in place in the United Kingdom, the "Food Safety Act of 1990," which makes product wholesomeness the responsibility of the producer. In the case of an outbreak of illness under such a policy, a producer without a HACCP plan and a third-party audit program is considered guilty of producing a contaminated product by default (95).

In general, methods for minimizing contamination risks include sanitary handling and control of the storage room, transportation and marketing environment. Commodity specific guidelines for general GAPs and GMPs exist in several publications (64;66;67;84;85;105;134). Critical control points in the production of fresh tomatoes include (108): employee hygiene; sanitation in the field including irrigation water, pesticide use, animal exclusion and culling defects; preventing fruit injuries; packinghouse sanitation; and storage and keeping records.

Employee hygiene

Worker contact with the product is the single most important critical control point in any food handling process (95), as people are, without question, the primary source for human pathogens. Managing this critical control point involves providing sanitary conditions throughout production, including available, well-stocked and continuously maintained facilities for hand washing, restroom use and the exclusion of any workers

that are ill, have recently been ill, or have open injuries from coming in direct contact either with commodities or any handling equipment that comes in contact with the commodity. Supervision and training workers in matters of personal hygiene and the use of such facilities is also important. Properly trained workers are not only less likely to cause a contamination event, but are also more likely to recognize a hazard and be able to report or neutralize it (21).

Sanitation in the field

Sanitary practices begin in the field, before a commodity is harvested. If a commodity is contaminated before or during harvest, it may become irreversibly contaminated or carry pathogens to the packing facility and cross contaminate otherwise sound fruit. Acceptable field sanitation includes exclusion of domestic or wild animals from the field, avoidance of the use of raw manure for fertilization, and avoidance of the use of non-potable water in pesticide applications or overhead irrigation (108).

Water, particularly surface water, is a source of contamination. Certain steps reduce the hazards associated with the use of surface water in crop production. Generally, the crop canopy should never become moistened with water from ponds, lakes or streams. As such, irrigation should be by drip or furrow; never a method by which the foliage is moistened with the water. Pesticides should never be diluted with surface water. Pesticide active ingredients are rarely designed to kill all forms of life even when concentrated as in the spray tank (21;24).Wells used for water sources should be either designed for production of potable water or should have periodic tests for coliforms.

Since many species of animals can act as a source of microbial contamination and damage to field grown commodities, an exclusion plan is beneficial. Measures to restrict

wild animals may or may not be practical, but the exclusion of domestic animals is essential.

Culling, the removal of damaged, disfigured or diseased fruit from a fruit lot prior to packaging is an important aspect of marketing high quality, risk-free and decay-free products. Injured fruit are usually predisposed to decay. Moreover, human pathogens often survive quite well in wounds and certain ones can multiply in fruit tissues. Corky areas or other rough areas on fruit also provide sites for survival of both decay and human pathogens. Theoretically, culling fruit as they are harvested, before they are flume dumped and their pathogen load is suspended in transfer water, would be best. However, piece-rate wage scales, as used in the tomato industry, are not compatible with field sorting. Consequently, sorting occurs in the packinghouse. To assist in this critical handling step, managers should ensure the workers are in a pleasant well-lit environment. Bender et al. (23) found that while differences in grades did not affect fruit flavor, fruit of a lower grade did show increased incidence of wounding, bacterial soft rot and fungal decay.

Bins, buckets and any other harvest and handling containers should be cleaned and sanitized regularly. Surfaces are not easily sanitized unless they are first cleaned. Disposable and reusable shipping containers should both be kept free of plant or fruit debris while stored before to use. Proper cleaning and sanitization of bins, buckets and other harvest equipment completed as part of a control program can eliminate several sources of microbes and avoid subsequent outbreaks (21).

Preventing fruit injuries

Fruit may be injured in any number of ways at several points during production, harvest and handling. Animal or insect feeding or environmental damage in the field,

harvester negligence, transport injury, damage from grading and packing lines and even injury after being packed are all potential hazards. Most mechanical injuries can be avoided through use of facility design and procedural standards that take into account a commodity's physiology and are then adjusted accordingly to minimize abiotic stresses. Style of packing materials, method of transport and cushioning of packing lines are all some of the potential ways to alleviate injury (24).

Use of a wet dump system for transfer of fruit from field bins to a packing line lessens impact injuries compared to dry-dump systems (118). As such, fewer fruit are lost and fewer wounds are created. Sargent et al. (111) reported that a spherical sensor packed with fruit and dropped a maximum of 30 cm from a carton to a conveyor belt multiple times in a tomato repack operation reached an average maximum acceleration of 113 G (178 G maximum, where G = Gravity and 1 G = 9.81 m/s^2). Due to the size of a field bin, dumping such a container onto a conveyer would likely produce higher acceleration force. By contrast, fruit dropped into a water dump tank from a field bin were only exposed to an average acceleration (over two surveyed packinghouses) of 38 G (76 G maximum). Thus, tomatoes in the water dump systems should receive fewer and less severe impact wounds.

Fruit flushed from gondolas experience a shorter drop than do those from bins (111), but streams of water with pressures as high as 77 kg/cm^2 (1100 psi) flowing at 121.13 L/min (32 gal/min) are used to break up and then suspend mounds of fruit. The impact of such streams on wounds and stem scars on fruit is believed to cause infiltration and water congestion. The resulting water channels enable microbes to internalize (become lodged into tissues below the fruit surface) (41;70).

Packing line sanitation

Dump tanks need to contain a biocide level sufficient to prevent the accumulation of microorganisms as well as cross contamination, which is the movement of microbes from fruit to fruit. Harvesting after prolonged rain events, for example, can lead to cross contamination of fruit that move through the flume system (118). Surfaces along the entire packing line must also be regularly sanitized to avoid any biofilm or litter build up that could serve as a point of contamination. Sponge and brush rollers may retain some water that, if contaminated with biological materials (juice from broken fruit, leaf litter, etc.) and a microbial population, may also develop biofilms that could harbor pathogens capable of inoculating any fruit passing over them. Sanitation (94) and frequent replacement of the rollers is advisable to avoid and remove, respectively, any biofilm buildup.

Storage and record keeping

Proper storage temperatures and humidity control help to prevent decay development and water loss. Additionally, keeping storage rooms free of standing water will prevent the growth of fungi, like *R.s*. Records of steps taken to clean, sort, and handle fruit properly all provide evidence that reasonable efforts to producing a clean and sanitary product have been utilized. Such records would be an extremely valuable defense should a trace-back from a human disease outbreak occur, or if a shipment is dumped due to excessive decay at the receiver's end.

Preventing Inoculation and Contamination

Microbes that contaminate intact surfaces of fruits and vegetables can be removed through standard cleaning and chlorine treatments. If a pathogen becomes internalized, however, it is protected from biocidal treatments and adverse environments; treatment of

tomatoes with chlorine after exposure to inoculum did not prevent decay (115). Once a pathogen is internalized and past the fruit's protective waxy cuticle, fruit cells are exposed to attack by catabolic enzymes or other pathogenic interactions.

Cuticle Breaks and Other Wounds

Most postharvest pathogens of tomatoes are unable to penetrate intact fruit surfaces, but fruit cells below the surface are susceptible. Initial infections occur at wound sites or through internalization via natural openings of the cuticle (8;12;37;70;119;140). These points are significant in bacterial decays, as Bartz (6) found in one survey that only 2% of primary decay lesions were connected directly to surface wounds; most bacterial decays are due to some form of infiltration at the stem or blossom scar. Fungal decays, however, are more prevalent at points where the cuticle is damaged through abrasions and/or cuts (23). Fruit may have any number of wounds from the picking and handling procedure, or from field damage, prior to harvest (144).

Infiltration

The most common event leading to pathogen internalization is when microbes contact fresh wound surfaces or other water channels connecting the fruit surface to intercellular spaces within the fruit. Fresh wounds contain cell sap that is released by the injured cells. This fluid floods the wound surface and adjacent intercellular spaces, providing a channel by which microbes can diffuse into or be otherwise drawn into intercellular spaces below the wound. Wounds are most often a result of handling and harvesting, but may occur prior to harvest. Internalization also occurs when water infiltrates (is forced into) fruit tissues. Infiltration of water may not itself be detrimental to a fruit. However, if excessive quantities penetrate openings on the fruit surface, the

fruit may crack (20;119). If internalized water harbored any decay pathogens, the infiltrated fruit will quickly develop symptoms of infection.

Infiltration is a particular hazard that may affect fruit handled through wet dumps (72;144). Water can enter intercellular spaces, which are air passageways that are necessary for tissue respiration (12). When fruit are submerged in or covered by water, air in the intercellular spaces and hydrophobic forces associated with waxy surface layers usually prevent water intrusion. However, under certain conditions pressure differentials develop that force water through surface pores into adjacent intercellular spaces (8). This happens due to the effects of a temperature differential between the fruit and the environment, direct water pressure (38;40), or capillary attraction between intercellular spaces and water channels in the fruit surface (69).

Temperature differentials cause infiltration as predicted by the general gas laws (8;20). If fruit cool while submerged in water, air trapped in intercellular spaces contracts, creating an internal vacuum which draws water into surface apertures (11). The duration of exposure and the extent of the temperature differential both directly affect the amount of water absorbed (8;36;131). Predictably, as the temperature differential increases, the volume of water entering the fruit increases (11). This phenomenon also occurs in many other commodities. Warm strawberries that were hydrocooled increased in weight due to water uptake and became diseased if the water was not first disinfected through chlorination (47). Peppers that were hydrocooled also showed significant water uptake (36). If fruit are cooler than the surrounding water, the resulting expansion of internal gases may reduce or eliminates water uptake, but it will not cure fruit of previously internalized bacteria (20).

Water infiltration may also be caused by intermittent or continuous hydrostatic pressure. Full submersion was found to occasionally cause an infiltration of stomata or wounds on tobacco leaves (70). Droplets placed over open stomata or needle prick wounds quickly entered water congested leaves. By contrast, if the leaf was not water congested, the droplet water did not enter the leaf.

Thermal effects and hydrostatic forces, or any other cause of infiltration, are theoretically independent of one another, but may act additively or synergistically to increase total infiltration (8). All possible infiltration causes need to be identified and managed to maintain a decay-free and wholesome product.

If a tomato fruit is infiltrated with enough liquid to increase its weight by at least 3%, cracking and water soaking of the fruit surface often develops (131), producing potential points of inoculation. In a test where fruit were infiltrated to an average weight gain of 0.1 g of an aqueous bacterial suspension, most of the fruit gaining less than the average remained healthy in storage, whereas those gaining more than 0.1 g all decayed (8). The fruit in this test were from the same lot and treated the same to induce infiltration, showing that infiltration rates can differ between fruit of the same age and variety, even when harvested from the same field.

Water congested leaves have been shown to readily internalize surface bacteria (70). Tobacco leaves that were water congested and then exposed to drops of an aqueous suspension of bacteria, plant viruses, India ink, or Gram-positive cocci, rapidly internalized the treatment liquid (40;41). The ink penetrated through stomata. Thousands of Gram-positive cocci were isolated after surface disinfection of the leaf surface. Leaves that were not water congested failed to absorb these suspensions. Channels of water in

surface apertures, therefore, provide a means whereby capillary forces can draw in aqueous suspensions of particulate matter (40). When tomato stem scars were treated with a liquid fruit wax formulation, the wax quickly penetrated into the fruit (8). When these fruit were subsequently submerged in water, the wax-treated stem scars absorbed more water than those not previously treated. Soft rot pathogens in the water also caused a higher incidence of decay in the wax treated fruit. This was an unexpected result, as a waxy, hydrophobic coating should seal a stem scar from infiltration. These unanticipated results may be a result of an incomplete seal, whereby the wax evacuated any air bubbles in the stem that would block infiltration, or may have reduced water adherence to the cell walls thus reducing surface tension and allowing for increased capillary reactions to occur.

Bartz (7) reported that tomatoes inoculated with bacterial suspensions by vacuum infiltration developed internal pockets of decay. Water soaking from bacterial soft rot was most prevalent at the shoulder of the fruit, emanating from the stem scar. Fruit with stems still attached showed incidence of infiltration no different from those fruit that had their stems removed before infiltration, indicating infiltration may occur prior to harvest if sufficient free moisture is present (7). Recent tests utilizing water-soluble marking dyes suggest that while the presence or absence of stems may have little effect on the incidence of water soaking, stem attachment seemed to affect where the dye solution penetrated (Bartz, *unpublished*). Fruit infiltrated with water while their stems remained attached were soaked through the edge of the stem scar, the dye becoming positioned along the shoulder of the fruit. By contrast, dye movement in fruit with stems removed prior to infiltration progressed into the fruit's core. Any form of infiltration, regardless of

its cause or location, can allow of the internalization of large microbial populations (8). Further studies in this area will be needed to determine the actual role of the stem on infiltration and the significance of that role.

As stem scars dry after stems are separated from the fruit, they form a barrier of air pockets between the environment and the intercellular spaces. Stem scars with this air barrier tend to be less porous and are less favorable as a site for multiplication of decay causing bacteria. Conversely, fresh stem scars are more likely to have water penetrate deeply into xylem vessels thereby enhancing the survival of suspended microbes (8), even after prolonged storage when the stems remained attached until just prior to testing (20).

Although green fruit are less prone to injuries during harvest and handling, riper fruit are less likely to be infiltrated. Red fruit are much less likely to absorb detectable water than are green fruit (10). However, red fruit are still vulnerable to infection beneath the stem scar if exposed to a temperature differential while submerged in a suspension of soft rot bacteria. Regardless of fruit ripeness or age of the stem scar, infiltration due to water pressure has the potential to be nearly instantaneous (8), as evidenced when wounded tomato fruit (15) or congested tobacco leaves (41) were treated with drops of an aqueous dye solution, followed by an immediate washing, and did not visibly remove all dye. Therefore, biocides in a flume, hydrocooler or dump tank must be able to inactivate microbes immediately upon their entering the water (8).

When infiltration is caused by temperature differentials instead of water pressure, chlorination tends to be a more effective biocide, suggesting differences in the rates of infiltration. This was also noted in potato tubers (18) and grapefruit (46). In both cases,

control of environmental and handling conditions are important in combating infiltration, including use of a water sanitizer, maintaining higher water temperatures than incoming fruit and minimizing time and pressure from a water treatment, as observed by Bartz (8), pg 305, "…one may prevent infiltration of tomatoes that are handled in water by keeping exposure times to less than 2 min and immersion depths to less than 17 cm (7 in.)..."

Factors other than temperature, pressure and time may aid in infiltration as well. Surfactants are one such factor and should be avoided in a flume system. Surfactants decrease the surface tension of water and, thereby, enhance its ability to enter pores that are normally protected from water intrusion by a combination of air bubbles and surfaces covered with a waxy cuticle (7;20;119). Bartz (20) found that at 40^{o}C, tomato fruit infiltrated with a surfactant solution absorbed an average one g of water more than those treated with water alone. On two different occasions, it was also found that potato tubers had significantly more soft rot when treated with surfactants (11;18). A related test found that several strains of *E.c.c.* normally failed to produce disease in live corn stalks, but the addition of surfactants predisposed the corn plants to decay (121).

Chlorine as a Sanitizing Agent in Packinghouse Water Systems

Chlorine in several forms, most commonly as sodium hypochlorite (NaClO), is commonly used as a biocidal treatment to combat a variety of microbial contaminants in water (143). Originally introduced to treat potable water (and still used for this purpose), chlorination is now used in many situations where water is used and the possibility of microbial contamination exists. For example, chlorine has been shown to effectively protect tomato fruit from cross inoculation by decay pathogens in dump tanks and flumes. However, treatment with chlorine does not eliminate microbes from inoculated wounds nor does it leave a residual on treated fruit (115). While the lack of residual chlorine may

be beneficial from a food toxicology standpoint, this means fruit are not protected from any subsequent exposure to pathogens.

Mode of Action

The mechanism of inactivation by chlorine may include any of several different reactions. Chlorine may react with cellular membranes, altering their permeability (31). The inability to maintain or restore energy charge may be affected by membrane disruption or by eliminating a cell's ability to generate ATP. The acidic hypochlorite acid component of chlorinated water is a strong oxidant and will react with any number of cellular components, deactivating or destroying them (102). Most likely, it acts similar to other oxidizing agents, owing its antimicrobial activity to its oxidizing effects on proteins, in particular upon the thiol groups of cysteine residues, which are important determinants of protein structure and function (53). Each of these effects or any combination of them may be responsible for cellular inactivation.

Several environmental factors influence the activity of chlorine in water, most notably the pH of the solution, and to a lesser extent, the temperature. In water over a pH range of about 5.0 through 9.0, unreacted chlorine, also called "free chlorine," exists as a combination of hypochlorous acid and hypochlorite ion. At approximately pH 7.5, these two forms are present in equal amounts (figure 3-1). At pH 6.0 roughly 97% of the free chlorine is in the hypochlorous acid form (HOCl), whereas at pH 9.0, 97% is in the form of hypochlorite ion (OCl^-) (31;143). White (143) estimated that HOCl acid is 20 to 300 times more effective as a biocide than is OCl^-. Additionally, White also suggested that the ion probably has to be converted to the acid in order to kill microbes. At a constant chlorine concentration, spores of *Alternaria tenuis* were inactivated more rapidly at pH 6.0 than at pH 8.0 (113;114). A diluted household bleach solution (1.3%, or ~13,000

mg/L) buffered to pH 7.0 or lower effectively removed all bacterial cells from submerged rice seed, while fungi were eliminated only when the same solution was brought to pH 5.0 or below (34). This is also consistent with findings that chlorine solutions above pH 8.5 tend to be relatively ineffective biocides (25;102;143). The negative charge on OCl^- ions prevents them from crossing the negatively-charged cellular membranes to react with proteins, genetic material, or any other intracellular components (44), whereas HOCl freely crosses such membranes. This mechanism is similar to cyanide (HCN) and ammonia (NH_3), in that each is also more biocidal than their ionic counterparts (31).

Recommendations

In Florida, 150 to 200 mg/L free chlorine with a pH in a range of pH 6.5 to 7.5 and a temperature at 5.5^{o}C (10^{o}F) greater than the pulp temperature of incoming fruit is recommended for dump tank and flume waters (19). A water temperature of about 41^{o}C (~100^{o}F) is considered a good target temperature for most water systems (20;100;101;118). These recommendations are meant to maximize chlorine's efficacy and maintain its concentration over time, while minimizing the likelihood of equipment corrosion, water infiltration or microbial decay.

Problems with Chlorination

While the proper chlorination of packinghouse water systems has been effective, inexpensive and not hazardous, this practice does not solve all sanitation problems. There has been concern about the production of hazardous disinfection byproducts that may create problems in water disposal. Storage decays were still observed after flume tests where chlorine concentrations were 2.5 to 9 times greater than those needed to completely inactivate spores in a 30 to 45 s exposure (15). Moreover, microbes lodged in intercellular spaces below the surface of a wound may survive treatments by being

protected from contact with a biocide (87;114). This possibility was directly demonstrated by Bartz et al. (15) using water blue dye as a marking agent.

A major concern with chlorine is its rapid reaction rate with organic material and certain chemicals, which dissipates its antimicrobial efficacy. In a test with *G. candidum*, Brown and Warowski (25) suggested, page 100, "much or all of the chlorine is tied up by the soil and organic matter which prevents the chlorine from reacting with fungus propagules." Similarly, as the amount of organic materials in a chlorine solution increases, a proportional amount of residual chlorine inversely decreases (76).

Chlorine that has reacted with certain chemicals may retain some oxidative potential as chloramines, chlorimines or chloroalkyl amines. Collectively, these forms are called "combined chlorine" (143). If C × T values are high enough, combined chlorine will inactivate most bacteria and likely certain fungal spores. However, combined chlorine is not considered an effective sanitizer for packinghouse water systems due to having only minimal to very slight anti-microbial activity (143). Despite this, chloramines have been shown to retain some biocidal activity, but require up to four times the exposure time to inactivate a microbial population than would HOCl (143). Certain disinfection by-products of water chlorination may have undesirable health effects in the environment, such as production of halomethanes, which are carcinogens. For this reason alternatives that do not produce hazardous disinfection byproducts are needed.

Bacterial cells have developed survival mechanisms for various environmental stresses. Certain of these processes are also effective in protecting the microbe against chlorine treatments. For example, attachment of bacterial cells to hard surfaces decreases

their sensitivity to free chlorine. Bacteria that attach to moist, hard surfaces may be able to multiply, forming aggregates and producing contiguous glycocalyx layers (a biofilm) (49). Microorganisms embedded in such films are difficult to treat successfully with chlorine because they are protected from contact (77). Biofilm age and inherent roughness of the surface on which the biofilm forms are each apparently directly relevant to increasing bacterial survival, post-biocide treatment (75). Mir et al. (93) and Carlson (31) each concluded that microorganisms embedded in bacterial aggregates are protected against the action of chlorine. Other studies show organisms can be protected from biocidal treatments by 'hiding' in and on vegetable material, such as within growth ridges, stomata, or other physical recessions (75). In alfalfa seed, for instance, 1,000 µg/ml chlorine did not reduce *Salmonella* cells below detectable limits and treatments of up to 4,000 µg/ml did not guarantee sprout surface sterility (69).

Reports of bacterial resistance to chlorination have appeared (93). This resistance is only evident at very low chlorine concentrations, however. In three separate studies, with different bacterial organisms, resistant strains survived exposure to 0.01 to 1.0 mg/L free chlorine, while treatments with higher concentrations produced complete inactivation of the "chlorine resistant" strains (33;106;109). Chesney et al. (33) concluded that, page 2131, "no significant bacterial resistance to chlorine compounds has been found despite decades of water treatment with powerful chlorine-based oxidants."

