EFFICACY OF ELECTROLYZED WATER AS A SANITIZER AND CLEANING AGENT AND ITS EFFECT ON FOOD CONTACT SURFACES

by

BEATRICE AYEBAH

Under the direction of YEN-CON HUNG

ABSTRACT

Biofilms are potential sources of contamination to food in processing plants, because they frequently survive sanitizer treatments during cleaning. This research investigated the sequential use of alkaline and acidic electrolyzed oxidizing (EO) water in the inactivation of *Listeria monocytogenes* biofilms on stainless steel surfaces in the presence or absence of organic matter. Alkaline EO water did not exert any bactericidal action on the *L. monocytogenes* biofilms, however, acidic EO water produced a reduction of 4-5 log CFU/ coupon and the sequential treatment resulted in additional inactivation. Results suggested that alkaline and acidic EO water can be utilized together to achieve a better inactivation of biofilms than when applied individually. The addition of organic matter in the form of chicken serum, to acidic EO water, decreased its oxidizing capacity and chlorine concentration. Organic matter reduced the

bactericidal activity of acidic EO water on both plantonic cells and biofims of *L. monocytogenes* and the extent of reduction was dependent on the organic load.

The effect of EO water on various materials which can be found in food processing environments was also investigated. ASTM A-36 medium carbon steel, 110 copper, 3003-H14 aluminum, polyvinylchloride (PVC) type 1, and 304 stainless steel were subjected to standardized corrosion tests in acidic EO water, chlorine water, modified EO water and deionized water. Carbon steel which had a fair corrosion resistance to acidic EO water, was the most affected material. Stainless steel, which is the most commonly used material for food processing equipment fabrication, had an outstanding corrosion resistance to acidic EO water.

A laboratory scale conveyor system was evaluated for the application of acidic EO water in the inactivation of *L. monocytogenes* biofilms on teflon and stainless steel surfaces. Biofilms on teflon were much more resistant than biofilms on stainless steel. Acidic EO water maintained a large part of its bactericidal efficacy even after being reused several times. This research demonstrates that acidic EO water can be reused several times during immersion cleaning without significant reduction in its bactericidal efficacy or the probability of recontamination from the used water.

INDEX WORDS: Electrolyzed oxidizing water, *Listeria monocytogenes*, Biofilms, Sequential treatment, Organic matter, Corrosion, Surface materials, Stainless steel, Teflon, Mixed biofilms, Conveyor system

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DEDICATION

To Charles, the Love of my life

&

To the most precious gifts God gave me,

Kaitlyn and Lady Zuriel

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Consumer awareness of the health effects of food additives used in the preservation of foods has increased, leading to the consequent demand for fresh and healthy foods with minimal alteration. This has resulted in more pressure on the food industry to limit the use of chemical preservatives and processing methods which cause gross losses in the nutritional constituents of the raw food materials. Current eating habits in developed and developing countries, which focus largely on the consumption of convenient processed foods have placed even more demands on the food industry with the increasing interest in fresh fruits and vegetables and fresh-cut produce. Since most of these are consumed raw or without significant further processing, food processors are required to modify their processing and handling protocols to meet very stringent safety requirements.

In the wake of these new trends, cleaning and sanitation has become one of the most critical operations in the food processing industry; responsible for the prevention of post process contamination and ensuring the microbiological safety and reasonable shelf life of ready-to-eat foods. Formation of biofilms on food processing equipment and food contact surfaces in the food industry presents an additional challenge in food plant sanitation, as food processors explore new sanitizers and methods for efficient chemical disinfection. These sessile communities of microorganisms have been shown to exhibit significantly higher resistance to sanitizers than their planktonic counterparts (Frank and Koffi, 1990; Norwood and Gilmour, 2000; Ayebah et al., 2006), making them potential sources of contamination that may lead to spoilage of food or the transmission of foodborne pathogens. Among the many foodborne pathogens responsible for the estimated 76 million illnesses and 5,000 deaths annually, in the

U. S. (Mead et al., 1999), *Listeria monocytogenes* is of particular interest due to its high mortality rate among those at risk, ubiquitous nature, ability to form biofilms, and the U. S. Department of Agriculture's U. S. Food Safety and Inspection Service and the U. S. Food and Drug Administration's requirement for the absence of this pathogen in a 25g sample of a given production lot of ready-to-eat foods.

Many researchers have demonstrated the ability of *L. monocytogenes* to establish itself in food processing facilities and remain members of the resident microbial flora for long periods of time, even years (Miettinen et al., 1999, Autio et al., 1999, Gunduz and Tuncel, 2006). A large potential exists for *L. monocytogenes* to contaminate food, in processing environments where they may persist in the form of biofilms. Due to the difficulty of biofilm removal and the associated high costs, the control and prevention of biofilm formation through sustained efficient chemical disinfection presents one of the practical ways of ensuring a safe food supply. The use of sanitizers however, does not come without its limitations. Several factors including, the presence of organic matter, the composition of the surface to be sanitized as well as the types of microorganisms that may be found in the processing plant, affect the efficiency of sanitizers. Chlorinated compounds in particular, may cause corrosion of food processing surfaces and equipment and their bactericidal efficacy is also reduced by organic matter. These factors, among others, must be considered in order to select the most appropriate sanitizers for food processing environments.

Among the different sanitizers recently researched and applied for microbial inactivation, acidic electrolyzed oxidizing (EO) water has been demonstrated to exhibit a strong bactericidal effect on various pathogenic bacteria. Generated on site, from a dilute solution of sodium chloride (NaCl), EO water is easier to produce and safer to handle than other chlorine sanitizers

which involve the handling and storage of high concentrations of the stock chemical. With its low pH (~ 2.6), strong oxidizing potential (>1100mV) and chlorine content as well as the advantage of being able to use it in sequential treatment with alkaline EO water without any additional costs, acidic EO water provides an alternative for efficient and economical biofilm control.