A cellular product with strong reducing potential, bacterial glutathione (GSH), is produced by certain strains of *E. coli*. This reducing agent is capable of protecting cells by irreversibly reacting with chlorine or other oxidizers. However, only a limited amount is produced and it can not be excreted (can not cross the cellular membrane). Release of

the product into the environment to react with chlorine requires cellular lysis. A sacrificial resistance such as this is only significant at very low concentrations of HOCl (or other oxidative agents) where enough cells remain intact after the biocide has been inactivated for the population to survive. Cultures with GSH producing cells had increased survival rates when chlorine concentrations were from 0.25 to 0.75 μM, but at 1.00 μM or greater (50 μM HOCl ≈ 2.6 mg/L HOCl (44)), microbial kill was near 100% for all strains. Similarly, Dukan et al. (45) showed that lethal damage to bacterial (*E. coli*) DNA by chlorine could be reduced by certain environmental factors, but again, only at very low concentrations of free chlorine; differences were seen with treatments around 57 μM, but from 3 to 6 $\log_{10}$ reductions occurred at 76 μM.

Reliable studies suggesting consistent bacterial survival in HOCl concentrations of ≥ 10 mg/L have not been reported. Recommended flume system chlorination rates are up to 100-fold greater than rates used in the reports of bacterial resistance. Consequently, bacterial resistance to chlorine should not be considered a significant issue for packinghouse water systems.

The relatively high concentrations of chlorine recommended for packinghouse water systems are required to inactivate fungal decay pathogens (15). Additionally, a high concentration helps to off-set the loss of free chlorine to reaction with materials having a chlorine demand, such as organic (76). Increased chlorine concentrations also decrease the time of exposure required to attain microbial kill (114), as predicted by the general C x T formula.

Chlorine Alternatives

Due to the potentially hazardous disinfection byproducts associated with water chlorination, several alternatives for water sanitation have been examined (143). These

have included aqueous solutions of ozone, chlorine dioxide (ClO_2) or cupric ion, as well as irradiation with ultraviolet (UV) light. In general, these treatments may be good at sanitizing water under particular conditions (143), but the constant and dynamic demands of the dump tank do not appear to be adequately addressed. For example, use of cupric ion requires larger doses or longer treatment times than would be practical in a flume system to be effective. UV radiation has little ability to penetrate turbid water. Even with clear water, UV light can interact with bacteria to only a depth of about 5 cm. Suspended particulate matter large enough to be seen with the naked eye absorbs UV irradiation, thereby protecting adjacent or embedded microbes (143). A tomato dump tank often has extensive amounts of suspended particulate matter in the water, which would block UV irradiation. Similarly, bacteria in protected locations on fruit could not be effectively treated with UV because the light waves would not penetrate fruit tissues. Finally, UV would be difficult to apply where fruit enter the water and it is at this point where cross-contamination is most likely to occur.

Hydrogen peroxide (H_2O_2) is a naturally occurring biocide that is a component of cellular metabolism and breaks down to form water and oxygen gas. It is readily available and easily and safely disposed. However, many microorganisms have developed methods of dissipating H_2O_2; their populations are more readily reduced in halide-based oxidizers. As Chesney et al. (33) observed, page 2134, "…HOCl is much more toxic (500 to 1,000 times) to *E. coli* cells than is H_2O_2." Dukan and Toutati (45) also noted, page 6149, "for the same concentration, killing is more drastic with HOCl than with H_2O_2." H_2O_2 also reacts quickly, readily and irreversibly with many different compounds and, thus, is relatively unstable. It is a functional sanitizer, but must either be

used in a clean environment, or after a cleansing agent has been effectively applied. Neither of these situations exists in tomato flumes.

Peroxyacetic acid (PAA; CH_3CO_3H) is the peroxide of acetic acid. It is a broad-spectrum oxidizing agent, has no hazardous decomposition products and is soluble in water (54). It is a more powerful sanitizing agent than H_2O_2 because it has a higher membrane lipid solubility and is not deactivated by the enzymatic cellular defense mechanisms, peroxidase and catalase. It is relatively stable in the presence of organic materials (101). It is most active in an acidic environment with an activity that declines rapidly above pH 7, under high temperatures, or in the presence of metal ion. In its concentrated formulation, it is a hazardous material (129) with a potentially offensive aroma (vinegar). Tests on the airborne concentration of PAA and the amount required to decrease the respiratory rate of mice 50% (RD_{50}) show (54) that PAA (as the commercially available aqueous mixture of PAA, H_2O_2 and acetic acid) has an RD_{50} value of 3.8 mg/L, with a long term exposure limit of 0.2 mg/L. By comparison, the RD_{50} of chlorine gas is 3.5 mg/L (53). Use of PAA in packinghouse water systems would likely require periodic additions of fresh PAA. An accumulation of acetic acid, the breakdown product, in the water or a build-up of off-gassing PAA could lead to worker discomfort.

Ozone (O_3) is one of the strongest sanitizing agents available (143). It is highly reactive (with a half life of only around 15 to 20 min, or less than 1 min in water containing suspended or dissolved organic materials) and is not particularly water soluble, diffuses rapidly, is more stable at neutral pH levels (6-8) and is poorly characterized for any potentially hazardous disinfection byproducts. It will rapidly break

down at higher pHs and is lethal to humans at 4 mg/L during prolonged exposures (101). OSHA limits workers to 0.8 mg/L over an 8 h period to avoid risk of pulmonary edema, etc. (129).

Chlorine dioxide, $ClO_{2(aq)}$, must be generated on-site (143) due to its explosive nature if concentrated or compressed. *G. c.* p.v. *citri-aurantii* spores can be eradicated when suspended in clean water for 90 s with 2 µg/ml of ClO_2 in water (25). However, when used on fruit contaminated with *G.c.c.* spores from grove soils, up to 35 µg/ml reduces the viable spore population by only about 50%. In a dump tank at a packinghouse for apples, 1 µg/ml of ClO_2 was not enough to control spore loads. If the concentration is bumped up to 3 to 5 µg/ml of ClO_2, an effective residual concentration can be maintained for spore control (103). ClO_2 is several times more expensive than are the various forms of HOCl (143). ClO_2 will react with any reducing agents present in solution, although to a lesser extent than will HOCl. ClO_2 is used in poultry processing facilities to disinfect chill water, which has a high organic matter content (143). $ClO_{2(aq)}$ is used as a vapor dissolved in water. As such, splashing or other movement in the water will lead to rapid off-gassing.

ClO_2 may also be utilized as a vapor treatment, $ClO_{2(g)}$. Significant reductions in populations of *L. monocytogenes* and *E. coli* O157:H7 on contaminated apple surfaces accompanied treatment with $ClO_{2(g)}$ (42;43;59). Several spoilage organisms, including lactic acid bacteria, yeasts and fungi that had been dried on stainless steel strips were successfully inactivated by the gas treatment (57). Treatment with $ClO_{2(g)}$ produced greater reductions in seeded *E. coli* populations on uninjured apple surfaces compared with aqueous solutions of several other sanitizers (42;109;145). These levels of control

required concentrations from 0.62 to 1.24 mg/L of ClO_2 vapor, which exceed both the current Occupational Safety and Health Administration (OSHA) short term exposure limit of 0.3 mg/L and the 8 h time-weighted average concentration of 0.1 mg/L (97). The use of ClO_2 vapor treatment has not been cleared for commercial use as of this date. Cheaper and safer methods of on-site generation and delivery would enhance practical use of this product.

Table 2-1. Tomatoes consumed in the U.S. from 1960 to 2000, in kg per capita*

Year	kg
1960	5.72
1965	5.44
1970	5.49
1975	5.44
1980	5.80
1985	6.76
1990	7.03
1995	7.76
1996	8.03
1997	7.76
1998	8.12
1999	8.07
2000	8.07

*Source: United States Department of Agriculture Economic Research Service. http://www.ers.usda.gov/Briefing/tomatoes/tomatopdf/TomatoConsumption.pdf (82).

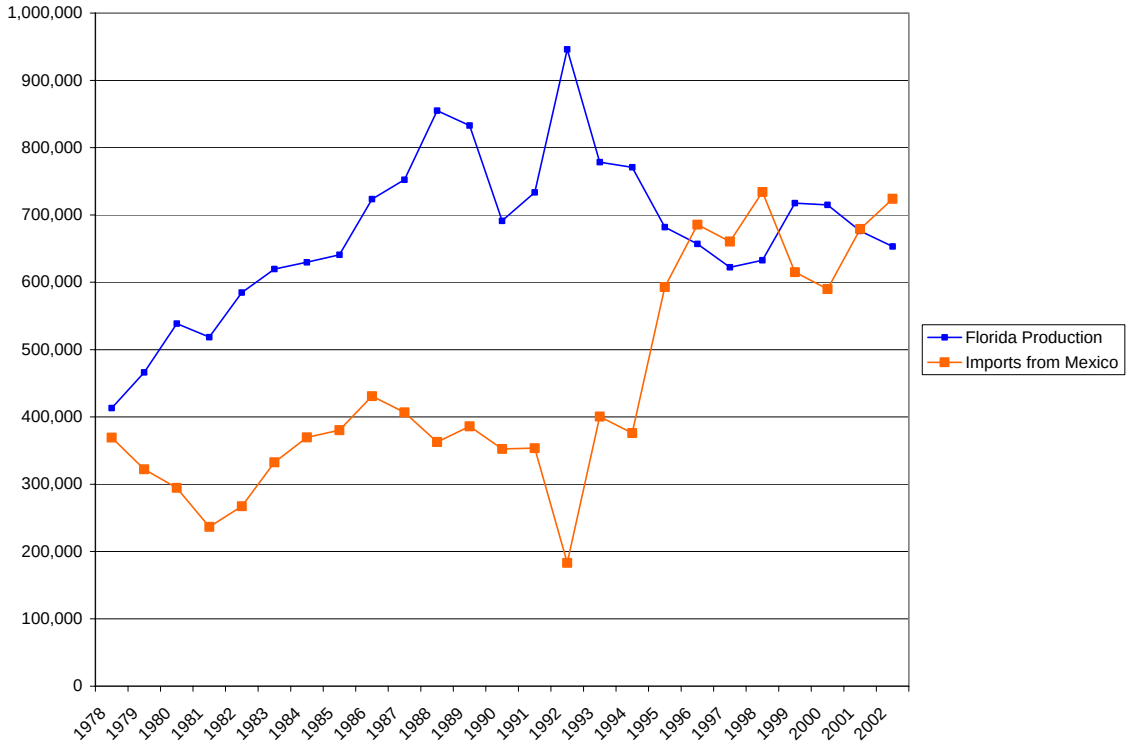

Figure 2-1. Comparison of Florida tomato production with U.S. imports from Mexico (1000kg). Source: Data compiled from the National Agricultural Statistics Service, http://www.nass.usda.gov:81/ipedb/ (146).

CHAPTER 3
BIOCIDES TESTED

Introduction

The standard water treatment for sanitation in tomato packinghouses is currently chlorination of the water supply with chemicals that form the combination of hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) (143). The more effective killing agent in this solution is HOCl, which has been estimated to be 20 to 300 times more active as a biocide than OCl^-. Chlorinated water has a high oxidation/reduction potential (ORP), which makes the solution unstable in the presence of reducing agents such as organic matter, microorganisms, etc. HOCl has a good general biocidal activity and, at appropriate concentrations, kills most bacterial and fungal cells rapidly (15). The specific mechanism of biocidal activity of HOCl is unknown, but reactions with essential structures or enzymes are presumed. This activity occurs inside the cell. The similarity of HOCl to water (net neutral charge, small molecular weight) allows it to penetrate cell walls whereas the negatively charged OCl^- ion is repelled (44). The ratio of acid to ion in chlorinated water is primarily controlled by solution pH (figure 3-1).

The instability of chlorinated water results from the oxidative nature of both HOCl and OCl^- (143) and is highly influenced by solution pH (15;114;131;143). They are each very reactive with chemicals such as ammonia, amino acids, proteins, organic carbons, nitrites, iron, manganese, organic nitrogen and others (143), all of which may be washed into flume systems from incoming tomato fruit . Most of the products of these reactions are poor sanitizers (chloramines) or have no sanitizing activity (chloride ion). As a result,

fresh chlorine products must be added continuously or intermittently to maintain an effective free chlorine residual.

The reaction of chlorine with aromatic chemicals associated with dissolved tannins and lignins from plant tissues leads to the production of trihalomethanes, such as chloroform (143). Trihalomethanes are considered hazardous, with a potential to cause cancer in humans. The reaction of chlorine with various amino acid or ammonium chemicals produces chloramines, which are quite toxic to aquatic life forms. Thus, spent chlorinated water from packinghouses requires special handling and may be considered hazardous. The EPA's desire to reduce the risk associated with water treatment at various facilities has led to a search for alternatives.

Aqueous Chlorine Dioxide ($ClO_{2(aq)}$)

Chlorine dioxide is a gas at room temperature and pressure. It is more soluble in water than sodium hypochlorite (NaClO) (at 20^{o}C; about 70 g/L versus about 7 g/L, respectively) (143), although at the pH level used in packinghouses (6.0 to 7.5), NaClO quickly dissociates into HOCl and OCl^- as discussed above. ClO_2 does not dissociate in a liquid, but instead exists as a dissolved gas. It will readily leave solution if it is stored in an open container, or faster if used in agitated, aerated water, such as seen in a flume or dump tank. Additionally, dissolved ClO_2 is also subject to a rapid decomposition if it is exposed to ultraviolet light, such as from sunlight or fluorescent lighting.

A major benefit of ClO_2 is that its reactions are more specific than are those of HOCl. For example, ClO_2 does not react with exposed amino groups or with ammonium ion (143). Thus, reactions of ClO_2 and humic materials do not lead to the production of trihalomethanes as HOCl may.

Acidified Sodium Chlorite (ASC)

When solutions of sodium chlorite ($NaClO_2$) are acidified (<pH 2.4), the chlorite (ClO_2^-) slowly coverts to chlorous acid ($HClO_2$) and then eventually to chlorine dioxide gas ($ClO_{2(g)}$). Other oxidative species may be intermediates. Both $HClO_2$ and ClO_2 have anti-microbial activity. The slow conversion may enable ClO_2 to penetrate wounds better than HOCl or other oxidative chemicals. ASC concentrations are reported as milligrams per liter, mg/L (ppm), or the millimolar values, mM, of sodium chlorite present in the base solution, prior to acidification.

ASC solutions are not highly stable and will convert to chloride ion (Cl^-), citric acid (as used in this system as the activator) and sodium ion (Na^+) within 24 h. Thereby, each work day in a packinghouse the solutions must be refreshed, but this also suggests that residues of the active chemical will disappear from treated fruit (143). Rinsing with potable water followed by storage for 24 h is still required after application, however (39).

Peroxyacetic Acid (PAA)

Two products were used, both based on acetic acid stabilization of hydrogen peroxide in solution: Oxidate™ (BioSafe Systems, Glastonbury, CT) and Tsunami® (Ecolab Inc., St. Paul, MN). Tsunami® is a peroxyacetic acid solution labeled for use at 120 mg/L (1.58 mM). Oxidate (or StorOx, when labeled for postharvest applications) is ‘hydrogen dioxide’, a synonym for ‘oxygenated water’ or, most commonly, ‘hydrogen peroxide’ (2), with a small concentration of peroxyacetic acid present to stabilize the solution. Oxidate is labeled for use by diluting the stock solution to 54 ppm for fruit applications or 128 ppm for tank applications. The exact concentrations of chemicals in

Oxidate are unknown, so millimolar values cannot be reported. All concentration values are reported as ppm dilutions of the stock solution's label rate 27% hydrogen dioxide.

Test Pathogens

The chlorine alternatives were tested for preventative and curative activity against the three most important pathogens of Florida-grown tomatoes. *Erwinia carotovora* subsp. *carotovora* (*E.c.c.*), the causal agent of bacterial soft rot, is the most important pathogen in terms of decay losses (102). *Geotrichum candidum* (*G.c.c.*) is a yeast-like fungus that may cause decays in stressed green fruit or in ripe fruit (32). *Rhizopus stolonifer*, (*R.s.*) a true fungus with pigmented spores, causes watery soft rot and can produce nesting in boxes of fruit at any stage of ripeness (9).

Materials and Methods

Pathogen Preparation

Bacteria were cultured on nutrient agar (NA, Difco™ Laboratories, Detroit, MI) and regularly re-isolated from inoculated fruit to maintain pathogenicity. Cells of the bacterium prepared for stock solutions were either washed from up to 3-day old petri-dish cultures with sterile tap water (STW, municipal water that is autoclaved for a time based on volume, usually 15-20 min, to eliminate any microbes present, followed by cooling to room temperature) or grown for 12-24 h in nutrient broth plus 2% dextrose solution on a shaker (~50rpm) at room temperature. Cells in the initial suspensions were pelleted by centrifugation at ~3,000 rpm for 10 to 20 min in an IEC HN-SII Centrifuge (Damon/IEC Division, Needhamhts, MA). The supernatant was discarded and the pellet was suspended in fresh STW, thereby removing spent growth medium. Cells produced from broth culture were treated three times in this manner, whereas cells from plates were washed once. The concentration of bacteria in the final suspension was estimated by

turbidity at 600 nm in a spectrophotometer (Spectronic 20; Baucsh & Lomb, Inc., Rochester, NY), based on a previously determined regression of optical density over colony forming units per ml (cfu/ml) (102).

Rhizopus stolonifer and *Geotrichum candidum* p.v. *candidum* were maintained on acidified potato dextrose agar (APDA, Difco™), containing about 0.2% lactic acid to discourage growth of bacterial contaminants. When the cultures were 2 weeks old or less, the surfaces of *G.c.c.* plates were flooded with STW. A bent-glass spreader that had been soaked in ethanol and then flamed was brushed over the culture surface to suspend the conidia (102). With *R.s.*, a flamed metal transfer loop was used to collect sporulating mycelia from a dry plate; the resulting mycelia/spore mass was placed into a 150-ml glass flask containing STW and a small amount of Tween-80 (~0.1% v/v). For both fungi, a magnetic stir bar was added and the suspension was stirred until it appeared homogeneous. The suspensions were then poured through a square of sterilized cheesecloth into a second flask to separate out spores. The stock concentration of conidia was based on the turbidity of the suspension as measured on the spectrophotometer set at 540 or 600 nm for *R.s.* or *G.c.c.*, respectively, and compared against previously prepared regression graphs of propagules/ml vs. turbidity (15;102). With *G.c.c.*, Tween-80 was not necessary. Both fungi were periodically re-isolated from a lesion on an inoculated fruit.

Biocide Preparation

Each biocide used was prepared as directed by the product label or instructions supplied directly by the manufacturer. Unless otherwise specified, the concentration applied to fruit was the maximum rate specified on the product label.

Hypochlorous acid

Standard laundry bleach ("Ultra Clorox® Regular Bleach", 6.00% sodium hypochlorite, Clorox Co., Oakland CA) was diluted with STW as needed for testing. The free-chlorine concentration in the stock solution was periodically determined by titration with a Hach Digital Titrator (Model 16900-01, Hach Company, Loveland CO). Prior to each test, the pH of the diluted bleach solutions was reduced to 6.5 to 7.0 by the addition of N/2 HCl. In certain tests, phosphate buffer (pH 7, 0.5 M) was added to stabilize the pH of the solution, whereas in others, natural bicarbonate salts present in city treated water provided stability.

Acidified sodium chlorite

A solution of $NaClO_2$ was combined with a diluted solution of citric acid (Sanova®; supplied by the Alcide Corporation, Redmond, WA, a subsidiary of Ecolab Inc., St. Paul, MN) at labeled ratios to produce desired concentrations of ASC (pH 2.4). Excess citric acid was used to lower the solution pH to 2.4 (or less) as needed.

Aqueous chlorine dioxide

$ClO_{2(g)}$ was generated from a dry product, provided by the ICA TriNova Corporation (Forest Park, GA), activated and diluted into deionized water ($DI{\cdot}H_2O$). The product was a folded sachet that contained $NaClO_2$ in one side and an activator in the other. The sachet was unfolded, the contents mixed and then the sachet was submerged in $DI{\cdot}H_2O$ in a tightly capped and covered (to protect the solution from light) flask. The final solution of $ClO_{2(aq)}$ was stored in amber tinted glass containers that were sealed from the atmosphere with a ground glass stopper and stored at 5°C. The pH of the test solution was measured using a digital pH meter (Accumet® AR20 pH/Conductivity

Meter) and the concentration of ClO_2 in solution was measured by iodometric titration procedures provided by the sachet supplier.

Peroxyacetic acid

For each trial, a sample of stock biocide solution was drawn from the provided container and diluted to the needed concentration. A fresh dilution was made for each trial, from the stock solution. A hand-held, test-strip activated spectrophotometer (Reflectoquant® System, Merck KGaA, Darmstadt, Germany) was used to determine PAA concentrations of Tsunami®.

Plate tests

A 250 ml beaker containing a test solution was placed on the top of a magnetic stir bar plate (Corning Laboratory Stirrer/Hot Plate, Models PC-420 or PC-220, New York, NY). A magnetic stir bar was added to the solution, stirring was commenced and a sample of the test microbial suspension was then added. Timing of exposure began when the inoculum contacted the solution. In tests where temperature was a variable, the magnetic stirrer/hot plate was used to warm the solution to the desired temperature prior to the addition of the inoculum. After set exposure intervals, 1 ml samples were removed from the solution with a pipette and transferred to a 16x100 mm glass test-tube (Kimble Glass Inc., Vineland, NJ) containing 10 µl of 20% sodium thiosufate. A fresh, sterile pipette tip was used for each sample. The thiosulfate solution was used to inactivate residual oxidizer in the samples. After all test samples were taken at the desired intervals, a 10 µl sample from each tube was spotted in a radial pattern on a NA plate for bacteria, or on APDA plate for fungi. Plates used for *R.s.* also contained 0.01% Tergitol™, a nonylphenol ethoxylate surfactant (Dow Chemical Company, Midland, MI) by volume,

to reduce mycelial growth so that individual colonies formed. One spot from each time sample was placed on each of three plates.

A petri-dish template of pathogen concentration was created prior to each test. Ten µl of each concentration in a serial 10-fold dilution of the pathogen stock suspension was placed around the edge of the recovery medium. With *E.c.c.* there were seven spots beginning with the sample from 8 $\log_{10}$ cfu/ml and concluding with that from 2 $\log_{10}$ cfu/ml. With *R.s.* and *G.c.c.*, five spots ranging from 6 $\log_{10}$ to 2 $\log_{10}$ spores/ml were plated. Growth in test sample spots was compared to template growth to determine the $\log_{10}$ number of cells recovered.

Specific experimental parameters were as follows: with chlorinated water, 8 $\log_{10}$ cfu/ml *E.c.c.* were exposed to 1.11 mg free chlorine/L at room temperature and at 40^{o}C and at pH 7.5 or 9.0 for 0 (control), 10, 20, 40 or 60 s; 5 $\log_{10}$ spores/ml of *G.c.c.* were exposed to 25 mg of free chlorine/L at room temperature and pH 6.0, 7.5 or 9.0 or at 40^{o}C and pH 6.0 or 9.0. Samples were removed at 15 s intervals beginning as soon as possible after treatment onset and concluding 120 s later. In tests of PAA, an aqueous cell-suspension of 7 $\log_{10}$ *E.c.c.* per ml was exposed to 80 mg/L (1.05 mM Tsunami™) at pH 4.0 or pH 9.0 at room temperature and 40^{o}C for 15 s intervals from 0 s to 120 s; with *G.c.c.* 6 $\log_{10}$ spore/ml were exposed to 80 mg/L in all combinations of two pH levels (pH 4.0 or pH 9.0) and two temperatures (22 or 40^{o}C); and with *R.s.* PAA was also tested against 6 $\log_{10}$ propagules/ml *R.s.* at room temperature and 40^{o}C, at pH 4.0. $ClO_{2(aq)}$ at 10 mg/L (0.148 mM) was tested against: 8 $\log_{10}$ *E.c.c.* at pH 10.0 or 9.0; against 6 $\log_{10}$ *G.c.c.* at pH 9.0 and against 4 $\log_{10}$ *G.c.c.* at pH 9.0 and 7.0; and against 6 $\log_{10}$ *R.s.* at pH 9.0. All $ClO_{2(aq)}$ tests were performed at 15 s intervals, from 0 to 120 s and at room

temperature or 40°C. In all tests with ASC, cells were exposed to 1200 mg/L (13.3 mM) biocide at pH 2.4 and room temperature or 40°C for 0 through 120 s; *E.c.c.* was at 8 $\log_{10}$ cfu/ml and both *G.c.c.* and *R.s.* were at 6 $\log_{10}$ propagules/ml.

Fruit tests

Fruit were donated by the DíMare Corporation, Tampa, FL and were mature-green or riper, depending on the test. Fruit were of variety Florida 47, or unknown. Fruit in 11.34 kg (25 lb) boxes were sampled from regular shipments of fruit that had been processed through a fresh market tomato packinghouse. For each test, fruit were selected based on similar size, freedom from defects and similar ripeness. Negative control fruit were wounded, but not inoculated. Likewise, positive control fruit were wounded and inoculated, but remained untreated.

For inoculation, a section of cuticle tissue approximately 1-2 cm in diameter, and to a depth of ~1 mm or less, was removed with a scalpel. Fruit were usually wounded five times each, at positions roughly halfway between the equator of the fruit and the stem scar, below the shoulder. Wounds were then inoculated by either dropping 10 µl of inoculum directly on the wound surface, or immersing the fruit in an aqueous cell suspension for ~3 s. Fruit were allowed to dry at room temperature for 30 min prior to treatment.

A simulated water flume system was created in a 14 L plastic bucket, containing tap H_2O and an aquarium pump (~3 L/min. flow rate, Beckett Corporation, Irving, TX). In tests on the curative properties of the test biocides, a desired amount of concentrate biocide was then added to the flume. After the biocide had visually dispersed uniformly (~20 s), wound-inoculated fruit were added. In tests on the protective properties, sanitizers were added to the water first, followed by the wounded fruit and then a

measured sample of the stock inoculum. After the set time interval (usually 120 s), the fruit were removed and then stored in a humid chamber (on a lunch tray, placed inside of a plastic autoclave bag, with a moist paper towel) at room temperature for observation.

Tests were performed to explore the effect of sponge or brush rollers by dipping fruit in biocide for brief time intervals (to simulate spray applications), or by manually rubbing fruit surface wounds (shave wounds) with gloved hands (Diamond Grip™ latex gloves, #MF-300, Microflex Medical Corporation, Reno, NV) or dishware cleaning brushes (to simulate sponge or brush rollers, respectively), while still in biocide suspension. After treatment, fruit were rinsed in DI·H_2O then stored for observation. Tests were repeated for curative treatments: fruit were wounded, inoculated with 6 $\log_{10}$ *E.c.c.* and allowed to dry for 15 min, then dipped in biocidal solution for a designated time interval, with or without manual rubbing of the wounded surfaces of each fruit while wearing fresh, textured, gloves. After treatment, fruit were transferred to a humid chamber and observed for decay.

Storage and reading

Treated fruit were stored in humid chambers at room temperature. Fruit were either placed in snap-lid type containers with moist paper towels to maintain high relative humidity, or were on trays that were placed in loosely sealed autoclave bags along with either standing water or moist paper towels to reach an maintain a relative humidity of near 100%. Fruit were rated for decay incidence at 24 h intervals. Fruit that showed decay progressing beyond the originating point to overcome other negative wounds were removed, as further observations would be indistinguishable between point decay and spread from adjacent wounds. When readings were unable to be taken at 24 h intervals,

fruit were stored in 5°C chambers to slow pathogen growth, until readings could be resumed.

Results

Plate Tests

Erwinia carotovora subsp. *carotovora*

With 8.0 $\log_{10}$ cfu *E.c.c.*/ml, an initial free chlorine concentration of 1.1 mg/L (21.2 µM) at room temperature and pH 7.0 produced a 2-log reduction (99%) in viable cfu recovery in less than 10 s (Fig. 3-2). At pH 9.0, a 99% reduction was also seen within 10 s of treatment. Although viable cells were still detectable after 60 s of treatment, room temperature treatments had consistently one or two log cycle higher recovery than 40°C treatments. No cells were detectable after 1 min of treatment.

Treatment of 8 $\log_{10}$ cfu/ml *E.c.c.* with 80 mg/L PAA (1.05 mM) at 40°C and pH 9.0 showed 99% reduction in recoverable cells in less than 10 s. If cooled to 22°C prior to treatment, the same log reduction required an exposure of about 20 s. Viable bacteria were not detected in the treated suspension after 20 s exposure at 40°C or 90 s exposure at 22°C (figure 3-3). The minimum viability detection limit was 2 $\log_{10}$ cfu/ml. All plates were examined after 48 and 72 h and the counts were similar at each observation.

At pH 4, (80 mg/L) PAA at 40°C reduced 7 $\log_{10}$ *E.c.c.* below the detection limit within 15 s of exposure time. At room temperature, a 99% reduction was seen at 15 s and no cells were recoverable after a 30 s treatment. StorOx® was also able to reduce 7 $\log_{10}$ cfu/ml of *E.c.c.* below detectable limits, when used at the labeled rate for use on hard surfaces (270 mg/L, or 3.55 mM).

ASC was able to reduce the initial 8 $\log_{10}$ cfu/ml *E.c.c.* to below detectable levels at room temperature in less than 15 s. Similarly, instead of being produced through the

reactions of ASC, ClO_2 vapor in aqueous solution at 10 mg/L (0.148 mM) and pH 9 was able to reduce 8 $\log_{10}$ cfu/ml *E.c.c.* below detection limits, at either room temperature or 40^{o}C, within 15 s of exposure. Temperature did not affect kill rates against bacterial pathogens.

Geotricum candidum* p.v. *candidum

Initial 5 $\log_{10}$ concentrations of *G.c.c.* propagules were reduced 99% in 25 s by 25 mg/L HOCl at pH 6.0 at either room temperature or at 40^{o}C. Increasing the pH at room temperature did not affect the time required to reduce 99% of the inoculum or the time to which no propagules were recoverable. At pH 9.0, increased temperature reduced recoverable cells 99% after 75 s, but at room temperature only 1.5 log cycles of recoverable cells were reduced after 2 min of treatment (figures 3-4 and 3-5).

An initial concentration of 6 $\log_{10}$ propagules/ml of *G.c.c.* were unrecoverable after 15 s of treatment at 40^{o}C with 80 mg/L (1.05 mM) PAA (Tsunami®). At room temperature, a pH 4 solution was able to reduce the same initial *G.c.c.* inoculum 99% within 15 s; a pH 9.0 solution required 75 s. Neither pH 4 nor pH 9 solutions at room temperature were capable of reducing 6 $\log_{10}$ propagule/ml *G.c.c.* below detection limits after 2 min of treatment (figure 3-6).

In some tests of ASC vs. *G.c.c.*, a 5 $\log_{10}$ reduction was seen in vitro within 60 s of treatment. In another test 2 min did not reduce *G.c.c.* growth. In tests where ASC was allowed to react for 15 min after it was prepared and prior to treatments, *G.c.c.* was reduced over 99% from initial inoculum concentrations in less than 15 s.

At room temperature, 6 $\log_{10}$ propagules/ml of *G.c.c.* were reduced 99% in less than 15 s of exposure to 10 mg/L (0.148 mM) $ClO_{2(aq)}$ at pH 9 (figure 3-7); at 40^{o}C, a

99% reduction required 45 s of treatment. At room temperature, a neutral (pH 7.0) solution on of 10 mg/L $ClO_{2(aq)}$ (0.148 mM) reduced a 4 log_{10} concentration to unrecoverable within 15 s. In another trial at room temperature and pH 7.0, 6 mg/L (89 μM) ClO_2 took over 1 min to reduce a 4 log_{10} initial concentration of *G.c.c.* below detectable limits. When the temperature was increased to 40°C, 2 min of treatment in 10 mg/L (0.148 mM) ClO_2 at pH 9 was not sufficient to completely reduce 6 log_{10} propagules/ml of *G.c.c.* to undetectable levels, where treatment at 6 mg/L (89 μM) reduced 4 log propagules/ml to the point of none being recoverable, at either the recommended pH 9 or neutral pH.

Rhizopus stolonifer

HOCl treatments required 45 s and 25 mg/L concentration to reduce 3 log_{10} *R.s.* to unrecoverable, as previously reported (15).

PAA (Tsunami®) at 80 mg/L (1.05 mM) and 40°C was able to reduce 5 log_{10} propagules/ml of *R.s.* below the detection minimum after treatment for 30 s (15 s treatments did not achieve 99%). At room temperature, a 1 log cycle reduction required 90 s; longer treatment time did not reduce populations further. At pH 3 or pH 6, PAA (Oxidate®) at 270 ppm dilution had no effect on 6 log_{10} propagules/ml *R.s.* at either room temperature or at 40°C, when treated for up to 2 min.

$ClO_{2(aq)}$ at pH 9 and 10 mg/L (0.15 mM) reduced recoverable *R.s.* propagules 99% when treated for 2 min at room temperature, or 90% over the same time interval at 40°C (figure 3-8). Results were similar if treated at pH 7, or if ASC was used.

Fruit Tests

Protective: *E.c.c.*

Total fruit decay observed after 72 h of incubation was reduced from over 60% in untreated positive controls to 15% or less when treated with 2 to 5 mg/L HOCl (figure 3-9) as a protectant in flume inoculation tests. Uninoculated control fruit showed no decay. Fruit treated with 1 mg/L chlorine showed 30% decay, which was not significantly different from the untreated, inoculated control. Using arcsine transformation on all 72 h data, concentrations over, 1 mg/L were significantly different from untreated positive controls ($P>0.05$). Only 3 mg/L and 1 mg/L were different within treatments. These concentrations and results were similar to those seen in plate tests. When ASC was used in protective treatments, the incidence of *Erwinia* soft rot on wounded tomato fruit was reduced to 0%. PAA treatments were capable of reducing decay rates in wounded fruit to 20%.

Curative: *E.c.c.*

In curative tests, HOCl treatments at 200 mg/L (3.8 mM), the maximum recommended application level for fruit use, showed little to no efficacy (figure 3-15). Even in inoculated wounds that were negative for decay 24 h after HOCl treatment (only 9%), viable *E.c.c.* cells were still recoverable when using the recovery procedures according to Lukasik et al. (83) and as described in appendix A. In a second test, HOCl treatment delayed onset of decay, but did not prevent it. In control fruit 90% of the wounds decayed at 24 h and 100% at 48 h; test fruit had 64% then 94% decay, respectively, and 100% by 72 h. The 48 h readings were not significantly different from the control readings at $P<0.05$.

Treatment with 1200 mg/L ASC (13.3 mM $NaClO_2$) was capable of curing fruit of dip or direct inoculated *E.c.c.* cells (figure 3-15), if treated before soft rot decay was visible internally or externally on fruit (internal decay was determined by sacrificial observation of similarly treated fruit). When treated for shorter periods of time (figure 3-10), fruit that were rubbed had slightly less decay 48 h after treatment than those that were dipped. Fruit dip treated for 30 s had 60% decay, where fruit rubbed for 30 s only had 30%. By 72 h after treatment, differences were no longer significant. While all treatments were significantly less than untreated positive controls, all treatments were also significantly worse than fruit treated in a 2 min bath, which showed no decay. Fruit exhibited phytotoxicity at wound sites when treated for two min with 1200 mg/L ASC (figure 3-11).

When stored in a vented flume hood with fluorescent lighting prior to treatment of *E.c.c.* inoculated fruit, $ClO_{2(aq)}$ had reduced efficacy after 6 h of storage and was undetectable with no protective biocidal efficacy after 24 h (tables 3-1 and 3-3). Chlorination treatment was no different after storage than when freshly used at 6 or 24 h.

Figure 3-11. Phytotoxicity of ASC on wounded tomatoes. Effect of ASC (1000 mg/L, left) on wounded tomato tissue, compared to wounded fruit treated with 200 mg/L NaHOCl (right).

Table 3-1. Comparison of $ClO_{2(aq)}$ and HOCl stability as protective treatments, over time. Inoculations were ~6 log_{10} cfu/ml *E.c.c.*, unless otherwise noted.

$ClO_{2(aq)}$ vs. HOCl as *E.c.c.* flume test protectant, trial 1								
Biocide	time between preparation and treatment	concentration detected mg/L (mM)[a]	calculated concentration in flume	% decay[b]				
				24 h	48 h	72 h	96 h	6 days
HOCl (mg/L)	~2 h	112 (2.14)	2.24 (0.028)	0	16	28	28	28
	6.5 h	108 (2.06)	2.16 (0.041)	0	0	4	4	4
	24 h	103 (1.96)	2.06 (0.039)	0	4	4	4	12
ClO_2 (mg/L)	~2 h[d]	93 (1.38)	1.86 (0.028)	0	0	8	8	8
	6.5 h[d]	18 (0.27)	0.36 (5.34 µM)	0	20	24	32	32
	24 h	[c]	NT	68	96	96	96	96

[a]Biocides initially made to 100 mg/L, via stock calculations
[b]5 fruit with 5 wounds each (% = (# decayed)/(25 total wounds))
[c]Undetectable by titration method
[d]*E.c.c.* inoculum depleted; half concentration used (~5.5 log_{10} cfu/ml)

Table 3-3. Second test of comparison of $ClO_{2(aq)}$ and HOCl stability and as protective treatments. Inoculated with ~5 log_{10} cfu/ml *E.c.c.*, unless otherwise noted
[a]Undetectable via colorimetric ampoule system (detection minimum = 0.2).

$ClO_{2(aq)}$ vs. HOCl as *E.c.c.* flume test protectant, trial 2						
Biocide	time between preparation and treatment	concentration detected mg/L (mM)	calculated concentration in flume mg/L (mM)	mg/L (mM) detected in flume after treatment	% decay[b]	
					24 h	6 days
HOCl (mg/L)	Fresh	153 (2.92)	3.06 (0.058)	1.25 (0.024)	0	0
	6 h	120 (2.29)	2.40 (0.046)	1.67 (0.032)	0	0
	24 h	141 (2.69)	2.82 (0.054)	1.93 (0.037)	0	0
ClO_2 (mg/L)	Fresh	100 (1.48)	2.00 (0.030)	1.2 (0.018)	0	0
	6 h	12 (0.18)	0.24 (3.56 µM)	0.2 (2.97 µM)	12	52
	24 h	[a]	NT	[a]	60	92

[b]5 fruit with 5 wounds each (% = (# decayed)/(25 total wounds))

Fruit treated with PAA as a dip to cure inoculation of 8 log_{10} cfu/ml of *E.c.c.* developed 100% decay (figure 3-15). In treatments with 15 or 30 s of rubbing instead of dip treatment, decay rates remained 100%. Differing severities of decay prompted a reading based on a Horsfall-Barratt (65) type scale (figure 3-12). None of the treatments had statistically significant differences from the control; although not significant, the 30 s

rub treatment rated worse than the control. Up to 50% reduction in decay was observed if initial inoculum levels were reduced to 6 $\log_{10}$ cfu/ml (figure 3-13; curative). Utilizing a different PAA product, from label rate of 54 ppm to a product with 80 mg/L (1.05 mM) PAA, instead of reducing the inoculum concentration, still showed 100% decay in inoculated fruit within 24 h. When inoculum levels were reduced to 6 $\log_{10}$, StorOx® at 54 ppm was able to reduce the incidence of decay in tomatoes when used as a curative treatment from 100% decay in control fruit to 60% (figure 3-13).

Curative: *G.c.c.*

Fruit inoculated with *G.c.c.* then treated with ASC as a curative agent were observed to only develop decay in a maximum of 10% of the wounds. Some trials had as little as 0% decay. Inoculated but untreated positive control fruit never developed more than 5% decay in all trials.

Discussion

As reported in other studies and also seen in this one, even large populations of *E.c.c.* are readily controlled in aqueous solutions (10). When inoculated on wounded fruit, however, applied biocides are reduced in efficacy (13), possibly because bacterial cells are capable of using the tissues for protection. Due to the importance of control of *E.c.c.* and the lower concentrations of chlorine required to reduce populations of the bacteria below recoverable levels, it was used as a minimum standard; if a tested biocide was incapable of controlling *E.c.c.*, that biocide was presumed incapable of controlling an organism which required greater concentrations of chlorine, such as a fungus (*R.s.*) or a yeast (*G.c.c.*). Also, a biocide incapable of log reductions of a test organism in an aqueous solution was not tested against propagules protected by fruit tissues.

Conventions of Reporting Data (mg/L vs mM)

Each of the biocides used had a known formulation, as per the MSDS sheets provided by the manufactures, except for Oxidate® (StorOx®). For ease of convention, mg/L (equivalent to parts per million) were reported as the major units of concentration. Values of millimolar concentrations were also given, where known. In the case of unknown Oxidate® product chemistry, mM values could not be calculated and parts per million based on the label values were given.

Minimum Detection Limits

As populations of a pathogen are treated with various biocides, in vitro or on wounded tomato fruit, surviving cell populations are reduced to differing degrees. The growth of cells on media after treatment was used to determine the surviving fraction. If cells were not seen to grow, this may or may be representative of a complete elimination of the population. In series dilutions, plates created with 10 µl spot inoculations of 2 $\log_{10}$ propagules per ml showed minimal growth, if any. This was expected, as a 10 µl sample taken from a sample with only 100 cells per ml should have but one cell, assuming complete homogeneity. Bacterial cells often clump or are not perfectly defined by precise mathematical predictions.

In test solutions, lack of cellular growth may be due to complete cellular deactivation or reduction, or may be indicative of a concentration of propagules that is low enough that a test sample has a low probability of detecting remaining cells. In all tests reported herein, lack of growth on recovery media was attributed to a reduction of cells to at or below this detection minimum and complete elimination of pathogen cells is not assumed.

Fruit Tests

Fruit are recommended to remain in a dump tank for no more than 2 min in a packinghouse, based on work that fruit submerged in bacterial suspension for 2 min had significantly less decay than those submerged for 20 min (8). In actual packing line situations, a wide range of potential time fruit spend in flumes was observed, from 30 s to 2 min (Mahovic and Sargent, *personal observations*). Thus the maximum 2 min was used as the standard timing for bath tests.

Packing lines may have sponge or brush rollers anywhere along the line and usually have a spray rinse after fruit exit the flume. Some biocides may have efficacy at these points, when applied as a spray treatment or when brushed or sponge rolled onto fruit instead of as a tank treatment. Tests to explore this possibility suggested that although a rub for a shorter time period was more effective than a short dip time period, such a treatment alone was less effective than a 2 min bath. In subsequent multiple hurdle tests (discussed in chapter 4), such treatments are suggested to be added to bath treatments as a 'last ditch' effort for removing previously missed pathogens.