The overall objective of this study was to investigate the cleaning and disinfecting effect of EO water on *Listeria monocytogenes* biofilms and to determine its corrosive effect on food contact surfaces. This dissertation consists of six chapters. The first chapter presents an introduction and literature review. The second chapter explores the possibility of enhancing the bactericidal efficacy of electrolyzed water through the application of both the alkaline and acidic fractions of EO water. The third chapter investigates the ability of EO water to inactivate *L. monocytogenes* planktonic cells and biofilms in the presence of organic matter. Chapter four examines the potential of EO water to cause corrosion of various materials commonly found in the food processing environment. The fifth chapter involves the application of EO water in the inactivation of duospecies biofilms, the fabrication of a laboratory scale conveyor system and its use in the application of EO water for the inactivation of biofilms. Chapter five also investigates the effect of the continuous use of EO water on its bactericidal efficacy. Chapter six presents an overall summary of findings and conclusions. A collection of the bibliography used is included in each chapter and the various chapters are set forth in accordance with the style of the journal to which it was submitted.

LITERATURE REVIEW

Biofilms

Biofilms may be defined as a community of microbes embedded in an organic polymer matrix, adhering to a surface (Carpentier and Cerf, 1993, Davey and O'Toole, 2000, Bryers, 2000). This community of microbes consists of both viable and non viable cells and forms microcolonies with "water channels" between them (Davey and O'Toole, 2000). The organic polymer matrix, usually referred to as extracellular polymeric substances (EPS) is said to be microbially produced (Costerton et. al., 1987, Zhang and Bishop, 1994) and may contain polysaccharides, proteins, phospholipids, techoic and nucleic acids and other polymeric substances.

The formation of biofilms involves the initial attachment to a surface, followed by the formation of microcolonies and finally the maturation of microcolonies into an EPS-encased mature biofilm (Davey and O'Toole, 2000). In nature and food systems, microorganisms are more often than not found in the form of biofilms. Their ubiquitous nature has led to the speculation with regard to why bacteria may form biofilms instead of living as individual cells. These include i) protection from hostile environments, ii) a means of entrapment of nutrients from the environment and iii) a means of acquiring new genetic traits (Poulsen, 1999; Davey and O'Toole, 2000).

Biofilms in the food processing environment

The food processing environment particularly presents an excellent opportunity for the formation of biofilms because of the existence of conditions that are favorable for their development. The presence of microorganisms in raw foods to be processed, food and non-food

contact surfaces to which microorganisms can attach, and the availability of water and food or food residues which serve as nutrients for microbial growth and metabolism, all seem to enhance the possibility of biofilm formation.

Spoilage organisms (eg. *Pseudomonas* spp.) as well as food borne pathogens like *Salmonella* spp. (Joseph et al., 2001; Ronner and Wong, 1993), *Listeria monocytogenes* (Kim et al., 2001; Blackman and Frank, 1996; Kim and Frank, 1994) and *Escherichia coli* O157:H7 (Dewanti and Wong. 1995) possess the ability and have been documented to form biofilms on stainless steel, plastics, glass, teflon, and buna-n rubber (Blackman and Frank, 1996; Norwood and Gilmour, 1999; Kim and Frank, 1994; Allison et al., 1998; Joseph et al., 2001; Ronner and Wong, 1993).

The attachment of microorganisms and subsequent development of biofilms in food processing environments are potential sources of contamination which may lead to major food spoilage problems or transmission of food borne diseases. Miettinen *et al.,* 1999, in their work in an ice cream plant from 1990-1997, isolated *Listeria moocytogenes* from the environment (floors and floor drains) and the equipment (outer and inner surfaces of the whipping, filling, molding and packaging machines) used for processing. *L. monocytogenes* has also been isolated from processing equipment, the processing environment, raw material and finished products from meat, poultry and seafood industries in the Faroe Islands, Finland, Iceland, Norway and Sweden (Suihko et al*.,* 2002). In these and other processing facilities where pathogens have been isolated from the environment and equipment, it is believed that these pathogens might have existed and persisted in the form of biofilms

Control and removal of Biofilms

Cleaning and sanitizing are important parts of the processes that are carried out in food processing plants. The aim of these processes is to remove all food residues present after processing, as well as to reduce and / or eliminate spoilage and pathogenic microorganisms in any shape or form, be it planktonic cells or biofilms. Most chemical cleaning agents used in the food processing industry are alkali compounds which act as detergents for fat and protein (Chmielewski and Frank, 2003). These can be used in combination with wetting agents which wet and penetrate the soil making it easier to remove, sequestrants and chelating compounds which bind and remove minerals, dispersing agents which prevent redeposition and acids which remove deposited minerals (e.g. milkstone) (Chmielewski and Frank, 2003; Zottola and Sasahara, 1994)

Typical sanitizers that are applied in the food industry include chlorine compounds (hypochlorites, chlorine dioxide), organic acids (peracetic acid), trisodium phosphate, iodophors and quaternary ammonium compounds. Chlorine compounds are often the most effective and least expensive, although they may be more corrosive and irritating than alternatives like iodine and quaternary ammonium compounds (Giese, 1991; Marriot, 1999). Several factors affect the selection of appropriate sanitizers for food processing plants and these may include, the composition and amount of soil present, the types of surfaces to be sanitized as well as the types of microorganisms that may be found in the plant. The application of sanitizers is central to the hygienic control of biofilms, and several of these, including chlorinated compounds, peracid sanitizers, quaternary ammonium compounds and iodophors, have been investigated in the inactivation of biofilms (Fatemi and Frank, 1999; Frank and Koffi, 1990; Somers and Wong, 2004; Joseph et al., 2001; Ronner and Wong, 1993).