Plate tests

Each of the biocides used was able to reduce 7 $\log_{10}$ *E.c.c.* below recoverable limits in vitro, when used at the maximum label rates and under any label-specified conditions (requisite pH, temperature, etc.). Although log reductions were seen, none of the biocides used, including HOCl, were able to completely and reliably eliminate all recoverable propagules of either of the fungal pathogens, *R.s.* or *G.c.c.*

Initial in vitro test results with ASC against fungal pathogens were inconsistent. Further research into application methodology (143) suggested that the preparation of the solution through mixing of the base and acid solutions requires an incubation time for

generation of ClO_2. Based on laboratory observations, 15 min or more of incubation was needed, prior to application. The product label did not specify this action. This was based on ClO_2^- reactions to produce $HClO_2$, which in turn generates ClO_2 (143). For bacterial cultures, this incubation time seemed to be unnecessary as the $HClO_2$ was effective against bacteria. Repeating initial tests, but utilizing a time delay to allow for ClO_2 generation to begin, produced consistent data. Values of pH were significant for ASC, not in product efficacy but for chemical generation; thus, ASC treatment solutions were brought to and maintained at the label recommended pH of 2.4. Similar to results seen with $ClO_{2(aq)}$, as would be expected, temperature affected kill rates with ASC by increasing the required C x T values to effect similar log reductions.

$ClO_{2(aq)}$ shared similar results with ASC in controlling fungal propagules, with solution temperature effecting results significantly more than pH. Control was similar to chlorine treatments and expected results for a reaction with an oxidizer (51). However, although effective against *E.c.c.* and *G.c.c.* in vitro, $ClO_{2(aq)}$ was ineffective against *R.s.* Inability to affect viability of spores of *R.s.* in vitro eliminated the need for testing this system on fruit.

In one test, one instance of *G.c.c.* cells recoverable after ClO_2 treatment at 75 s (figure 3-7) suggests that the population was not completely eliminated, but was reduced below the detectable limit of 2 $\log_{10}$ propagules per ml. This is consistent with the previously discussed minimum detection limits and sample size.

Tests utilizing $ClO_{2(aq)}$ displayed a lack of residual biocidal ability (tables 3-1 and 3-3) shows that even if a solution had only pathogens and no alternate sink for the biocide to react with, efficacy is dramatically reduced over time. Over the course of a 6 h work

day in a packinghouse, $ClO_{2(aq)}$ solution's efficacy would be reduced to a point where it would not have a biocidal effect, even if no sinks were present.

PAA seems to be less effective as temperatures decrease, which has been suggested previously (51;87). However, in tests against *G.c.c.* at 40°C, where 6 $\log_{10}$ reductions were observed, control groups showed a 5 $\log_{10}$ reduction of initial inoculum in one of three trial runs; the other two had no recovery. In other biocidal tests at 40°C, however, control groups of *G.c.c.* showed no growth reductions. It is unclear if higher temperatures assisted the efficacy of PAA, induced a propagules reduction against *G.c.c.*, or if experimental error occurred.

R.s. was almost unaffected by PAA treatments at 270 mg/L (3.55 mM). At both room temperature and 40°C at pH 6, there was no significant difference in recovery rates of treated versus untreated suspensions. When treated with a lower concentration of PAA 80 mg/L (1.05 mM) at pH 4, however, heating to 40°C reduced 5 $\log_{10}$ propagules/ml below detection limits (figure 3-14), whereas room temperature treatments were still insufficient. These variations were similar to those seen in *E.c.c* and *G.c.c.* tests, except that control treatments were unaffected by the heat. It is again possible PAA required elevated heat to become effective against this pathogen. Treatments with both *G.c.c.* and *R.s.* suggest this, but both are inconclusive. Further trials would be required for verification.

Protective tests

While 1 mg/L (19 μM) HOCl treatment was able to eliminate *E.c.c.* cells in vitro as predicted (51;51;106), as a fruit protectant it was not statistically different ($P<0.001$) from decay seen in an inoculated and untreated control. At slightly higher concentrations (from 2 mg/L to 5 mg/L, or 38.2 to 95.4 μM, respectively), HOCl was able to reduced

decay in fruit from cross contamination to almost zero. Some wounds still decayed, but the number was not significantly different from zero decay, as seen in uninoculated controls.

ClO_2 used as a vapor dissolved in water generated from either dry production sachets, or from ASC solutions, was able to completely reduce decay from cross contamination of bacterial cells to zero percent incidence. PAA was not complete in its ability to protect fruit from bacterial inoculation, but did reduce the incidence of decay to 20% that of the positive control fruit.

Curative Tests

In inoculated fruit, ClO_2 treatment was significant as either a vapor dissolved in an aqueous solution or as ASC. The ability of ClO_2 to cure fruit of bacterial decay pathogen infestation in fresh wounds suggests that other bacteria that may be present in a tomato wound, such as those capable of causing human diseases, would also be cured. Advanced infections may be untreatable.

In some tests, but not all, fruit treated with 1,000 mg/L (11.1 mM) of ASC exhibited phytotoxicity at wound sites (figure 3-11). Phytotoxicity of a biocide toward its intended target usually eliminates use of the biocide in that it may cause more damage than it avoids. In this case, however, intact cuticles did not show damage and only wounds that would normally result in a fruit being culled from a packing line displayed phytotoxic effects.

Fruit tests of aqueous ClO_2 vs. *G.c.c.* showed little decay. However, based on observations of untreated, inoculated, positive controls, there was little conclusive evidence for or against efficacy. Further fruit trials of $ClO_{2(aq)}$ vs. *G.c.c.* were not

conducted because of the inability to promote regular decay of positive controls. Further tests are necessary, where inoculation of *G.c.c.* can be uniform.

Both the inability to cure fruit of bacterial pathogens and the inconsistent reduction of fungal pathogens in vitro precluded use of a PAA solution as a curative agent for wounded tomato fruit against fungal pathogens in this study. Variation in all PAA trials on fruit suggest that alternate treatment methods may be necessary to reduce experimental variation and discover the true efficacy of PAA on inoculated tomato fruit. Further tests of PAA at lower pH levels and higher temperatures also merit investigation. Differences in observed fruit decay rates in curative or protective tests may be due to fruit to fruit differences, inoculation methods, or varying environmental conditions.

Summary

Protecting a fruit from infection was shown to be more effective than curing an already infested/inoculated/infected fruit, as has been previously reported (15). Low concentrations of HOCl were nearly as effective in controlling cross contamination of wounded tomato fruit by bacterial pathogens when in a flume, as it was in eliminating the same bacterial cells in vitro. This is in stark contrast to the inability of 200 mg/L of HOCl to cure fruit of inoculated cells. Equally, PAA at 54 ppm used as a curative agent reduced decay to 50%, but use as a protectant reduced decay to 20%. This is not surprising, however, as microbes exposed to biocidal treatments in vitro, with no competitive sinks for the chemistries used, are the only reactive species a biocide interacts with, allowing for maximum exposure and subsequent kill rates. In tomato fruit, decay developed and viable bacterial cells were recoverable after treatment with chlorination or PAA, even at maximum label concentration under recommended pH and/or temperature levels and over 2 min exposure time.

While continued use of HOCl as a protectant in flume tanks is supported, this treatment should not be expected to cure fruit of bacterial or fungal contamination. A PAA solution may be a viable alternative, although its efficacy is not significantly greater than that of HOCl; ease of use and cost comparison should be the determining factor between continued HOCl use and replacement with PAA as a protectant. It is possible that a solution of $ClO_{2(aq)}$ will be beneficial as either a protectant or as a curative agent. The limiting factor in deciding between continued use of HOCl or replacement with a ClO_2 chemistry will be delivery methods, ease of use, cost and secondary effects on equipment (aqueous ClO_2 as a generated gas or as part of ASC is known to be more corrosive to metals than is HOCl, in any of it's salt or acid forms; the low pH required for generation and efficacy suggests this as well).

Figures

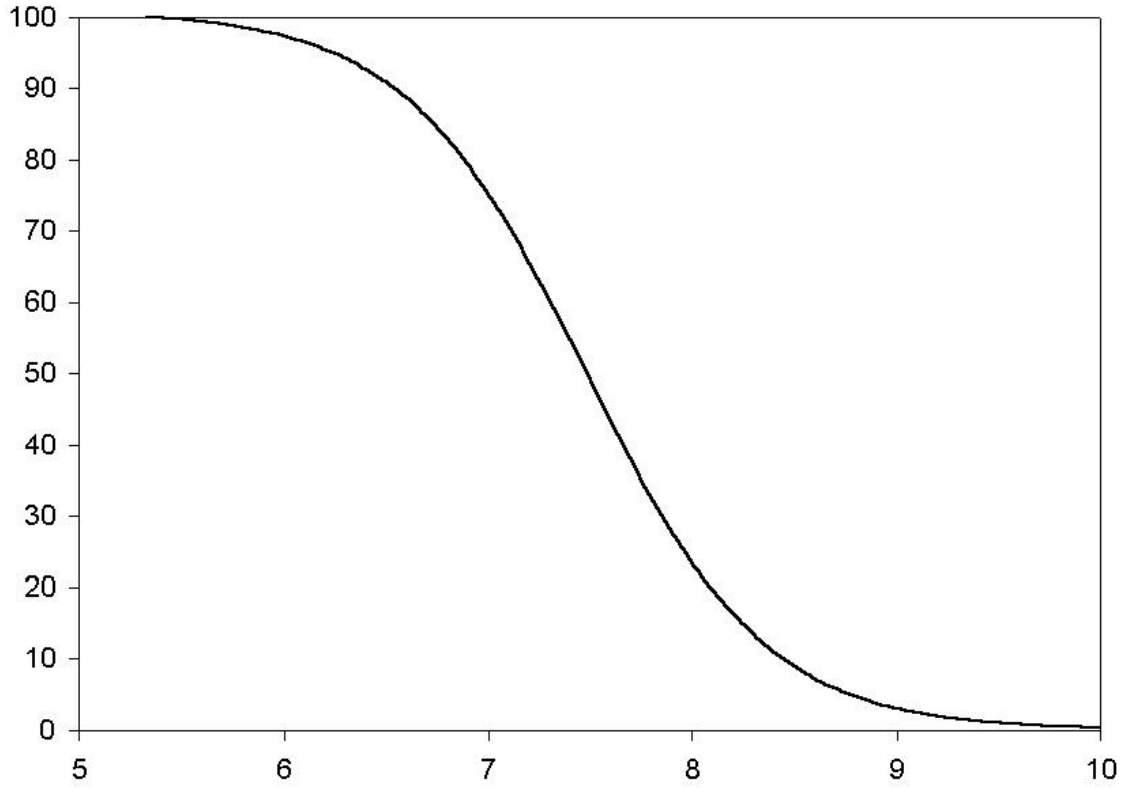

Figure 3-1. Effects of pH on chlorine at 20^{o}C. The x-axis represents solution pH; the y-axis represent total % of chlorine in the solution; area to the right of the curve is the fraction of total chlorine present as OCl^{-} and the area to the left of the curve represents the fraction present as HOCl.

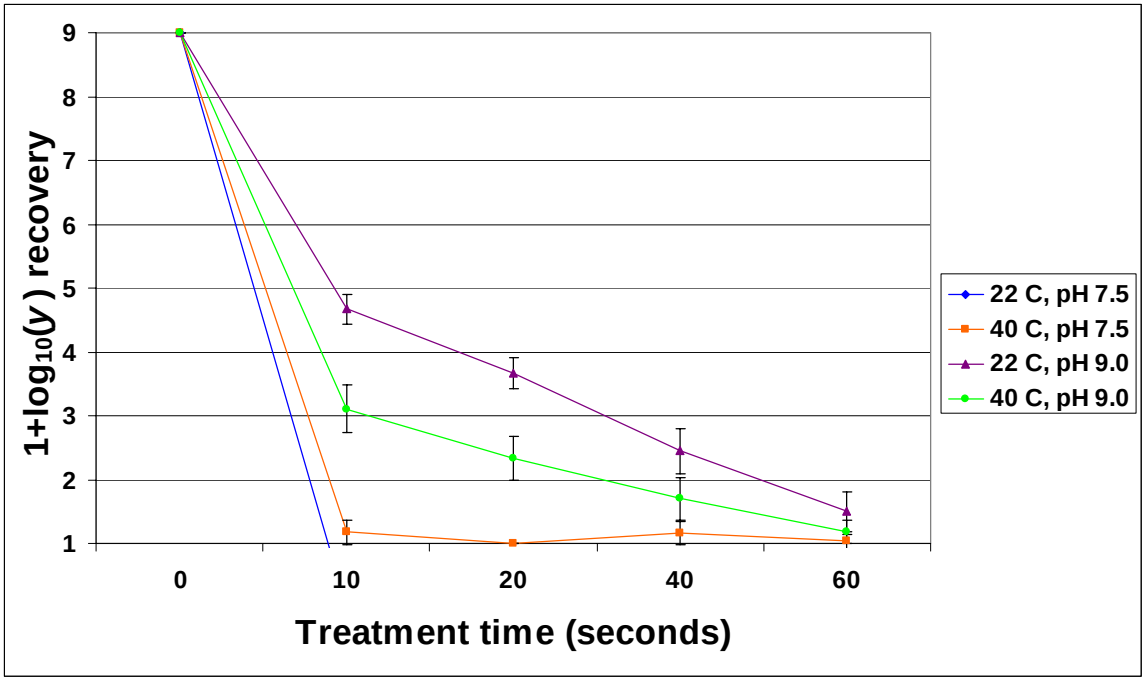

Figure 3-2. Efficacy of 1.1 mg/L HOCl solutions against 8.0 $\log_{10}$ cfu *E.c.c.*/ml at two temperatures (22 and 40°C) and at two pH levels (7.5 and 9.0). Tests show growth as compared to template plates after 48 h incubation on NA plates at room temperature.

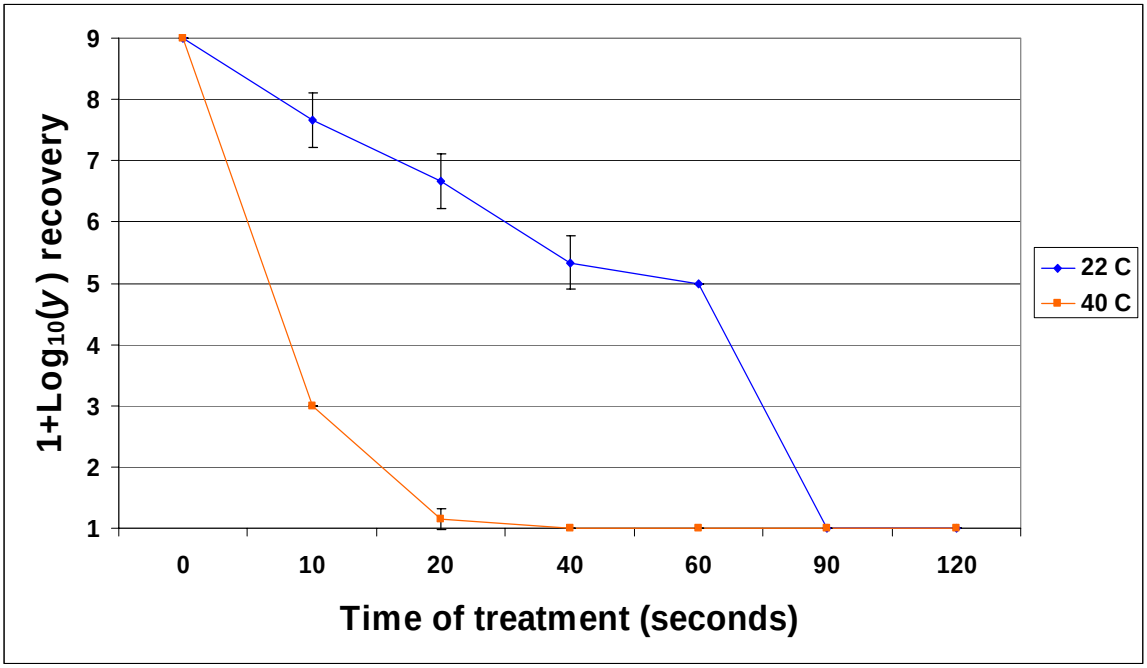

Figure 3-3. Efficacy of 80 mg/L PAA (Tsunami®) solutions against 8.0 $\log_{10}$ cfu *E.c.c.*/ml at pH 9.0. Tests at 22°C had three repetitions and tests at 40°C had six. Tests show growth as compared to template plates after 48 h incubation on NA plates at room temperature (growth at 72 h was the same). Missing error bars indicate uniform readings. $P>0.05$.

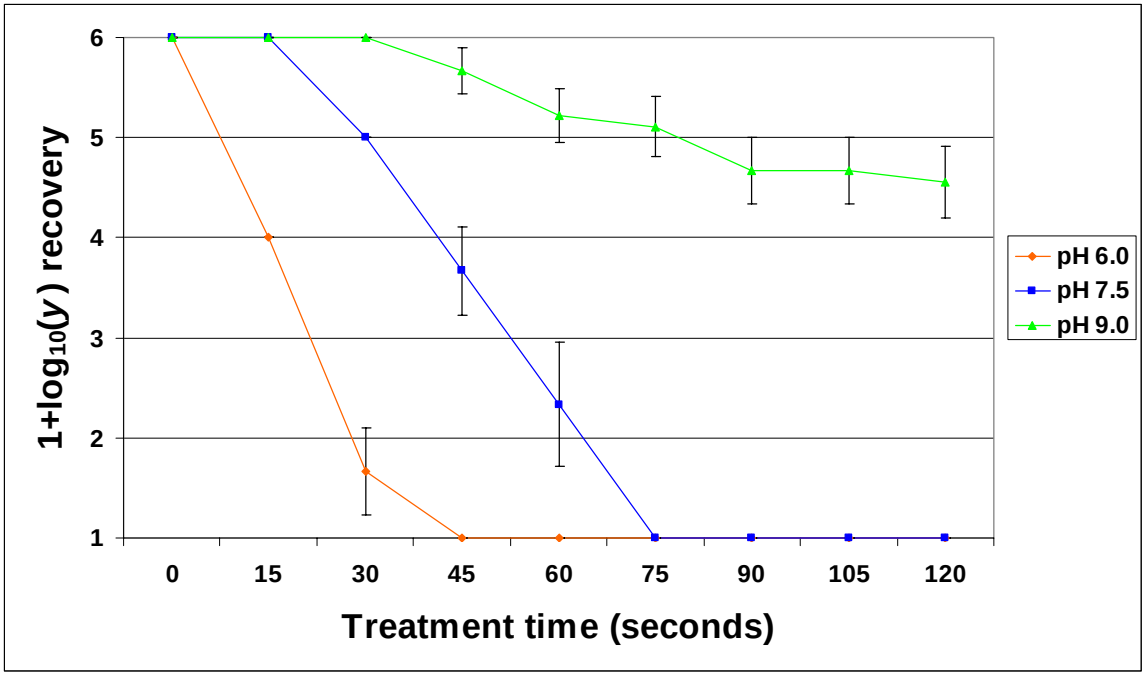

Figure 3-4. Efficacy of 25 mg/L HOCl solutions against 5.0 $\log_{10}$ propagules *G.c.c.*/ml at 22°C and three pH levels (6.0, 7.5 and 9.0). Tests show growth as compared to template plates after 48 h incubation on APDA plates at room temperature.

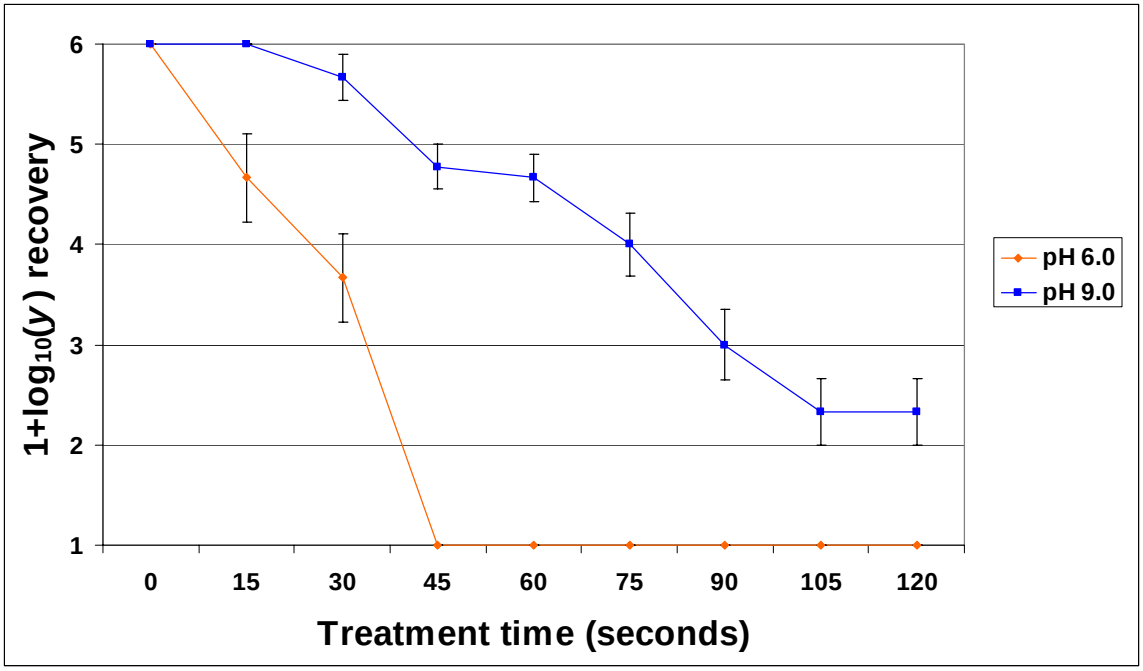

Figure 3-5. Efficacy of 25 mg/L HOCl solutions against 5.0 $\log_{10}$ propagules *G.c.c.*/ml at 40°C and two pH levels (6.0 and 9.0). Tests show growth as compared to template plates after 48 h incubation on APDA plates at room temperature.

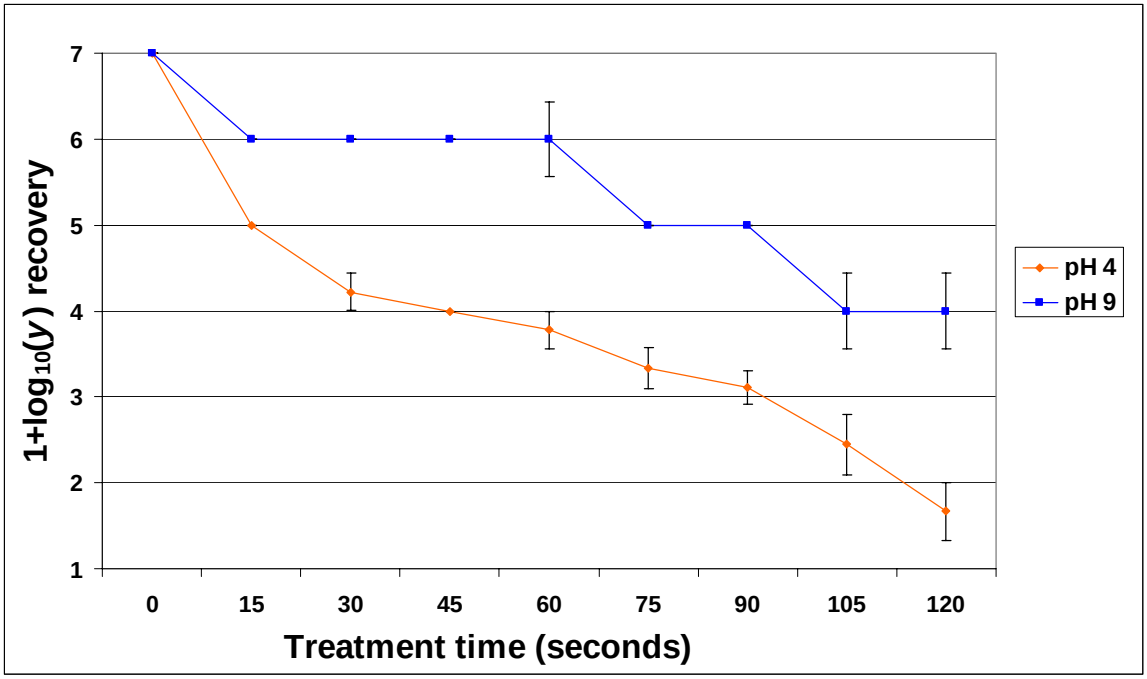

Figure 3-6. Efficacy of 80 mg/L PAA solutions against 6.0 $\log_{10}$ propagules *G.c.c.*/ml at 22°C and two pH levels (4.0 and 9.0). Tests show growth as compared to template plates after 48 h incubation on APDA plates at room temperature.

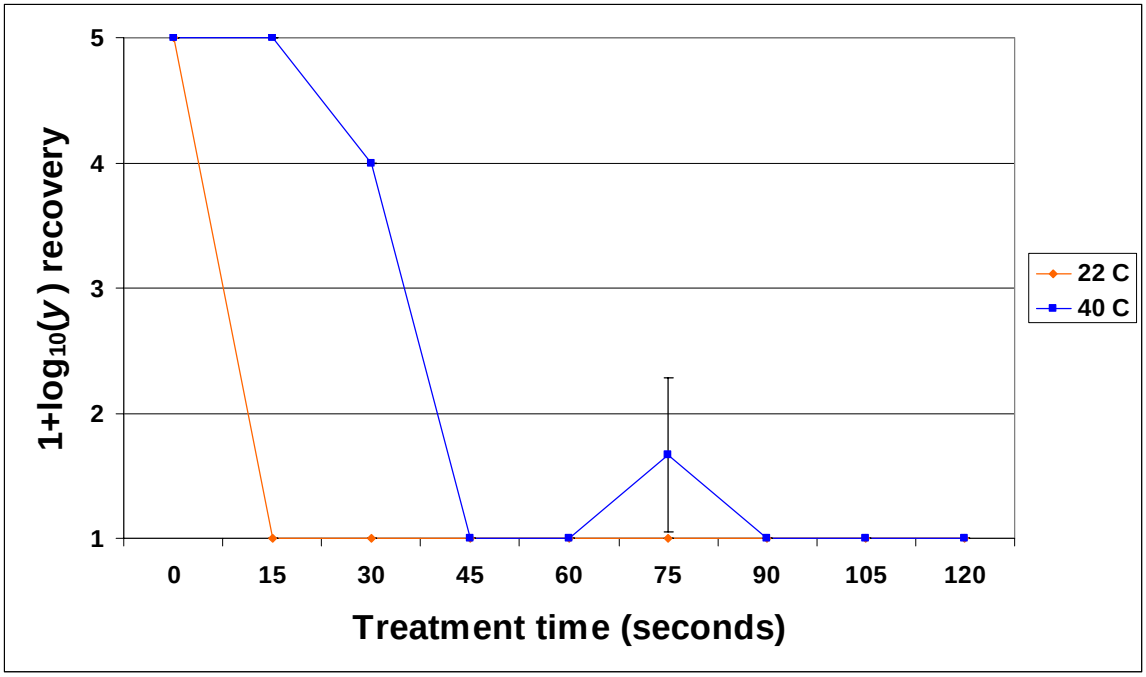

Figure 3-7. Efficacy of 10 mg/L $ClO_{2(aq)}$ solutions against 6.0 $\log_{10}$ propagules *G.c.c.*/ml at two temperatures (22 and 40°C) and pH 9.0. Tests show growth as compared to template plates after 48 h incubation on APDA plates at room temperature.

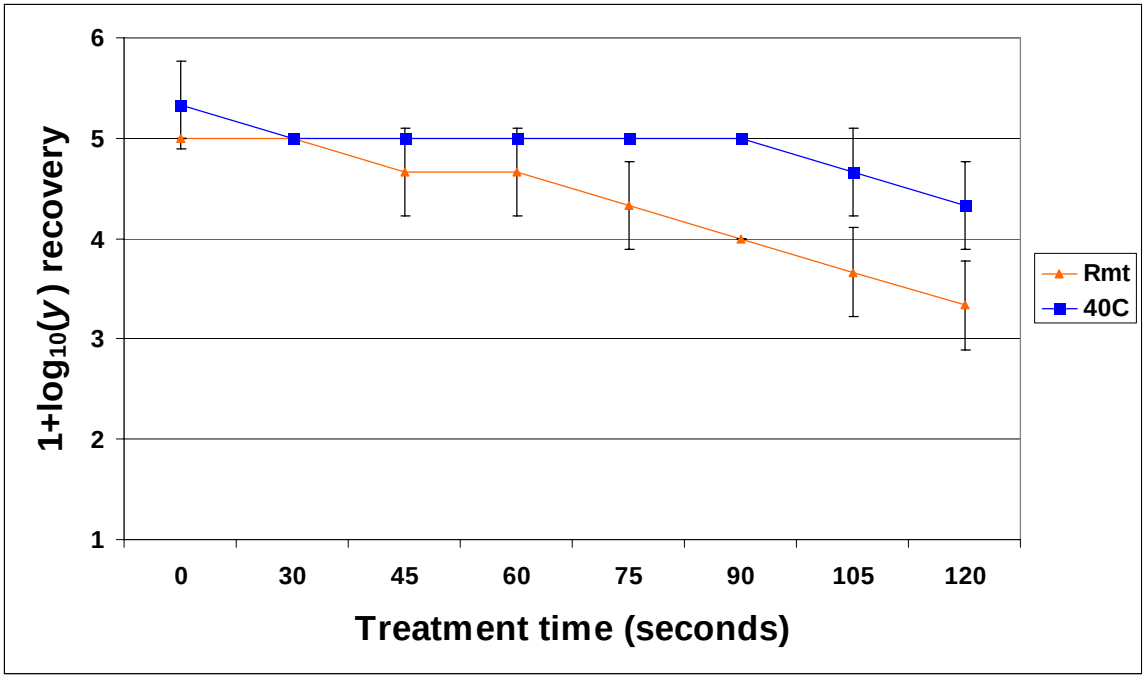

Figure 3-8. Efficacy of 10 mg/L $ClO_{2(aq)}$ solutions against 5 $\log_{10}$ propagules *R.s.*/ml at two temperatures (22 and 40°C) and pH 9.0. Tests show growth as compared to template plates after 48 h incubation on APDA plates at room temperature.

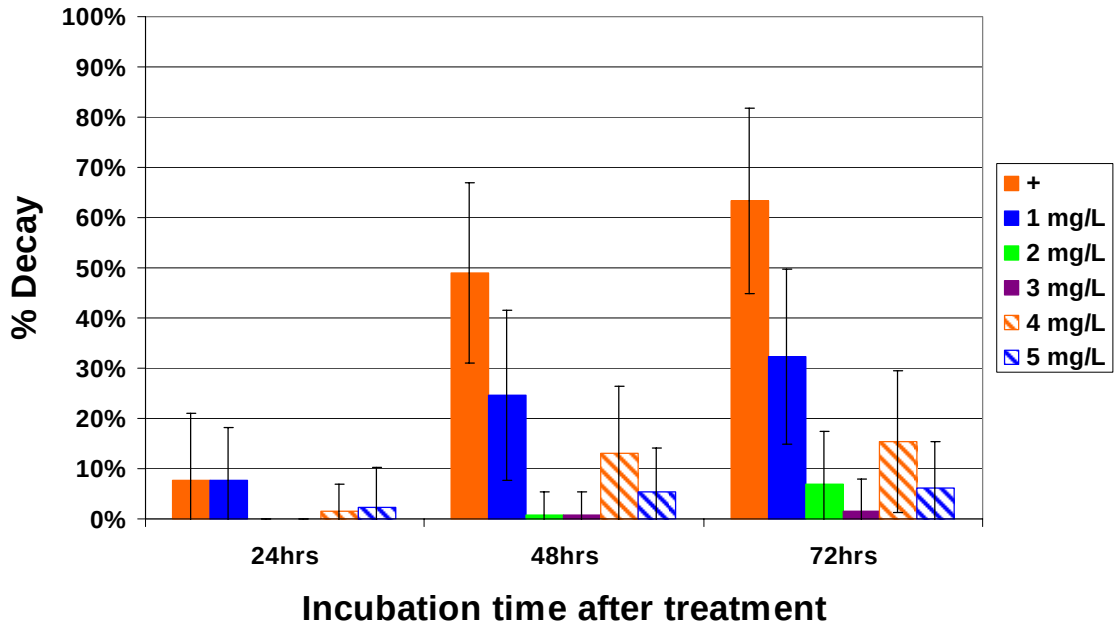

Figure 3-9. Efficacy of 1-5 mg/L HOCl solutions in a simulated flume system at protecting wounded tomato fruit from subsequent inoculation by 2 min exposure to 6 $\log_{10}$ *E.c.c.* After treatment, fruit were stored on a tray in a loosely sealed plastic bag to maintain high R.H. Percent values given as portion of all wounds. Positive control (+) consisted of wounded fruit in simulated flume water system and no biocide, followed by addition of inoculum.

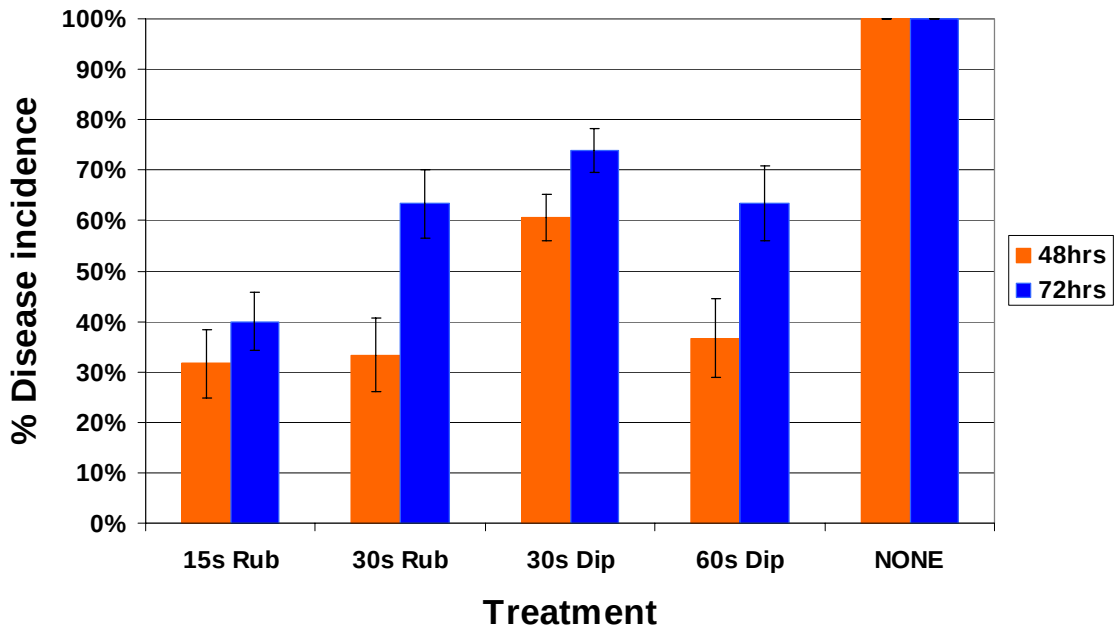

Figure 3-10. Efficacy of 1200 mg/L ASC applied as either a dip or as a simulated sponge roller application (rub) in curing wounded tomato fruit of inoculation by 6 $\log_{10}$ *E.c.c.* After treatment, fruit were stored on a tray in a loosely sealed plastic bag to maintain high R.H.

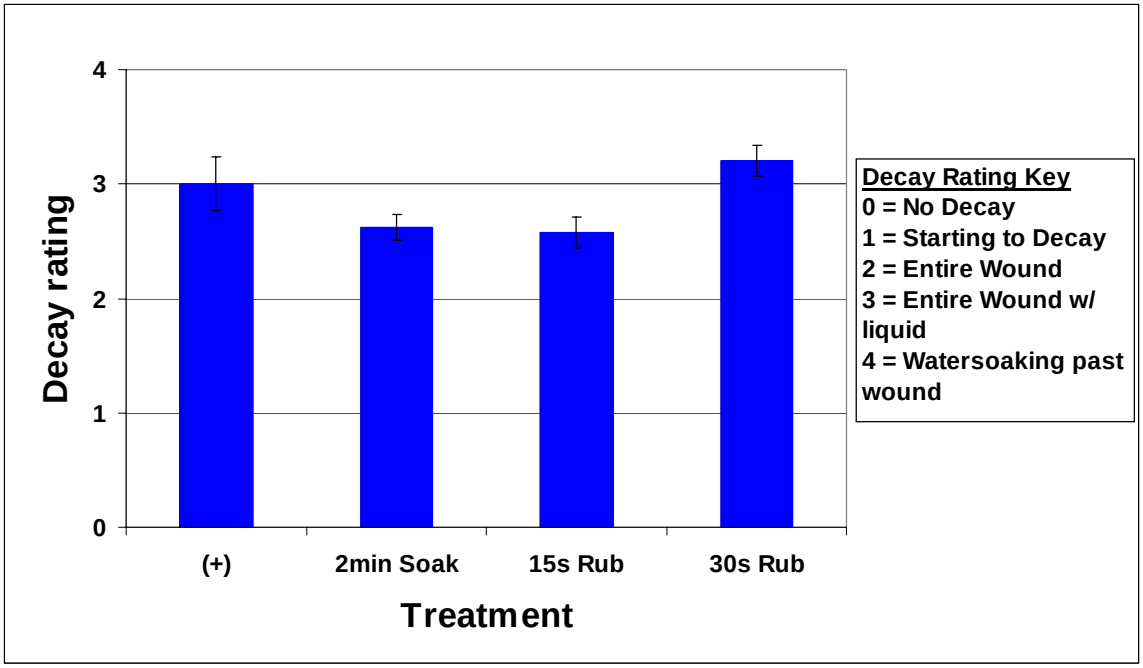

Figure 3-12. Horsfall-Barratt style readings of fruit inoculated with 8 $\log_{10}$ cfu/ml *E.c.c.* after various treatments with PAA.

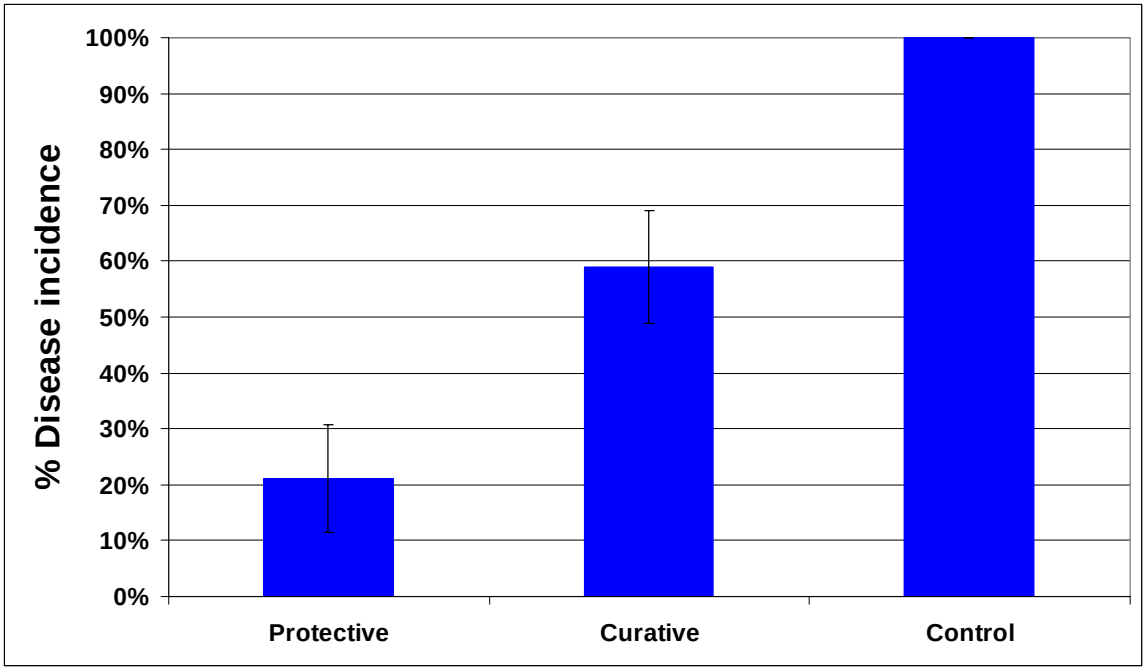

Figure 3-13. Efficacy of 54 ppm PAA (StorOx®) as either a protective or a curative agent of tomatoes inoculated with 6 $\log_{10}$ cfu/ml *E.c.c.* at 22°C.

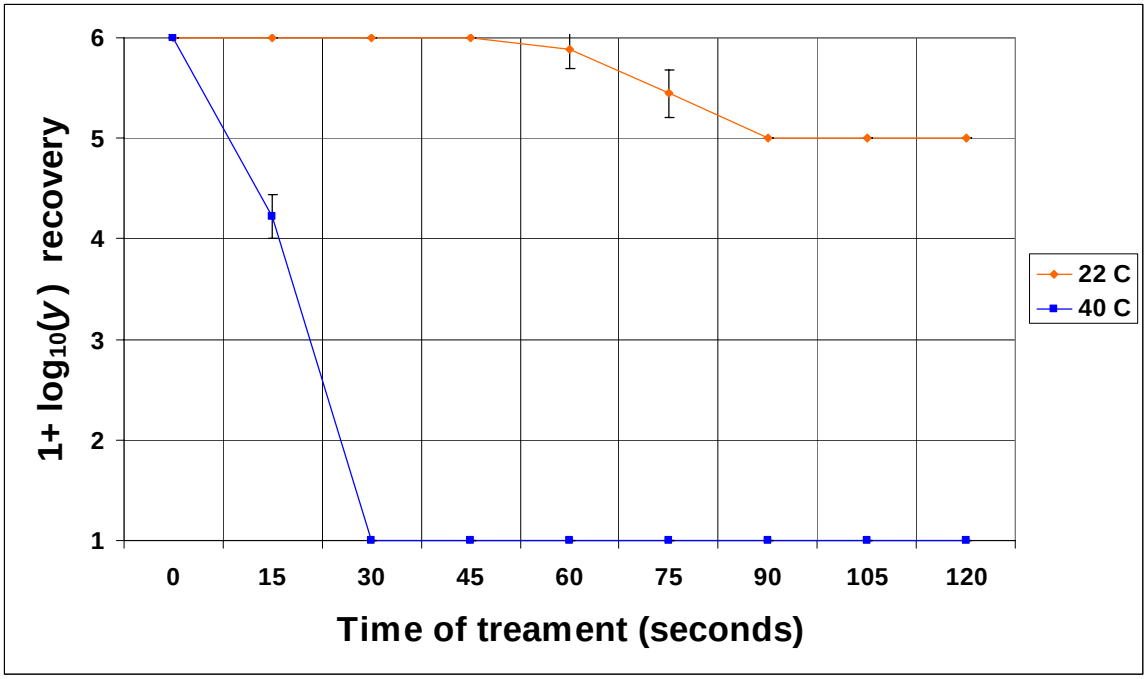

Figure 3-14. Efficacy of 80 mg/L Tsunami® brand PAA at pH 4 against 5 $\log_{10}$ propagules/ml *R.s.*, in vitro, at two temperatures (22 and 40°C). Tests show growth as compared to template plates after 48 h incubation on APDA plates at room temperature.