Typical sanitation programs involve the use of detergents to remove soil and the application of sanitizers to inactivate bacteria and prevent recontamination (Giese, 1991). This same sequence of sanitation has also been suggested for the effective removal and control of biofilms by Frank et al, 2003, who observed a > 7-log reduction in *L. monocytogenes* bioflms when they were subjected to treatment with an alkaline cleaner followed by acidified sodium chlorite; and Ayebah et al., 2005a, who achieved higher log reductions (> 6-log) when *L. monocytogenes* biofilms were treated with alkaline EO water followed by acidic EO water.

The potential of using heat to inactivate biofilms has also been investigated. In a study in which a predictive heat inactivation model for *L. monocytogenes* biofilms on buna-N rubber was developed, *L. monocytogenes* in biofilms were adequately inactivated by heat ranging from 78 to 80°C from 10-15 min (Chmielewski and Frank, 2006). The authors suggest that with proper maintenance of time and temperature controls, hot water sanitation using a clean-in-place system could inactivate *Listeria* in biofilms formed on rubber. The successful inactivation of biofilms with heat also provides the opportunity for their control by sanitizing certain suitable equipment with steam. During cleaning operations in the food industry, mechanical energy is applied in the form of scrubbing, scraping, manual brushing, pressure washing, etc. to physically remove soil and food residues from surfaces. These mechanical methods can also be applied to remove biofilms. Gibson et al. 1999, reported that the mechanical floor scrubber and high pressure spray they employed were particularly effective and reduced the total viable count and percentage coverage (> 99%) of attached and biofilm populations of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. During disinfection and removal of biofims in meat processing plants, Jessen and Lammert, 2003 reported that scrubbing the equipment surfaces with a household sponge followed by disinfection and rinsing removed high counts of bacteria observed

in certain sites and increased the percentage of sites with < 1 aerobic CFU/cm^2. Although the use of mechanical means and pressure sprays may be very effective in removing biofilms, it is important to consider the possible spread of microorganisms and hence contamination, by these methods, through aerosol formation. The application of cleaners and sanitizers at high temperatures where possible, may reduce the need for physical force in the removal of adherent bacteria.

A major factor in the formation of biofilms and their effective control is equipment design. Processing plants with poor equipment layout and design will have major problems with formation of biofilms, inefficient cleaning and sanitization and consequent contamination of products. It is important to minimize and if possible avoid dead ends, 90-degree joints and food contact parts which need the use of gaskets such as joints, in the design of equipment. These points become places where soil, water and bacteria may accumulate, leading to the development of biofilms. Such locations usually fail to receive sufficient exposure to cleaning and sanitizing compounds which is required for the removal of soil and inactivation of microorganisms.
The level of resistance exhibited by biofilms show that they cannot be easily eradicated with one particular treatment method, cleaner or sanitizer. Instead, a combination of these may be required to bring about adequate control. It is important to also identify points that may be critical to biofilm formation in the food industry, and to pay greater attention in the sanitation of such critical points.

Resistance of biofilms to inactivation by sanitizers

Resistance has been defined as the temporary or permanent ability of an organism or its progeny to remain viable and /or multiply under conditions that would destroy or inhibit other

members of the strain (Cloete, 2003). Bacteria may be defined as resistant when they are not susceptible to a concentration of antibacterial agent used in practice. Several research efforts on the control of biofilms have shown that bacteria contained in biofilms are protected from the antimicrobial action of sanitizers and are killed only at concentrations orders of magnitude higher than what is required to kill planktonic cells (Frank and Koffi, 1990; Norwood and Gilmour, 2000; Park et al., 2002b; Stewart et al., 2001). Increased sanitizer resistance of biofilms has been attributed to a) protection of the underlying organisms by the glycocalyx by limiting the penetration of the sanitizer into the biofilm matrix; b) neutralization of the sanitizer inside the matrix; c) genetic induction resulting in modification to the cell wall; d) slow uptake of antimicrobial agents as a result of the significantly slow growth of biofilm-associated cells (Brown and Gilbert, 1993; De Beer et al., 1994; Donlan and Costerton, 2002; Stewart et al., 2001).

In a recent study of the chlorine susceptibility of two opportunistic environmental pathogens, *Mycobacterium avium* and *Mycobacterium intracellulare*, cells exposed in biofilms were more resistant than those exposed to chlorine in suspension (Steed and Falkinham, 2006). In their research, when cells grown as biofilms were liberated and exposed to chlorine in suspension, they were less resistant than their biofilm counterparts. In this and other studies (De Beer et al., 1994; Norwood and Gilmour, 2000), the resistance of biofilms due to the limited diffusion and penetration of chlorine resulting from layers of cells and extracellular materials, is demonstrated. A study by Stewart et al., 2001, which measured the penetration and disinfection efficacy of chlorine based biocides, achieved poor biofilm killing despite direct measurement of effective physical penetration of the antimicrobial agent into the biofilm. They concluded that bacterial biofilms were protected by some mechanism other than simple physical shielding by

the biofilm matrix. Their research results, on the other hand, showed that a non reactive chloride tracer ion penetrated biofilms more quickly than alkaline hypochlorite, lending support to the theory that the penetration of antimicrobial agents into microbial biofilms is controlled by the reactivity of the antimicrobial agent with biofilm components. One of the new hypotheses for the resistance of biofilms to inactivation by antimicrobial compounds is that the development of biofilms invokes the formation of persister cells (Spoering and Lewis, 2001). Persister cells are thought to be microbial cells that have differentiated into an inactive, but highly protected state (Roberts and Stewart, 2005), and these are thought to be responsible for the resistance of biofilms to killing by sanitizers, however, there is currently not much evidence to support this theory.

In the study of the resistance of biofilms to inactivation by sanitizers, it may be important to consider the possibility that their resistance at any particular time, is likely to be due to a combination, and not just one, of the number of factors believed to be responsible.

***Pseudomonas* spp**.

Pseudomonas spp. are Gram negative, aerobic rod-shaped bacteria. They are one of the most diverse and ecologically significant groups of bacteria (Spiers et al., 2000), capable of utilizing a wide range of organic and inorganic compounds and of living under diverse environmental conditions (Palleroni and Moore, 2004). They are ubiquitous in nature, and are found in large numbers in the soil, freshwater and marine environments and also form intimate associations with plants and animals (Spiers et al, 2000). *Pseudomonas* spp are globally active in aerobic decomposition and biodegradation, and hence, they play a key role in the carbon cycle. Certain species of *Pseudomonas* are pathogenic for humans (eg. *P. aeruginosa*),

cultivated plants, (eg. *P. syringae*), and domestic animals, while others are a regular component of microbial food spoilage in the field, market place and in the home. Spoilage is characterized by any change in the food product that renders it unacceptable to the consumer from a sensory point of view. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), as textural changes (degradation of polymers) or as off-odours and off-flavors (Gram et al., 2002). *Pseudomonas* spp. are the most common spoilage organisms (Huis in't Veld, 1996) and as such, they are responsible for significant economic losses in the food industry (Braun and Sutherland, 2003).

Pseudomonas spp. are psychotolerant and are known to dominate proteinaceous foods stored aerobically at chill temperatures (Gram et al., 2002; Hinton et al., 2004). They are particularly associated with the spoilage of fresh and refrigerated beef and poultry (Hinton et al., 2004; Ercolini et al., 2006, Arnaut-Rollier et al., 1999), fish and shellfish (Lalitha and Surendran, 2006; Tryfinopoulou et al., 2002), raw milk (Dogan and Boor, 2003) and pasteurized milk (Dogan and Boor, 2003; Eneroth et al., 2000), as a consequence of post-pasteurization contamination. Pseudomonads are frequently isolated from the processing environment of food industries where they gain access through their presence on raw materials (Dogan and Boor, 2003). Their wide distribution in the environment also gives them access to the food processing environment from a myriad of sources. *Pseudomonas* spp. have been documented to form biofilms (Werner et al., 2004; Vanhaecke et al., 1990; Antoniou and Frank, 2005) on stainless steel and polycarbonate materials. Their ability to attach and grow as biofilms on various surfaces is of particular concern to public health, since it gives them the opportunity to persist in the role they play in human infections (eg. *P. aeruginosa*), and food spoilage.

In nature, most bacteria do not exist as pure cultures but rather complex multipsecies communities. When *Pseudomonas* spp. are isolated form processing plants and food, they are usually found in association with other bacteria such as Enterobacteriaceae, Aeromonadaceae, *Shewanella* spp and Lactobacillus spp. (Lalitha and Surendran, 2006; Ercolini et al., 2006; Geornaras and Von Holy, 2000). Several research studies have been conducted to investigate the nature and effect of interactions of *Pseudomonas* spp. biofilms with biofilms of other spoilage and pathogenic bacteria (Kives et al., 2005; Bagge et. al., 2001; Hassan et al., 2004). In mixed biofilms, *Pseudomonas* spp. may enhance, reduce or have no effect on the growth of other bacteria. Hassan et al, 2004 reported in their work on biofilms formed on condensate forming surfaces, that *L. monocytogenes* attached in significantly greater numbers to surfaces with preexisting *P. putida* biofilms than to *Pseudomonas*-free surfaces. In another study, *E. coli* PHL565 alone was unable to attach to solid surfaces however in mixed cultures with *Pseudomonas putida* MT2, it was able to attach and form a mixed *E. coli* / *P. putida* biofilm. Similar results of the enhancement of the growth of other bacteria in mixed biofilms with *Pseudomonas* spp. have been reported by other researchers (Lindsay et al., 2002). On the contrary, Norwood and Gilmour, 2001, reported that in monoculture biofilms consistently contained greater *L. monocytogenes* numbers than when in multispecies biofilms with *P. fragi* ATCC 4973 and *Staphylococcus xylosus* DP5H. The influence of *Pseudomonas* spp. on other bacteria in mixed biofilms not only affects their adhesion and growth but also may lead to changes in how these bacteria react to extrinsic factors in their environment, such as increased susceptibility (Lindsay et. al., 2002) or increased resistance (Ammor et al., 2004) to sanitizers. From the food safety standpoint, the favorable colonization of pathogenic bacteria such as *L. monocytogenes* as a result of the presence of *Pseudomonas* spp. is a matter of concern.

Listeria monocytogenes

The genus *Listeria*, are bacteria which consist of small, non-sporeforming Gram-positive rods (Gahan and Collins, 1991). Presently there are six species named, including *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri and L. grayi*. Within the genus *Listeria,* only *L. monocytogenes* and *L. ivanovii* are considered to be virulent, with respect to both the 50% lethal dose in mouse and the ability to grow in mouse spleen and liver (Rocourt and Cossart, 1997) and of these species, only *L. monocytogenes* is recognized as an important human pathogen. *L. monocytogenes* causes listeriosis and the disease manifests itself in the form of septicemia, meningitis, encephalitis and abcesses (Lovett and Twedt, 1988). Fever is a common symptom and other complaints may vary from nonspecific fatigue and malaise to enteric symptoms. Even though persons with no predisposing underlying conditions may be infected, the immunocompromised, such as patients with cancer or those undergoing treatments with steroids or cytotoxic drugs, pregnant women, neonates and the elderly are typical targets of listeriosis (Donelly, 2001). Pregnant women have 20 times the risk of acquiring listeriosis as normal, healthy humans and their infection may result in abortions, stillbirths, severely ill infants or the death of the mother.