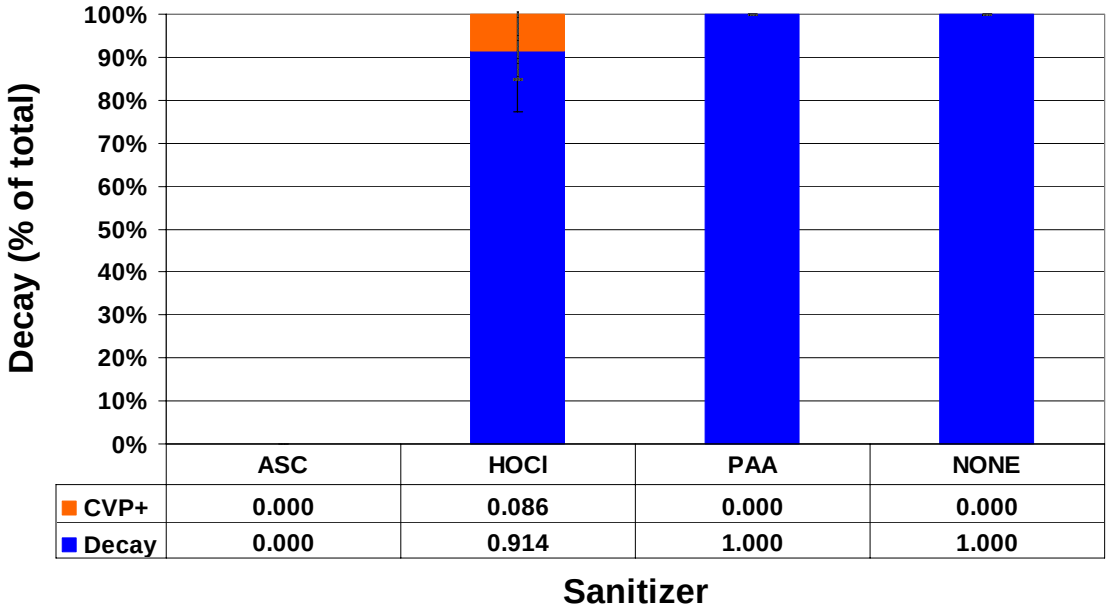

Figure 3-15. Efficacy of three biocides against further decay by 7 $\log_{10}$ cfu/ml *E.c.c.* inoculated tomato fruit wounds Biocide concentrations used were (mg/L): ASC = 1200; HOCl = 200; and PAA = 80 (Tsunami®). Negative wounds were extracted for recovery and plated to CVP. Positive CVP results were included as a percent of total wounds inoculated.

CHAPTER 4
CONTROL OF POSTHARVEST DECAY OF TOMATOES WITH FUMIGATION BY CHLORINE DIOXIDE

Introduction

A potential alternative, or addition, to use of HOCl as a sanitizing treatment in packinghouses is to directly treat fruit, either before or after packing, with a $ClO_{2(g)}$ treatment. ClO_2 is considered a free radical (143), too unstable to liquefy or handle as a concentrated gas. It must be generated on-site, from an acidification of $NaClO_2$ or $NaClO_3$, or by mixing elemental chlorine with solid $NaClO_2$. The ClO_3^- feed stock is much less expensive than ClO_2^-; however, only large-scale users such as the paper pulp industry can manage the ClO_3^- process, where elemental chlorine is produced as a contaminant. Small scale users, such as food processors, depend on $NaClO_2$ as a source for ClO_2. Although the ClO_2^- is relatively non-hazardous, the production process utilizes either concentrated mineral acids such as hydrochloric or sulfuric acid, or compressed chlorine gas. Additionally, the ClO_2 produced is concentrated enough to constitute an additional hazard. This generation is entirely contained and must be carefully and continually monitored.

A second way to produce $ClO_{2(aq)}$ is to acidify (to pH 2.4) a dilute aqueous solution of sodium chlorite with citric acid. Sanova® is described as acidified sodium chlorite (ASC) product. In a series of reactions, the ClO_2^- is converted first to $HClO_2$, which is then converted to ClO_2 (see discussion in chapter 3). The $HClO_2$ has considerable antibacterial activity, but there are questions about its activity as a fungicide.

A more recent development in ClO_2 production methods is the mixing of dry materials in a humid environment. A dry activator (ferrous salt) and sodium chlorite ($NaClO_2$, propriety process of ICA TriNova Corporation, Marietta, GA.) are held in separate sections of a porous, folded paper sachet. When the sachet is unfolded and then shaken to mix the chemicals, $ClO_{2(g)}$ is produced over a specified period of time, if the relative humidity is equal to or greater than 30%.

For a gas, ClO_2 is relatively soluble in water. Bacterial deposits or colonies are known to be hydroscopic and usually moist. When ClO_2 is released into an enclosed room, it appears likely to dissolve in water deposits and bacteria in those deposits are likely to be inactivated. ClO_2 is highly reactive to hydrocarbons, including ethylene (143), which is used in the tomato (*Lycopersicon esculentum* Mill.) ripening process. Therefore, a ClO_2 gas treatment will have to occur prior to application of ethylene, perhaps before being run along the packing line.

ClO_2 was tested for its ability to disinfect inoculated wounds on tomato fruit. The initial tests were conducted with a few fruit in an enclosed aluminum cooker/canner. Next, scale-up tests were done on boxes of fruit to determine the ability of the gas to penetrate fiberboard and packed fruit. Finally, tests were performed to evaluate the benefit of multiple biocide treatments to enhance curative effects.

Use of chemical control methods for the reduction of decay seen in tomato fruit, postharvest, is a common and essential method of maintaining fruit wholesomeness. Of interest would be to know if the use of multiple chemical methods, or other control methods, would have an additive, synergistic, or no effect on the decay seen in the final product.

Materials and Methods

Pathogen Preparation

Pathogens used and procedures for their preparation are described in the materials and methods section of chapter 3.

Fruit

Green tomato fruit were donated by the DíMare Corporation, Tampa, FL. (varieties were unknown; when reported, fruit were of the variety 'Florida-47'), a commercial fruit packer. Fruit were stored in a reach-in chamber at ~12.5^{o}C and 85% relative humidity prior to use. Fruit were stored at room temperature (ambient laboratory temperature was from 20-24^{o}C, most often 22^{o}C, depending on time of day and season) overnight before each experiment was run. Fruit used for *Rhizopus* and *E.c.c.* tests were green to pink, with preference given to green fruit, to match tomato growth stages seen in packinghouses. Light red to red fruit were used in tests with *G.c.c.* (28;29). Fruit stage determination was done according to a standard fruit color chart (Florida Tomato Committee, Appendix B) (48).

Inoculation

A scalpel was used to remove a strip of epidermis from the fruit equator in 4 to 10 evenly spaced locations. The resulting wounds were ca. 2 mm^2 with a depth of ~1 mm. One 10 µl drop of a test pathogen suspension was placed on each wound of all fruit for inoculation. Negative control fruit were wounded, but not inoculated. Likewise, positive control fruit were wounded and inoculated, but remained untreated. In certain tests, fruit were wounded and then briefly dipped into a pathogen suspension for inoculation. After inoculation, the fruit were allowed to dry for 30 min at room temperature before treatment.

Production of Chlorine Dioxide Gas

ClO_2 sachets were supplied by ICA TriNova Corporation, Marietta, GA. Mixing the contents of a sachet led to the production of ClO_2 over a 2 or 24 h period, depending on the design of the sachet used and the test for which it was intended. Release curves showing ClO_2 production, in mg, over time were supplied by ICA Trinova (figures 4-12 and 4-13).

Figure 4-1. Modified pressure cooker for gas treatments

Treatments

Fruit were placed in an aluminum 21 L pressure cooker, the lid of which had suspended from it a plastic box fan (figure 4-1). The wires for the fan were connected to a standard wall outlet through a rubber plenum inserted in place of the safety pressure-release value. In preliminary tests, a Watchdog temperature/relative humidity monitor (WatchDog™ model 250, Spectrum Technologies, Inc., Plainfield, IL) was placed inside of the chamber with five red or green tomato fruit. By 1 h, the R.H. within the chamber was 77%. With 10 fruit, the 1 h R.H. exceeded 80%. In both tests, the ambient R.H. in the

laboratory was ≥30% and was thus considered in the range for production of ClO_2. For experimental trials, fruit were placed in the chamber and the sachets were activated and then taped to the inside lid of the container. The lid was attached and then twisted to seal the chamber. A timer was started and the chamber was left undisturbed and unopened for the duration of the experiment. After the set time period expired, the chamber was opened and the fruit removed and placed on a tray for observation. The tray of tomatoes was stored at room temperature, inside of a plastic bag to maintain high humidity. Fruit were observed daily for decay and were removed as needed to prevent secondary spread of disease.

Tests with *E.c.c.* were repeated with six fruit per treatment, 10 wounds per fruit. The repeated tests were handled as replicates in statistical analyses. Fungal experiments consisted of three parts: five fruit at one ClO_2 concentration, five fruit at a second ClO_2 concentration, and three fruit that were left untreated. Untreated controls remained on the incubation tray during treatments. After treatment, the fruit were removed and placed on a tray stored in a humid chamber for incubation and observation.

As a known biocide treatment for comparison, three different sets of wounded tomatoes were inoculated with *E.c.c.* and then, 60 s later, flooded with ~5000 mg/L bleach solution (0.5%). These fruit were immediately placed on trays which were inserted in plastic bags, for observation.

Tests based on six fruit inoculations in the 21 L sealed container were scaled up to more closely simulate a packinghouse situation, with larger volumes of treated fruit and larger potential sinks for the ClO_2 gas. Wounded and inoculated fruit were randomly placed in a standard 11.34 kg (25 lb.) corrugated fiberboard shipping box, along with

enough unwounded fruit to fill the box, which was then placed in a 208.2 L (55 gal.) poly drum-liner. A small electric fan and an 88 mg ClO_2 sachet designed to produce a similar amount of product (figures 4-12 and 4-13), as compared to the volume of the bag versus the volume of the initial sealed test chamber, was activated and placed in an empty fruit box, in the drum liner, on top of the box containing the fruit for treatment. The drum liner was then filled with air, using a rubber tube and a lab air supply, to fill the volume of the liner before it was sealed for the test. The number of boxes and the number of sachets in the drum liner scale-up tests were varied. Tests were performed with one or three boxes of fruit, one or six of the 88 mg release sachets, at 2 or 24 h treatment times.

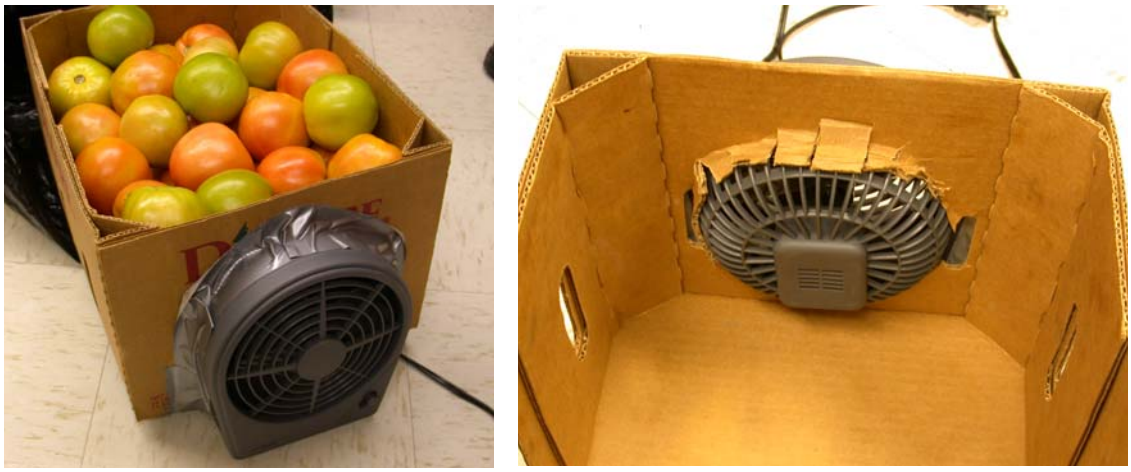

Figure 4-2. Design of forced air fan in corrugated fiberboard box.

Tests were performed to determine if the efficacy of ClO_2 is hindered by the corrugated fiberboard boxes used to pack tomato fruit. To simulate the physical barrier of a fiberboard box, as a scale-down test in a 21 L chamber, one of three sets of fruit were wounded, inoculated and placed in a brown paper bag that was loosely rolled shut and placed in the chamber. The second set of fruit was loosely placed on top of the paper-bagged fruit, in the same chamber. The chamber was sealed and the fruit treated with a 0.75 mg/2-hr sachet. A third set of fruit were treated in a second sealed container, also

with a 0.75 mg sachet, but with no paper bag, as a control. A fourth and fifth set of wounded and inoculated fruit were run along side the treatment as a set of controls; one remained untreated after inoculation and the other was treated with a 100 mg/L HOCl bath.

Figure 4-3. Forced air treatment system.

To verify that air movement was enough to bring vapor of ClO_2 in contact with boxed test fruit, a forced air movement system was designed. A single corrugated fiberboard box was excised at one end, where a six inch electric fan was taped to the box such that it sucked exterior air into the box (figure 4-2). Wounded, inoculated fruit were placed in the box with enough intact fruit to fill it. The box was then bagged in a 208.2 L (55 gal.) poly drum-liner with a sachet placed outside the box, directly in front of one intake vent (figure 4-3). The fruit were treated for 2 h with one 88 mg sachet.

Figure 4-4. Fruit treatment with corrugated fiberboard used as a competitive sink for $ClO_{2(g)}$, before or after fruit added to chamber.

To determine if air movement or interactions with the fiberboard had an effect on treated fruit, fruit were retested in the 21 L sealed chamber, with pieces of corrugated fiberboard also in the chamber. The first test had the fiberboard from one broken-down box, sans lid (figure 4-4) and as much fruit as the chamber would hold (almost all the fruit from one 11.3 kg box), and six wounded, inoculated fruit, randomly dispersed among the other fruit. A test with the same volume of fruit and the same number of wounded, inoculated fruit was also run, with only one piece of fiberboard among the fruit. A test with no fiberboard in the chamber with the fruit was also run as a control.

Tests were then performed to eliminate corrugated fiberboard as a variable from the larger scale test. Boxes were replaced with plastic onion bags, each still containing 11.3 kg (25 lbs) of fruit, including six that were wounded and inoculated with *E.c.c.* Different numbers of bags and different numbers of sachets were used: one or three bags; one, two or three sachets at 88 mg release over 2 or 24 h.

For multiple hurdle testing, three fruit were each wounded five times and inoculated at each wound with one 10 µl aliquot of 8 $\log_{10}$ *E.c.c.*, for each treatment. Fruit were then allowed to incubate for 30 min, followed by treatment for 2 min in a simulated

flume tank containing either DI·H_20, or 200 mg/L HOCl. Treatments were followed by either a 30 s rub treatment of 100 mg/L ASC, 1200 mg/L ASC, or no second treatment. Finally, two sets of fruit were fumigated using ClO_2 vapor at 99 mg release in 2 h as a final treatment, after being treated with water and (subscripts indicate concentration in mg/L) ASC_{100}, or $HOCl_{200}$ and ASC_{100}.

After treatment, fruit were placed on a tray, in a plastic bag that was loosely sealed. A moist paper towel was also placed in the bag, to maintain a high humidity (even though the respiration of the fruit tends to maintain 100% R.H. within the bag). The tray was stored at room temperature and fruit were observed for decay at 24 h and again at 48 h.

Fruit Evaluation

Fruit were observed and evaluated for decay at 24 hr intervals. Water soaked tissues with loss of structure (soft or sour rot), or with signs of the pathogen, were considered positive for decay. In certain tests, diseased tissues were removed from wounds to minimize spread of disease throughout the fruit. This technique was primarily required in tests with *R.s*. If decay had progressed such that the status of the non-infected wounds had been compromised, the entire fruit was discarded.

For tests in which no decay was observed in 7 days, a randomly selected representative population of the inoculated wounds was sampled by slicing a 1-2 mm thick piece of the tissue, and plating them on crystal violet polypectate (CVP) media, or acidified potato dextrose agar (Difco), to find if cells of *E.c.c.* or spores of *G.c.* or *R.s.*, respectively, survived the biocide treatment.

Results

Bacterial soft rot lesions were observed in all positive control wounds, save one (in one trial), by 72 h after inoculation with *E. c. c.* and storage at 22°C. In contrast, <5%

decay was observed among fruit treated with 7.5 mg ClO_2 released over 2 hr and ~10% was found among fruit treated with 0.75 mg over a 24 hr period (figure 4-5). These were neither significantly different from each other, nor from untreated control fruit (P<0.001).

G.c.c. inoculated controls did not statistically (P<0.001) produce 100% decay in red fruit, unless the inoculum concentration was at least 4 $\log_{10}$ propagules/ml. At this level, ClO_2 concentrations of 0.7, 1.095, 2.02 and 2.81 mg were able to reduce decay incidence to near zero (not significantly different from negative control) (figure 4-6). Treatment with 0.7 mg ClO_2 were observed to have 20% decay when *G.c.c.* inoculum concentration was 5 $\log_{10}$ propagules/ml. Likewise, inoculation of control fruit with *R.s.* was only able to induce 100% statistical decay if the initial inoculum was 4 or 5 $\log_{10}$ (figure 4-7). When treated with 2.81 mg ClO_2, these fruit showed no decay.

24% of the wounds on fruit that were in the paper bag (figure 4-8) had decay, when observation of the tested fruit was terminated five days after treatment. Within 24 h after treatment the untreated, inoculated, fruit were 100% decayed. The HOCl treated fruit showed 88% decay within 72 h. No decay was noted in the inoculated fruit treated in a gas chamber without a paper bag present, nor in the fruit treated in the chamber with (but not in) the paper bag.

When fruit were treated in scale-up tests (figure 4-9), 72 h after treatment all tests had 30% to 50% wound decay; none of these were statistically significantly different (P<0.001). This included the single box test with forced air delivery system of the ClO_2 vapor.

In the test with pieces of fiberboard in the 21 L chamber, 100% of the inoculated wounds decayed after treatment. A similar test with only one piece of fiberboard (~20 g) also showed 100% decay. The test with no fiberboard had no decay.

In tests utilizing onion bags instead of corrugated fiberboard, the one bag and two sachets with a release time in 2 h was the only treatment to show no decay after 72 h of incubation (figure 4-10). When there was more than one sachet, regardless of the number of bags of tomatoes tested, decay was 10% or less. If there was only one sachet in the treatment, decay was over 50% by 72 h.

Multiple hurdle tests with fruit treated with water control or with HOCl at 200 mg/L had 100% decay 48 h after treatment (figure 4-14). Fruit treated with 1200 mg/L ASC, regardless of if the initial treatment was HOCl or water, had 0% decay upon termination of the experiment. If fruit were treated with 100 mg/L ASC, half of the inoculated wounds were decayed at 48 h; the initial treatment did not make a difference on the results. Fruit treated with 100 mg/L ASC after treatment with water or with 200 mg/L HOCl, with a final treatment of $ClO_{2(g)}$, showed 10% decay if chlorinated or 20% decay if in water for the initial treatment. This difference was not significant at $P<0.05$.

Discussion

Any differences in the data from tests of *E.c.c.* vs. ClO_2 at low concentrations (figure 4-5), in the 21 L chamber, were based on concentration, not application time. Based on these results, for ease of future tests and considering time restrictions on packinghouse facilities limiting the practical use of a 24 h treatment, subsequent trials were performed as 2 h tests only, unless otherwise indicated.

The concentration of ClO_2 that reaches inoculated wounds of the product is in question. Sachets produce a known volume of ClO_2 over a given period of time; however,

the percentage of this quantity that dissolves in the cell sap over wounds is unknown. Theoretically, the volume of wounds per weight of tomato could be an important ClO_2 application factor.

ClO_2 readily dissolves in water (143). Fresh wounds on tomato fruit are moist, thus ClO_2 will dissolve into them. The rate of this is unknown and the rate of reaction with competitive sinks is unknown. In the tests reported here, the number of fruit and the number of wounds on each fruit were not necessarily consistent; this may affect the portion of ClO_2 delivered to each wound. Also, no data was taken to quantify the amount of moisture present in or on a given wound. Therefore, it is unknown if the same concentration of ClO_2 was introduced into each wound, for each test, even when the same amount of ClO_2 was introduced into similar systems. Any ClO_2 that was produced, however, likely did dissolve into tomato wounds, as there were no competing reaction sites. The exception to this is corrugated fiberboard, which was tested separately.

When corrugated fiberboard was introduced as a sink, the ability to reduce decay was itself significantly reduced, presumably due to less ClO_2 reaching the wound site to react and deactivate any pathogens present. Delivery of ClO_2 to wound locations was tested and, even through use of a forced air system, if fruit were in fiberboard boxes during treatment insufficient biocide was reacting with pathogens at inoculated wounds, allowing decay to occur.

Fruit treated in the 21 L chamber with any amount of fiberboard present showed decay. The same volume and density of inoculated fruit in the 21 L chamber, with no fiberboard present, remained decay-free after treatment. This suggests that air movement was sufficient to reach any wounds in the system, unless an alternate sink was present.

Corrugated fiberboard is a powerful enough sink, even in small volumes, to react with enough ClO_2 vapor to disallow sufficient treatment levels from reaching inoculation points to cure fruit of decay. Thus, effective treatment of fruit that have been packed is unlikely, as the high volume of corrugated fiberboard would react with the ClO_2, scrubbing it out of the atmosphere prior to any positive effects on wounded tomato fruit. Treating tomatoes in the ethylene chamber or in transport trucks, after packing into corrugated fiberboard boxes, would thus have little efficacy as a disinfectant. Treatment prior to the packing line may offer an opportunity. Tests on fruit still in field bins warrant investigation.

As more fruit are treated, the amount of ClO_2 that seems to be necessary to reach, affect and cure each inoculated fruit is also increased. Tests with larger amounts of fruit and altering concentrations of ClO_2 gas will be needed to determine the most effective concentrations to use when known levels of fruit are intended for treatment. Tests to compare the effect of excess wounds on fruit as added sinks for ClO_2 should also be carried out.