Listeria is ubiquitous in nature, occurring in the soil, vegetation and water and therefore is frequently carried by humans and animals. The organism can survive longer under adverse environmental conditions than many other non-spore forming bacteria of importance in food borne disease (Fenlon, 1999). *L. monocytogenes* has the ability to grow over a wide range of temperatures from -1.5 -50 °C and within pH ranges of 4.3-9.6. It survives freezing and drying, it is relatively resistant to heat, and has been reported to also survive salt challenges of up to 25.5 % NaCl (Lou and Yousef, 1999; Farber and Harwig, 1996). This resistance, together with its

ability to colonize, multiply, and persist on processing equipment, makes *L. monocytogenes* a particular threat to the food industry. Active surveillance data on food borne illnesses conducted by the Centers for Disease Control shows that *L. monocytogenes* causes an estimated 2,500 serious illnesses and 500 deaths in the United States each year (Centers for Disease Control and Prevention, 2000). Of all the food borne pathogens which cause food related deaths *L. monocytogenes* has the highest mortality rate (28 %) (Mead et al., 1999). Listeriosis has been associated with the consumption of foods such as milk, cheese, coleslaw and processed meats.

As a result of recurring outbreaks of listeriosis and the associated high mortality rate among those at risk, the U.S. Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS) and the U.S. Food and Drug Administration (FDA) established policies for the pathogen in ready-to eat foods (Shank et al., 1996). The policy requires the absence of *L. monocytogenes* in a 25g sample of every given production lot of ready-to-eat foods. This policy for *L. monocytogenes* in ready-to-eat foods has been highly debated in recent years. To date, the minimum infectious dose of *L. monocytogenes* is not known and it has been argued that perhaps regulators in the United States should establish tolerance levels for the presence of the organism in foods, especially for foods with very short shelf lives as well as those that are low risk and do not support the growth of the organism.

L. monocytogenes is able to attach and form biofilms on a wide range of surfaces found in the food industry and its presence in such environments may result in contamination of food. The pathogen has been reported to form biofilms on stainless steel (Frank and Koffi, 1990, Somers and Wong, 2004; Frank et al., 2003) which is the most commonly used food contact surface in the food industry, buna-N rubber (Somers and Wong, 2004; Mosteller and Bishop,1993) which is a common material used for gaskets on food processing equipment, glass,

which is used for bottling food, teflon (Mosteller and Bishop,1993; Bourion and Cerf, 1996) which is used in surfaces for food preparation and also for the fabrication of gaskets and conveyor belts in food processing plants. The biofilm forming ability of *L. monocytogenes* presents an additional challenge and makes it particularly difficult to completely irradicate it from food processing facilities as processors work to comply with the federally imposed policy for the absence of the microorganism in a 25g sample of a given production lot of ready-to-eat foods.

Association of *Listeria monocytogenes* with food processing facilities

Listeria monocytogenes has been isolated from a number of food processing environments and finished products and frequently the environment has been established as the source of the product contamination. The production line, environment and processed fish in a cold-smoked rainbow trout processing plant were sampled for *L. monocytogenes.* The frequency of contamination of the raw fish with *L. monocytogenes* was low; however, the frequency of contaminated fish rose after brining, and the most contaminated sites of the environment were the brining and post brining areas. Pulsed-field gel electrophoresis of the isolates showed that the contaminants of the final product were from the brining and slicing operations (Autio et al., 1999). In another study by Lunden et al, 2003, involving three meat plants and one poultry processing plant, *L. monocytogenes* was isolated from final products as well as the processing equipment. Several heat treated products were contaminated with the same PFGE type found on the processing equipment (slicers , dicers, freezers and conveyors) suggesting the equipment were poorly sanitized. Isolates of *L. monocytogenes* from fully cooked products have also been found to be indistinguishable by PFGE from isolates obtained from

drains and floor surfaces (Berrang et al., 2002). In cases where machines have been transferred from one processing plant to another, the problem of contamination has been transferred as well, emphasizing the role of equipment in the transfer of *L. monocytogenes* (Lunden et al, 2002). *L. monocytogenes* persistence and subsequent product contamination has been documented in other processing plants as well (Silva *et al*., 2003).

The annual incidence of listeriosis in the United States decreased by 44% between 1989 and 1993 and an analysis of the incidence and trend from 1996 to 2001 revealed a 35% decline (Anon, 2003). This desireable decreasing trend however, has not continued as expected. After an increase in cases in 2003, the incidence in 2004 was comparable to 2002 (CDC, 2005). The continual occurrence of outbreaks demands the continuation of efforts to prevent foodborne listeriosis.

Sanitizers

Cleaning and sanitizing are important critical control points in the food processing plant and if not properly done, may result in the contamination of products with food borne pathogens leading to food borne illnesses, recalls and economic losses. The frequent recovery of *L. monocytogenes* from drains, condensed and stagnant water, floors, fibrous conveyor belts, processing equipment and sections of equipment which are inaccessible to cleaning, demands the routine use of good cleaning and sanitizing practices and the continued search for effective sanitizers which will render food processing environments *Listeria*-free and decrease the overall microbial load. Various methods of disinfection have been applied in the food processing industry and new methods, new sanitizers, and new combinations of known sanitizers, which might perhaps do a better job are being sought for, each day in order to efficiently remove and

kill spoilage and pathogenic microorganisms found in the processing environment. Typical sanitizers that are applied in the food industry include chlorine compounds (hypochlorites, chlorine dioxide), organic acids (peracetic acid), trisodium phosphate, iodophors, peroxides, ozone and quaternary ammonium compounds (Giese, 1991; Marriot, 1999).

1. Iodine Compounds

Iodine-based disinfectants can be divided into three main groups according to the solvents and substances complexing with the iodine species: (a) pure aqueous solutions; (b) alcoholic solutions; (c) iodophoric preparations, and these exhibit intrinsic differences in their chemical and microbicidal properties (Gottardi, 2001; Marriott, 1999). Generally, free elemental iodine and hypoiodous acid are the active agents in microbial destruction. Iodine, mainly in its molecular form, can penetrate the cell wall of microorganisms rapidly, disrupt the bonds that hold cell protein together and inhibit protein synthesis (Fraise, 1999). Aqueous iodine and alcohol-iodine solutions are normally used as skin disinfectants. Iodophors are used as a skin disinfectant, used for water treatment and for cleaning and disinfecting equipment surfaces. The amount of free available iodine determines the activity of iodophors.

Iodine-type sanitizers are more stable in the presence of organic matter, less corrosive and less irritating to skin than chlorine compounds (Marriott, 1999). Iodine complexes are stable at a very low pH, hence they can be used at a very low concentration of 6.25 ppm and are frequently used at 12.5 to 25 ppm. Iodine compounds cost a little more to use than does chlorine and may cause off-flavors in some products, stain some equipment materials eg. polypropylene conveyor belts, plastic materials and rubber gaskets of heat exchangers, and react with starch to form a blue-purple color. Other disadvantages of iodine compounds are that they vaporize at

approximately 50°C, are less effective against bacterial spores and bacteriophage than chlorine, have a poor low-temperature efficacy and are very sensitive to pH changes.

2. Ozone

Ozone, a molecule comprised of three oxygen atoms, is naturally occurring in the earth's atmosphere (Marriott, 1999). Ozone (O_3) is one of the most powerful oxidizing agents known and is reported to be a stronger oxidant (52 % stronger) than chlorine and acts more rapidly against a broad spectrum of microorganisms (Robbins et al., 2005). At present, several thousands of plants use ozone in water and wastewater treatment. Ozone is also used for disinfection, mold control and preservation of food. Ozone is produced by applying energy in the form of radiation, electricity, or heat to gaseous oxygen. In general, ozone generation is more efficient at low temperatures as a result of thermal decomposition of O_3 at high temperatures. In the commercial generation of O_3, dry air or O_2 is passed between two electrodes separated by a glass or ceramic dielectric material. Concentrations ranging from 1 % to 3 % O_3 are produced if the feed gas is air and 2 % to 6 % if the feed gas is pure O_2.

Ozone exerts its antimicrobial effect by attacking the bacterial membrane at the glycoproteins, glycolipids, or at certain amino acids such as tryptophan. It also acts on the sulfhydryl groups of certain enzymes, resulting in disruption of normal cellular enzymatic activity (Greene et al., 1993). Bacterial death is rapid and this lethal effect of ozone is a consequence of its strong oxidizing power. Ozonation has been approved by the USDA Food Safety and Inspection Service for use in treating poultry chilling water. Recently small-scale ozonation units have been developed that can be used in food and dairy processing plants (Greene et al., 1993). These units use ambient air as an oxygen source, require only routine

replacement of the dessicant, and recirculate water through existing clean-in-place systems. Ozone is more stable in the gas phase than in the aqueous phase and its half life in the aqueous phase varies from hours to seconds depending on water conditions such as temperature, pH, UV light, O_3 concentration, and concentration of radical scavengers. One means of maintaining sufficient ozone concentration in water is by recirculating water through the ozonator. Treatment with ozone for 10 min produced a > 99 % reduction in adherent *Pseudomonas fluorescens* and *Alcaligenes faecalis* on stainless steel plates (Greene et al., 1993).

3. Organic acids

Acid sanitizers which are considered to be toxicologically safe and biologically active, are frequently used to combine the rinsing and sanitizing steps. Organic acids, such as acetic, peroxyacetic, lactic, propionic, and formic acid, are frequently used (Marriott, 1999). When acid sanitizers are used after the cleaning step, the acid neutralizes excess alkaline residues from the cleaning compound, prevents the formation of alkaline deposits and also sanitizes. These sanitizers destroy microbes by penetrating and disrupting the cell membranes, then dissociating the acid molecule and, consequently, acidifying the cell interior (Marriott, 1999). Acid sanitizers are especially effective on stainless steel or where contact time may be extended, with no danger of corrosion. The use of acid sanitizers is valuable in food processing plants with automated cleaning-in-place (CIP) systems, where the sanitizer is combined with the final rinse, after which the equipment may be closed to avoid contamination and held over-night.

Acid sanitizers act rapidly and are effective against bacteria, yeast and viruses; however, they are less effective with an increase in pH (beyond 3) or against thermoduric organisms.

The use of peracetic acid has gained a lot of interest as an alternative method of disinfection to chlorination, which produces harmful by-products such as trihalomethanes. When peracetic acid is used for disinfection, the breakdown products formed ie. acetic acid, oxygen and hydrogen peroxide, are not considered particularly harmful to the ecosystem (Stampi et al., 2002; Block, 2001). In addition to other advantages, hard water and residual organic matter do not have a major effect on the ability of acid sanitizers to inactivate microorganisms. Fatemi and Frank, 1999, treated adherent *Pseudomonas* and *L. monocytogenes* on stainless steel surfaces and reported that the peracid sanitizers were more effective than chlorine for inactivating biofilm in the presence of organic challenge (milk). Other comparisons of the ability of chlorine and peracid sanitizers to inactivate microorganisms (adherent and biolfims) indicate that chlorine is more effective (Trachoo and Frank, 2002; Rossoni and Gaylarde, 2000).