Fruit treated with ClO_2 at elevated concentrations may show a bleaching effect on stem scars, blossom scars and some surface imperfections of tomato fruit (such as “zippers” or “cat faces”, etc.). This was not seen in negative control treatments and is consistent with bleaching seen when using ASC, which also generates ClO_2. Wounds were first noted as sites of bleaching effects when tissues were observed to become desiccated, white and sunken during treatment, but only at wound sites or on residual stem or calyx tissue (figure 4-11). This was also seen in the calyx of strawberries treated with ClO_2 (*unpublished data*); (58). Stem scar whitening as a result of treatment suggests

high localized activity of ClO_2, resulting in fruit that may be considered 'cleaner' looking and may be more desirable to consumers. Consumer opinion surveys are warranted.

Figure 4-11. Phytotoxic effect of ClO_2 gas on fruit wounds. A) On wounded tomato fruit. B) Phytotoxcitiy causing bleaching of stem and stem scar (right), compared to control (left).

At higher concentrations, the stem scar bleaching may involve tissue losses. In any case, the intact cuticle was not affected by high ClO_2 concentrations. It can be hypothesized that the effect of the ClO_2 on the fruit tissue is a penetrating desiccation, depth and severity depending on C x T values. This seems to be consistent with the curative effects the treatment has on inoculated wounds. As the wound dries, pathogens may become trapped in tissue that it cannot metabolize, or the cells themselves may be killed.

Readings were difficult to make for *R.s.* tests due to this pathogen's aggressive growth. Positive wounds often would spread, via mycelium or airborne conidia, to other wounds that previously remained negative. Wounds that were positive, but suspected of being contaminated after treatment, were not counted as part of total decay. Removal of wounds decaying due to post-treatment cross contamination gave varying degrees of containment from further spread. Pathogens were not wholly excised from some of the wounds and were still able to spread to other, previously negative wounds. Alternately, in

several of the positive controls, decay from *R.s.* infection did not develop. Results suggesting that *R.s.* was well controlled are thus suspect. It is unknown at this stage if *R.s.* trials that showed poor control were factors of cross contamination from survivors, or if trials that showed good control were due to poor inoculation. Failure of aqueous ClO_2 to control *R.s.* in previous tests do not support successful curative effects here. Factors resulting from the different modes of delivery must be further explored before conclusions can be drawn.

G.c.c. results are similarly unreliable, in that inoculation results were inconsistent. It is not known whether the results suggesting good control of *G.c.c.* are due to the chemistry of ClO_2 or if they are due to poor inoculation, as evident in low decay rates of several of the untreated positive controls.

Compared to the results seen in the earlier tests in this paper, the multiple hurdle results were not unexpected (figure 4-14). Treatment with high levels of ASC was very effective at curing inoculated fruit, as were treatments with ClO_2 vapor; HOCl alone was not effective in curing fruit. The use of the most effective biocide alone was as effective as the use of that biocide, plus other biocides, in curing fruit. Based on these results, there were no additive or synergistic effects when multiple biocides were used against inoculated fruit as curative agents.

Figures

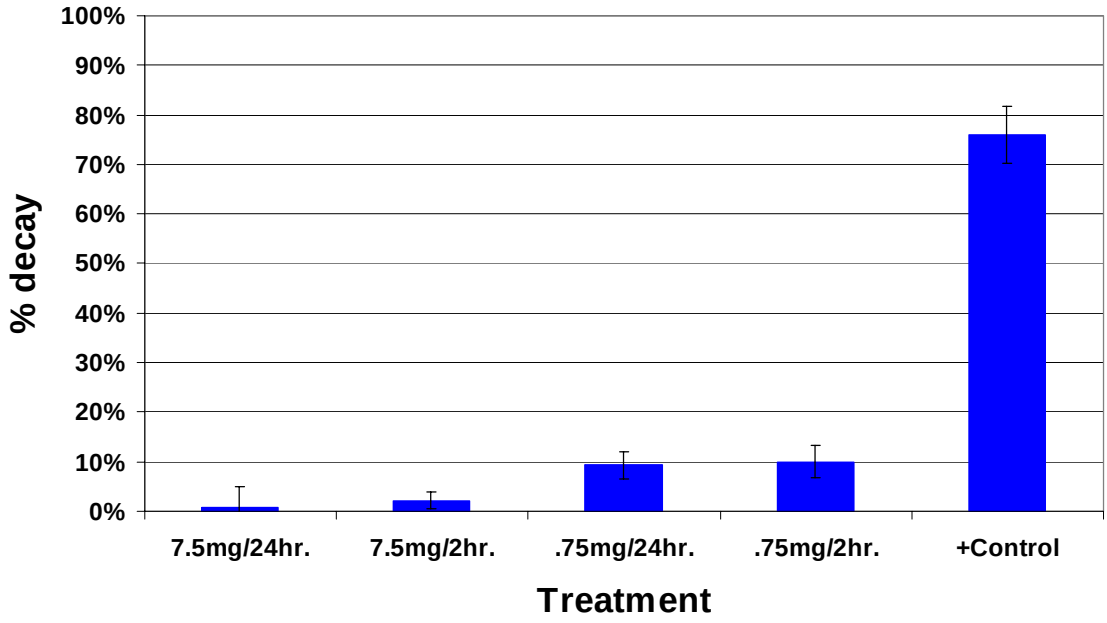

Figure 4-5. Wounded fruit inoculated with 8 $\log_{10}$ cfu/ml *E.c.c.* vs. ClO_2 gas used as a fruit curative agent. Treatments were performed in a sealed 21 L aluminum pressure cooker (figure 4-1), after which fruit were moved to trays in loosely sealed plastic bags to maintain R.H. ClO_2 gas applied to six fruit per rep at one of two concentration levels (7.5 mg and 0.75 mg) over one of two time periods (2 h or 24 h). Uninoculated control fruit showed no decay at termination of observations (72 h).

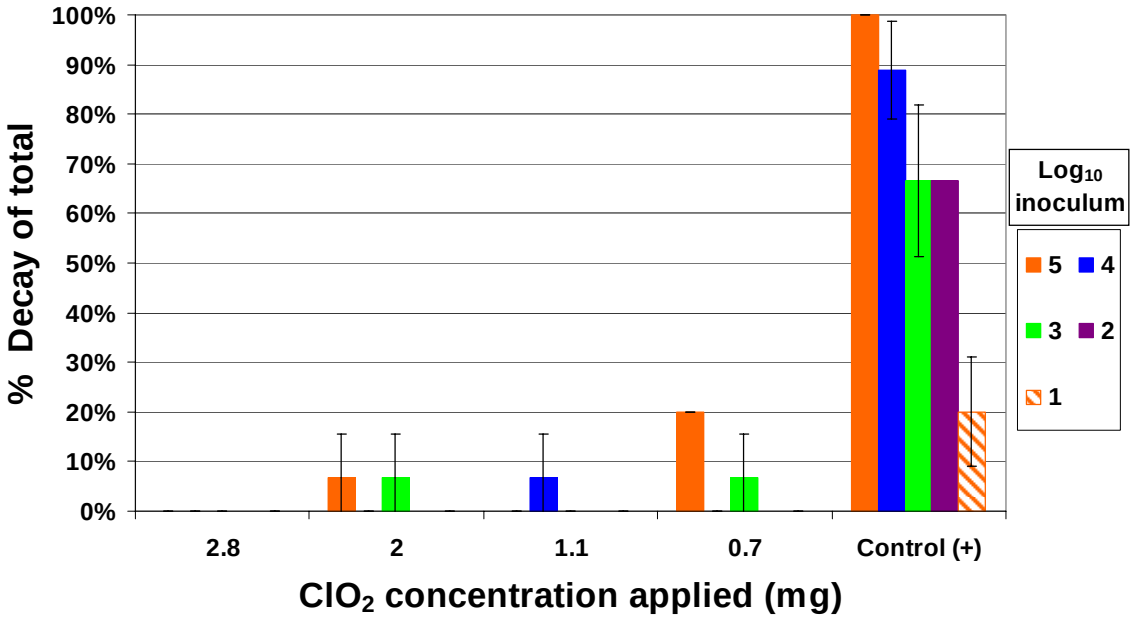

Figure 4-6. Efficacy of ClO_2 gas during a 2 h release as a fruit curative agent vs. 5 concentrations of *G.c.c.* (1-5 $\log_{10}$ propagules/ml) Fruit were wounded, inoculated, treated in a 21 L aluminum pressure cooker then incubated on a plastic tray stored in a loosely sealed plastic bag to maintain R. H. Uninoculated fruit showed no decay.

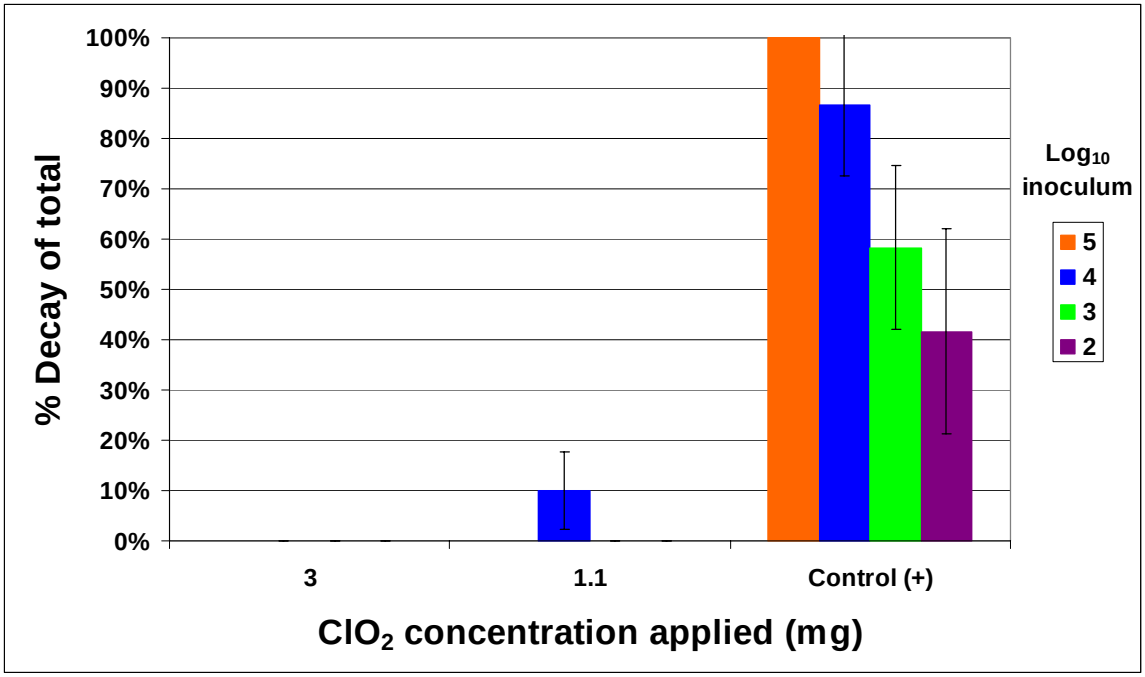

Figure 4-7.Efficacy of two concentrations of ClO_2 against four concentrations of *R.s.* (2-5 log_{10} propagules/ml), when used as a fruit curative agent in a 2 h release treatment. Fruit were wounded, inoculated, treated in a 21 L aluminum pressure cooker then incubated on a plastic tray stored in a loosely sealed plastic bag to maintain R. H. Uninoculated fruit showed no decay.

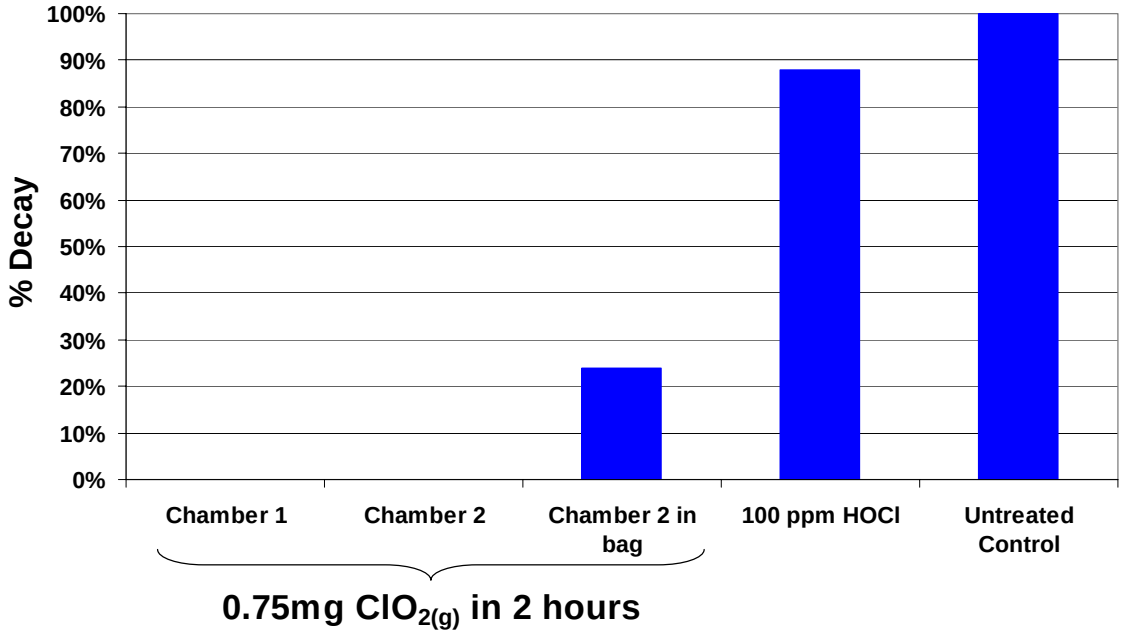

Figure 4-8. Efficacy of 0.75 mg ClO_2 against 8 $\log_{10}$ cfu/ml *E.c.c.* inoculated fruit treated in a sealed 21 L pressure cooker. Fruit were alone in the treatment chamber in one test. In the second test, fruit were in two sets, one in a loosely closed brown paper bag, the second resting on top of the brown paper bag the first set was in. Repeated three times, the first treatment had ten fruit with five wounds each and both sets in the second treatment had five fruit each, similarly wounded and inoculated. Decay given at termination (5 days after treatment).

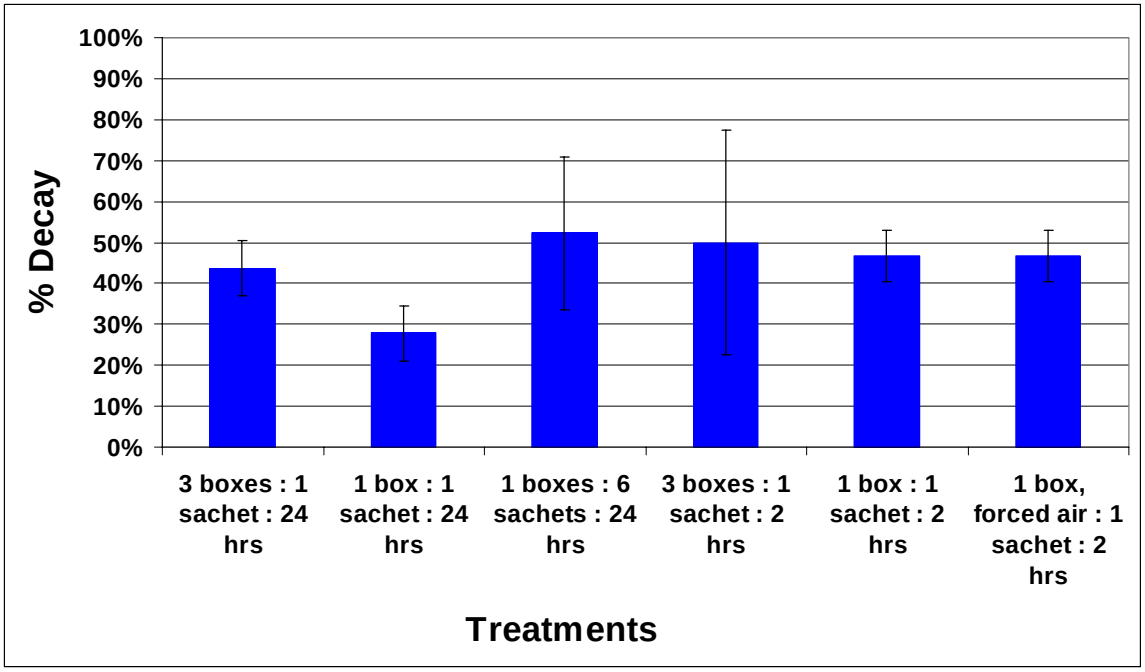

Figure 4-9. Efficacy of ClO_2 vapor as a curative agent of wounded green fruit inoculated with 8 $\log_{10}$ cfu/ml *E.c.c.* Test fruit were placed in corrugated fiberboard boxes, with enough unwounded fruit to fill each box, then placed in a 208.2 L (55 gal.) drum liner for treatment. Boxes were stacked up to three high, with an extra box containing a fan for air circulation and sachets as a source of ClO_2 vapor. Treatments consisted of sachets producing either 99 mg of ClO_2 vapor over 2 h, or 88 mg ClO_2 vapor over 24 h. After treatment, inoculated fruit were recovered and stored at room temperature on a tray in a loosely sealed plastic bag, to maintain R.H, for observation. Readings were taken 72 h after treatment.

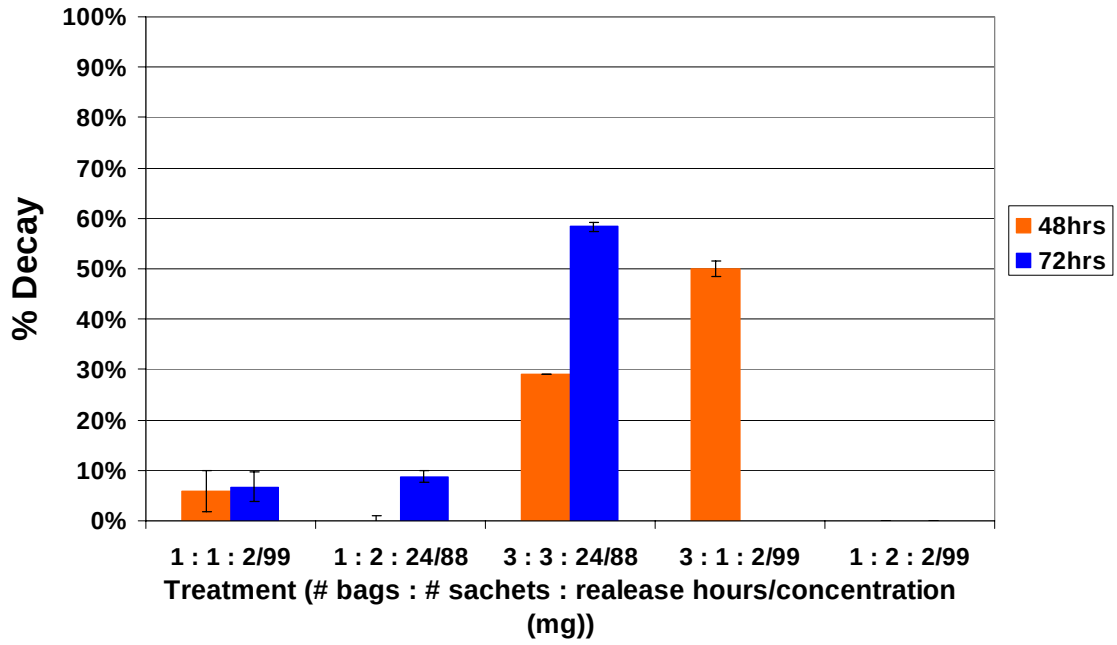

Figure 4-10. Efficacy of ClO_2 vapor as a curative agent of wounded green fruit inoculated with 8 $\log_{10}$ cfu/ml *E.c.c.* Test fruit were placed in mesh bags, with enough unwounded fruit to fill each bag, then placed in a 208.2 L (55 gal.) drum liner for treatment. Bags were stacked up to three high, with a fan for air circulation and sachets as a source of ClO_2 vapor placed on the top bag. Treatments consisted of sachets producing either 99 mg of ClO_2 vapor over 2 h, or 88 mg ClO_2 vapor over 24 h. After treatment, inoculated fruit were recovered and stored at room temperature on a tray in a loosely sealed plastic bag, to maintain R.H, for observation. Readings were taken 72 h after treatment.