It is apparent from these reports that certain sanitizers may be more effective against certain bacteria than others and, that must be considered as a factor in their selection. Peroxyacetic acid is effective against yeasts such as *Candida*, *Saccharomyces* and *Hansenula*, and molds such as *Penicillium*, *Aspergillus*, *Mucor* and *Geotrichum* and as a result, it has gained acceptance in the soft drink and brewing industry, where it is used for sanitizing aluminum beer kegs (Marriott, 1999).

4. Quarternary ammonium compounds

Quarternary ammonium compounds (quats) are cationic surfactants used largely to sanitize, floors, walls, drains, equipment and other food contact surfaces in processing plants (Beuchat, 2000; Marriott, 1999; Giese, 1991). Because of their surfactant activity, quats have good penetrating ability and appear to form a residual antimicrobial film when applied to hard

surfaces. They are therefore excellent for surfaces which can be sanitized for long contact times and for surfaces which do not require rinsing before production (Beuchat, 2000; Giese, 1991). Quats are not recommended for use in processing plants that use starter cultures because the residues inhibit these cultures (Chmielewski and Frank, 2003). Quats have the following major advantages. They are: (a) colorless and odorless when properly diluted, (b) stable against reaction with organic matter than are chlorine and iodine sanitizers although their bactericidal effectiveness is impaired by the presence of organic matter, (c) resistant to corrosion of metals and non-irritating to the skin, (d) stable against temperature fluctuation, (e) effective in a pH range of 6-10 and, (f) nontoxic. On the other hand, they are incompatible with anionic type synthetic detergents and because most detergents are anionic, surfaces must be rinsed well between cleaning and sanitizing with quats.

Quats are very effective against most microorganisms especially molds and Gram positive bacteria, however, they are ineffective against most coliform and Gram negative organisms (Marriott, 1999; Giese 1991). The mechanism of germicidal action is not fully understood but may be that the surface active nature of the quat surrounds and covers the cells outer membrane, causing a failure of the wall, which consequently causes leakage of the internal organs and enzyme inhibition. Several studies have been conducted using quats to inactivate planktonic and adherent spoilage and pathogenic bacteria. Tuncan, 1993, investigated the germicidal efficacy of a quaternary ammonium compound (25-200 ppm) on three *Listeria* spp. in suspension at 2 and 25°C. He reported that regardless of the concentration of quat used, a > 5.0 log CFU/ml reduction in *Listeria* was achieved in 30 s at 25°C. Sanitation with quaternary ammonium compound (200mg/L) reduced 4 h adherent *Staphylococcus aureus* populations on

new and abraded stainless steel and polycarbonate surfaces by more than 1,000-fold (Frank and Chmielewski, 1997).

5. Chlorine compounds

Chlorine has been widely used for many years to treat drinking water and waste water, as well as to sanitize food processing equipment and surfaces in processing environments (Beuchat, 2000). Chlorine compounds used as sanitizers have been applied in a variety of forms such as liquid chlorine, hypochlorites, inorganic chloramines, organic chloramine and chlorine dioxide. Varying antimicrobial acitivities are obtained with the different forms of chlorine (Giese, 1991). Chorine compounds are probably the most commonly used sanitizers. Hypochlorous acid (HOCl) is the most active form or residual (active killing agent) of chlorine compounds (Giese, 1991; White, 1999). It appears to kill the microbial cell though inhibiting glucose oxidation by chlorine-oxidizing sulfhydryl groups of certain enzymes important in carbohydrate metabolism.

Hypochlorous acid is similar in structure to water and its germicidal efficiency is due to the relative ease with which it can penetrate the microbial cell wall. This penetration is comparable to water, and can be attributed to both its modest size (low molecular weight) and its electrical neutrality (White, 1999). Other modes of chlorine action that have been proposed are: (a) disruption of protein synthesis; (b) oxidative decarboxylation of amino acids to nitrites and aldehydes; (c) reactions with nucleic acids, purines, and pyrimidines; (d) unbalanced metabolism after destruction of key enzymes; (e) induction of deoxyribonucleic acid (DNA) lesions with the accompanying loss of DNA-transforming ability; (f) inhibition of oxygen uptake and oxidative phosphorylation, coupled with leakage of some macromolecules; (g) formation of toxic N-chlor derivatives of cytosine; and (h) creation of chromosomal aberrations (Marriott, 1999).

In its application as a sanitizer, chlorine gas may be injected slowly into water to form the antimicrobial component, HOCl. HOCl is also formed when either calcium hypochlorite or sodium hypochlorite is dissolved in water. Hypochlorous acid in water dissociates to form hydrogen ion (H^+) and a hypochlorite ion (OCl^-) and the equilibrium between HOCL and OCl^- is maintained even though HOCl is constantly consumed through its germicidal function. All other things being equal, the germicidal efficiency of HOCl in chlorine sanitizers is a function of pH, which establishes the amount of dissociation of HOCl to H^+ and OCl^-. The OCL^- which is a result of the dissociation phenomenon, is a relatively poor disinfectant because of its inability to diffuse though the cell wall of microorganisms (White, 1999). Chlorine compounds are more effective antimicrobial agents at a lower pH (near pH 4) where the presence of hypochlorous acid is dominant. As the pH increases, the hypochorite ion, which is not as effective, as a bactericide, predominates. Sodium hypochlorites are the most widely used of the chlorine compounds. Chlorine dioxide, which is most often used in water and sewage treatment, has recently received attention as a sanitizer from the food industry. Chlorine dioxide is less affected by pH and organic matter, less corrosive to stainless steel and has been shown to have 2.5 times the oxidizing power of chlorine (Beuchat, 2000; Giese, 1991). Chlorine sanitizers are used extensively in the food industry to sanitize food processing equipment and food containers and they have been shown to be effective against Gram-positive and Gram-negative bacteria and conditionally against certain viruses and spores (Rossoni and Gaylarde, 2000; Joseph et al., 2001). The resistance of biofilms to sanitizers has been well documented (Frank and Koffi, 1990; Ronner and Wong, 1993; Norwood and Gilmour, 2000; Joseph et al, 2001) and several chlorine sanitizers have been evaluated in research efforts on the control of biofilms in the food processing plant.