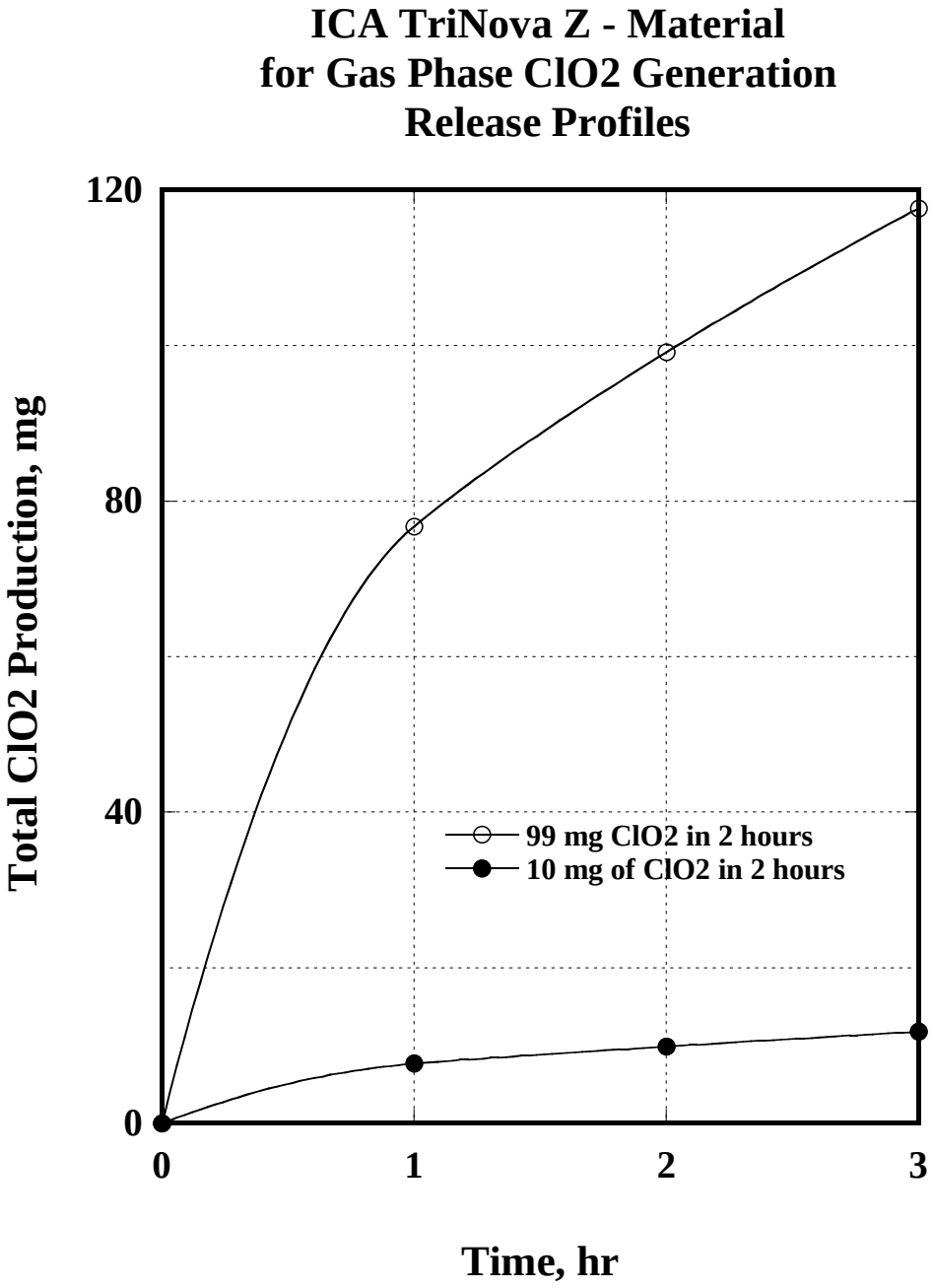

Figure 4-12. Release profile for 2 h ClO_2 sachets.

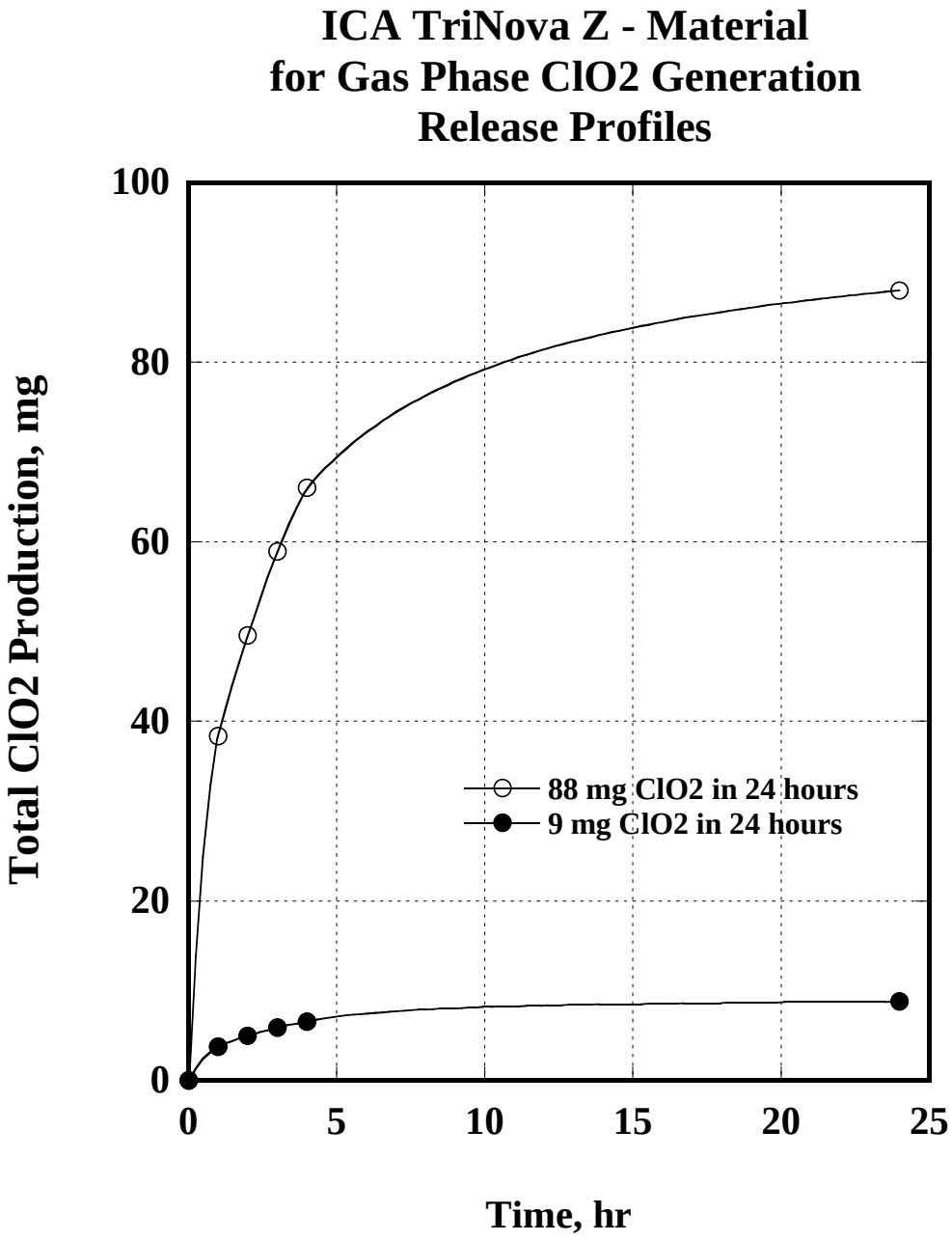

Figure 4-13. Release profile for 24 h ClO_2 sachets.

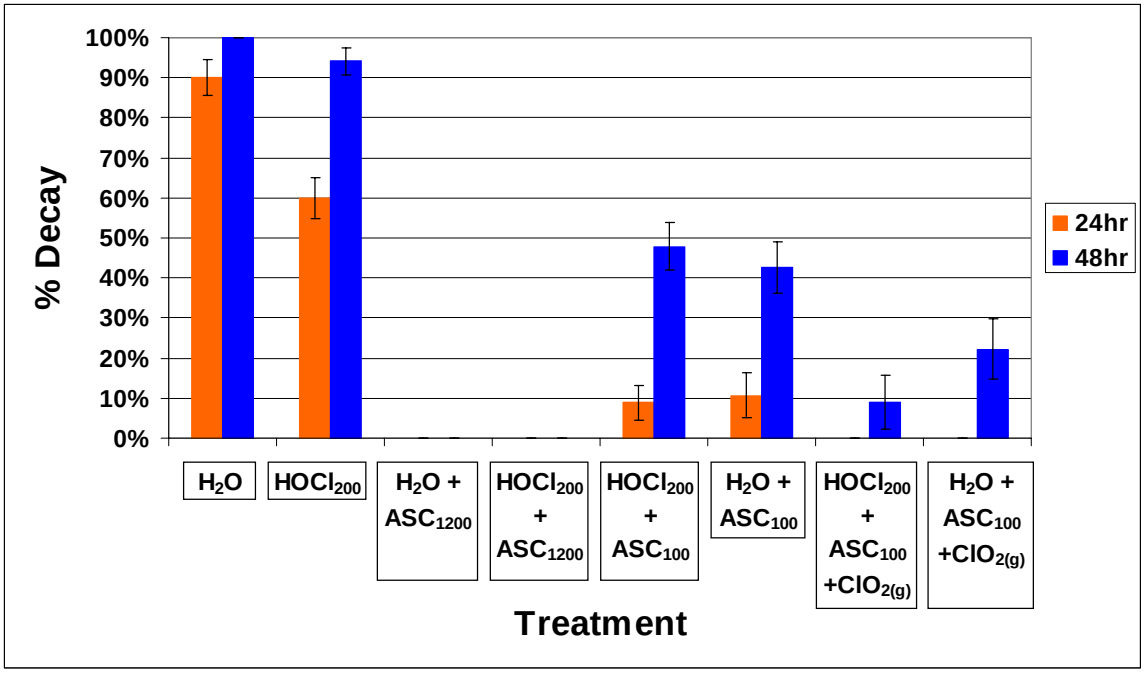

Figure 4-14. Multiple hurdle treatments. Wounded fruit inoculated with 8 $\log_{10}$ cfu.ml *E.c.c.* were treated with one or multiple biocidal treatments after inoculation. H_2O or HOCl treatments were simulated flume treatments for two minutes; ASC treatments were 30 s rub treatments and gas treatments were 88 mg over 2 h sachet treatments Fruit were stored on trays in loosely sealed plastic bags, to maintain R. H., after final treatment. Subscripts refer to a treatment concentration.

CHAPTER 5
CONCLUSIONS AND DISCUSSIONS

The summation of these tests suggests that continued use of chlorine as a protectant is a viable flume tank treatment, however, curing bacterial or fungal contamination in fruit should not be expected. Alternate chemistries, as studied within this paper, are not necessarily more effective than HOCl. With any potential biocide, above and beyond the efficacy of the chemistry, a cost and safety analysis, which is out of the scope of this work, should be considered as a factor to determine qualifications for use.

Specifically, a PAA solution may be a viable alternative, although its efficacy is not significantly greater than that of HOCl and its ability to control fungal pathogens may be less; further investigations into application methods of PAA are needed to determine if a method of delivery can be found to increase efficacy. Currently, ease of use and cost comparison should be the determining factor in deciding between continued HOCl use and replacement with PAA as a protectant.

It is possible that an aqueous solution of ClO_2 will be beneficial as either a protectant or as a curative agent. The limiting factor in deciding between continued use of HOCl or replacement with $ClO_{2(aq)}$ will be delivery methods, ease and safety of use, cost and secondary effects on equipment (aqueous ClO_2 as a generated gas, or as part of ASC, is known to be more corrosive to metals than is hypochlorite in any of it's salt or acid forms (143); the low pH required for generation and efficacy suggests this as well). The rapid off-gassing of ClO_2 from solution will have an effect on atmospheric conditions and must be addressed to comply with worker safety and environmental protection standards.

In these studies, the first tests consisted of a pathogen and a biocide in direct contact for different time periods, in vitro. Biocidal efficacy was maximized and kill rates were fast, as expected. Second, pathogens were inoculated on wounded fruit either prior to biocide application (curative), or while fruit were already suspended in a biocidal solution (protective).

In the first series of fruit tests, a biocide was present in an aqueous system with wounded fruit, prior to the introduction of a pathogen. When the pathogen was added to the system, the biocide was able to interact with the pathogen during the time period from when the pathogen was first introduced, until the point when the pathogen was able to internalize into a fruit's tissues via a wound. If this time was enough to fulfill the C x T value of the biocide/pathogen system, the biocide reduced the pathogen population by 99% or more and the fruit was protected from decay. If the biocide had a greater requisite 'T' value, either due to a lower 'C' value, a higher pathogen population, or due to a longer rate of reaction, the pathogen was often able to survive long enough to reach a wound, internalize, become protected and subsequently replicate and cause decay.

The second set of fruit tests involved a pathogen already inoculated onto fruit tissues, where the activity of a biocide may or may not allow contact between the pathogen and the biocide. For HOCl and PAA solutions, the biocide was incapable of coming into contact with the pathogen, or did not contact the pathogen for a long enough interval, effectively reducing 'T' to zero and thus reducing efficacy to zero: fruit decayed. $ClO_{2(aq)}$ was able to penetrate into the wounds on tomatoes enough to allow contact between the biocide and the pathogen, fulfilling the C x T values and reducing the pathogen population enough to avoid fruit decay.

The objective of utilizing gas phase ClO_2 as a separate treatment in lieu of relying on dump tank treatments alone has shown promising results. As was determined within this study, ClO_2 is highly effective in reducing decay in inoculated fruit, but certain compounds and materials appear to be ClO_2 sinks. For example, the corrugated fiberboard used for packing tomatoes greatly reduces the efficacy of the treatment against wound-inoculated tomatoes. The application of ClO_2 will therefore be highly effective when applied to fruit in environments free of alternate sinks, at the application rates appropriate to the volume of fruit treated.

Fruit sanitized prior to being run on a packing line would have a greatly reduced pathogen population. This is beneficial in reducing decay since pathogen population is directly related to incidence and severity of disease (17). The efficacy of ClO_2 is, however, not persistent and, as fruit exit the initial treatment, they are not protected from further infection (this does, however, also mean that there is less concern over effects on environmental safety). Upon dumping into flume tanks, if there has been an inoculum build-up in the water, fruit would be unprotected from becoming inoculated and subsequently decaying or causing human illness if consumed. Therefore, it is beneficial to have a treatment in the water flume to minimize or eliminate any pathogenic microbes that may be present.

As fruit are processed for packing and shipping from handling point to handling point, there are multiple points where infestation or inoculation may occur (at harvest, in a flume handling system, through contact with graders, transferred from moist and contaminated sponge or brush rollers, as they travel across either developed or developing biofilms, or other leaf litter or deposits of fruit wound exudates, etc.). A

multiple-hurdle approach to sanitation could be used to protect fruit from infestation at each of these control points.

Although a single biocide may be effective as a curative or preventive agent, it is likely to only be effective at the point of application (chemistries with residual action are undesirable on product destined for the fresh market or other unprocessed consumption). As fruit pass along a packing line and out of the application range of one biocide, a new biocide application would be necessary to avoid further infestation. An example of this is that, although highly effective, ClO_2 vapor treatments have no residual activity. Fruit treated with $ClO_{2(g)}$ prior to packing may still harbor inoculum deposits, such as any microorganisms protected in adhering leaf litter, etc. If such inoculum escapes deactivation and is subsequently deposited into a flume tank, the pathogen can there disperse and infest other fruit unless sufficient HOCl (or another biocide) is present.

After the flume handling phase, fruit are often rinsed with water that is usually chlorinated. If a disinfection failure occurs in a pre-flume gas treatment leading to contamination of flume water, or flume water is otherwise infested, a subsequent cross-contamination event is unlikely to be controlled by the HOCl rinse. Use of a more aggressive biocide in this rinse, such as ASC, may function as a viable “clean-up” application. This could serve as a chance to treat pathogens remaining due to the failure of a previous system, although test results suggest that such a treatment alone would not be effective (figure 3-10).

The multiple-hurdle concept was here only tested for use via one method: as a curative agent. Results in this study suggest that the use of one effective biocide as a curative agent is no different from the use of that same biocide in conjunction with other,

less effective, biocides. Also, a multiple-pathogen load was not tested and may have had different results: a single biocide may affect a single pathogen well, but not have activity against another pathogen. In the tests performed, only *E.c.c.* was used and all of the biocides tested showed efficacy against *E.c.c.* in vitro (Chapter 3). It is unknown if the same results would occur if a multiple pathogen cocktail was tested.

While a multiple-hurdle approach may be redundant for curing infested wounds, it can be useful as a protective measure. Also, given that it is possible for one treatment to fail throughout a production day, a multiple-hurdles approach to sanitation could serve as a fail-safe system.

The efficacy of the biocides tested here can be compared to potential use against human pathogens. Where bacterial plant decay pathogens were controlled, most likely human enteric pathogens would be controlled as well. *E.c.c.* is a member of the family *Enterobacteriaceae*, which also includes such human pathogens as *E. coli* and *Salmonella*. The control of other human pathogens may be presumed, but only direct testing will confirm this.

The morphological differences of agents such as viruses, parasites, spore-forming bacteria and others may also lead to differences in control levels. Fortunately, the only human outbreak of disease traced back to tomatoes, as of this writing, was of the genus *Salmonella*, which is very similar to *Erwinia*, as used in this study, in susceptibility to deactivation and in mode of transmittal.

APPENDIX A
LUKASIK EXTRACTION

Negative wounds in some tests were excised and checked for surviving cells that failed to initiate decay. Wounds that pitted on crystal violet polypectate (CVP) agar plates or showed visibly identifiable microbial growth on nutrient agar plates or acidified potato dextrose agar plates, depending on the microorganism tested for, were counted as positive. Positive extraction test samples were added to the experimental data as 'cells recovered'.

The recovery of cells from fruit tissue was according to the techniques of Lukasik et al. (83), who used the recovery of *Salmonella* Montevideo and *E. coli* O157:H7 from strawberries and tomatoes. *Erwinia* recovery from tomatoes was considered to be equivalent to *Salmonella* recovery, confirmed by lab testing (Mahovic and Schneider, unpublished data). Replicates were done using *Geotrichum* on tomatoes to determine validity of use for yeasts.

Materials and Methods

An agar plate of *G.c.c.* was flooded, scraped, filtered through cheesecloth and re-suspended in sterile tap water (STW). The suspension was then quantified by use of a spectrophotometer to produce an inoculum solution of 5 $\log_{10}$ propagules/ml.

Extraction tubes were prepared by combining 5 ml of phosphate buffer (pH 7) and 0.1% v/v Tween-80 in 10ml glass test tubes and autoclaving them for sterilization. Six red ripe tomato fruit were rinsed off and towel dried to remove any significant surface contaminants. Fruit were then shave-wounded five times each, approximately along the

equator, and inoculated with 10 µl of *G.c.c.* inoculum, introducing a calculated 3 $\log_{10}$ propagules to each wound (10 µl of 5 $\log_{10}$ propagules/ml). Fruit were then allowed to dry for 30 min.

After the drying period, each wound was excised from the tomato and placed in a separate extraction tube and briefly shaken on a vortex plate (Thermolyne "Maxi Mix II", type 37600 mixer, Barnstead/Thermolyne, Dubuque, IA). All of the tubes were incubated at room temperature (~21^{o}C) on a slow speed (~55 rpm) rotary shaker (Labline Dual Action Shaker, Labline Instruments, Inc.) for 20 min. After incubation in the tubes, all samples were again briefly vortexed and 100 µl of each tube sample was spread plated to APDA. Plates were then stored at room temperature and observed for growth. Colony forming units were counted and inoculum recovery as a percentage of the initial inoculum concentration was recorded. Colonies counted represent a 3.5 $\log_{10}$ dilution factor from initial inoculum, based on volumes in and added to test tubes and dilution of cells when added to agar plates.

A 10 µl sample of the initial inoculum was also pipetted directly into a 5 ml extraction tube, incubated and plated in the same manner as those recovered from fruit tissue. This will serve as the positive control and as a baseline count for the actual number of cells inoculated in fruit. This number will be used to determine percentage of cells recovered from fruit tissue.

Results and Discussion

Cell recovery from fruit and undiluted inoculum, as a control, is expressed as the number of colony forming units counted on spread inoculated APDA plates in Table A-1.

Table A-1. *G.c.c.* colony counts from APDA plates after extraction

Controls (2):	94	66								
Samples (30):	32	61	59	19	16	59	42	80	45	55
	56	30	45	44	25	13	23	70	31	52
	21	36	44	16	81	17	37	31	65	31

An average of 80 ±3.1 cells per control plate (standard deviation = 19.8, Sum of the square of the error = 3.1) was observed. Thirty test samples from fruit tissue had an average of 41.2 ±0.8 cells recovered (standard deviation = 19.3, sum of the square of the error = 0.8). Converting to inoculation concentrations calculated based on the dilution steps taken during testing (multiplying counted colonies by 3500), the average number of cells inoculated to fruit based on the control is $2.8x10^5$ (standard deviation = $6.9x10^4$). The average number of cells recovered from fruit is $1.4x10^5$ (standard deviation = $6.8x10^4$).

This suggests the loss of 0.1 $\log_{10}$ of the cells (51.50% recovery; or 48.50% loss) to error in this extraction method. When transformed to initial concentrations, this represents 94.6% recovery of inoculated cells. The viability of yeast-like fungal cell recovery utilizing this bacterial cell extraction method is considered acceptable for future cell recovery tests.

APPENDIX B
TOMATO FRUIT MATURITY CHART

GREEN
The tomato surface is completely green. The shade of green may vary from light to dark.

BREAKERS
There is a definite break of color from green to bruised fruit. Tannish-yellow, pink or red or 10% or less of the tomato surface.

TURNING
Tannish-yellow, pink or red color shows on over 10% but not more than 30% of the tomato surface.

PINK
Pink or red color shows on over 30% but not more than 90% of the tomato surface.

LIGHT RED
Pinkish-red or red color shows on over 60% but red color covers not more than 90% of the tomato surface.

RED
Red color shows on over 90% of the tomato surface.

As prepared by the Florida Tomato Committee (48)

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BIOGRAPHICAL SKETCH

Initially from Ohio, I moved to Florida with my family before I reached my first year. After high school, I attended the local community college while I worked to afford moving to Gainesville. The first summer after graduation I entered the University of Florida. Initially I was a microbiology major, but transferred to the Department of Plant Pathology to receive my bachelor's degree.

I continued with my education moving right into a master's program, working on tomato spotted wilt virus. After one year, my program was destabilized and I continued to work toward a mater's degree, non-thesis. Within a semester a new project opened up in postharvest plant pathology and I quickly started that work. I abandoned my idea of doing a PhD in Agriculture Extension Education, choosing to remain in a science-based program that would give me many more opportunities in the area that I truly love. This project became larger than a thesis and through available funds I extended it on to a dissertation.