Efficacy of chlorine sanitizers against pathogenic and spoilage bacteria in suspension or in the form of biofilms is affected by several conditions such as the presence of organic matter (Best et al., 1990, Frank et al., 2003; Somers and Wong, 2004; Fatemi and Frank, 1999; Peng, et al., 2002), the pH, concentration and time of exposure (Joseph et al, 2001, Rossoni and Gaylarde, 2000; Bremer et al., 2002). The type of surface on which biofilms are formed has also been reported to influence the efficacy of chlorine sanitizers to inactivate them (Bremer et al., 2002, Joseph et al, 2001; Mosteller and Bishop, 1993).

Various forms of chlorine and their definitions

A number of specific terms are used in the literature to identify various forms and aspects of chlorination. One of such terms is available chlorine. Available chlorine may be defined as a measurement of oxidizing capacity which is expressed in terms of the equivalent amount of elemental chlorine. Hence the concentration of hypochlorite, or any other oxidizing disinfectant, may be expressed as available chlorine by determining the electrochemical equivalent amount of Cl_2 to that compound (Dychdala, 2001). The term available chlorine is universally used to denote "parts per million, available chlorine".

Another term used in chlorination of water is chlorine demand. When chlorine is added to water, or in the application of chlorine containing solutions in disinfection, a certain part of this chlorine is consumed by impurities (inorganic or organic) present, and any unconsumed chlorine remains as "total residual (available) chlorine" without regard to the type of residual (Mercer and Somers, 1957). The "chlorine demand" of the water is the difference between the chlorine applied and the total residual chlorine. If the impurities present are organic in nature,

chlorine combines with ammonia and other nitrogenous compounds to form chloramines or N-chloro compounds which are referred to as "combined available chlorine".

"Free available chlorine", usually refers to that portion of the total chlorine residual remaining in water which may react chemically and biologically as hypochlorous acid (HOCl), hypochlorite ion (OCl^-) or elemental chlorine (Cl_2). These forms of chlorine may be found in water, provided there is no ammonia or other nitrogenous compounds to form chloramines and there is enough chlorine to satisfy the organic and inorganic chlorine demands (Dychdala, 2001). The free and combined available chlorine, when present in aqueous solutions, are collectively referred to as the total residual (available) chlorine. When chlorine compounds are used in solutions or on surfaces, where free available chlorine can react with cells, these sanitizers are bactericidal and sporicidal (Marriott, 1999).

Effect of organic matter on chlorine containing solutions

The available chlorine from hypochlorite and other chlorine-releasing chemicals reacts with and is inactivated by residual organic matter. This is evident especially in solutions with low levels of chlorine. If the recommended volume of chlorine solution and sufficient concentration is applied, a sanitizing effect can still be achieved in the presence of organic matter. The reaction of ammonia nitrogen produces inorganic chloramine compounds. Organic chloramines are formed through the reaction of hypochlorous acid with amines, imines, and imides. Chloramines are known to posses the capacity to exert bactericidal activity, however, they are very slow and require long exposure times, leading to their characterization as a poor disinfectant compared to free chlorine (White, 1999). The extent of reduction in available chlorine as a result of the reaction with organic matter is influenced by the amount of organic

matter present. The higher the amount of organic matter present, the greater decrease in the concentration of available chlorine (El-Kest and Marth, 1988; Ayebah et al., 2006). Bacterial spores and vegetative cells are more resistant to chloramine than to the hypochlorites (Marriott, 1999). Van de Weyer et al, 1993 also tested the efficacy of several disinfectants on *Listeria* in the presence of organic matter and reported that the bactericidal activity of the chlorine containing disinfectant was diminished in the presence of proteins.

Biofilms have been demonstrated to be more resistant to sanitizers than their planktonic counterparts and so for their control, the presence of organic matter presents an additional challenge. A recent report by Frank et al., 2003 showed that when cleaning and sanitizing were employed sequentially, using an alkali cleaner and acidified sodium chlorite, *L. monocytogenes* biofilms overlaid with chicken exudates and fat were reduced to nearly undetectable levels. However, when only sanitizers were used the organic load reduced the efficiency of inactivation. Somers and Wong, 2004 also reported that in the presence of meat and fat residue, the bactericidal efficacy of sanitizers on biofilms of *L. monocytogenes* was reduced.

The reduction in germicidal efficacy of chlorine sanitizers in the presence of certain types of organic matter is a limiting factor and requires adjustments in the chlorine concentration to allow for the organic chlorine demand during sanitation. The formation of potentially harzardous disinfection by-products such as trihalomethanes and haloacetic acids which are produced when chlorine and other disinfectants react with organic matter is also a public health concern because these compounds can cause cancer (Greene et al, 1993; Richardson et al., 1998; Wei et al., 1985). Because of these concerns, and others, the search for alternative disinfectants and the evaluation of other chemical sanitizers for disinfection of food processing facilities remains an area of active research.

Electrolyzed water

Electrolyzed oxidizing (EO) water, is produced from the electrolysis of a dilute (0.05-1%) salt (NaCl) solution in a cell containing inert positive and negative electrodes separated by a membrane. By subjecting the electrodes to a DC voltage, two types of water possessing different properties are generated. At the anode, i.e. the positive electrode, an aqueous acidic solution referred to as acidic EO water is produced and at the cathode, i.e. the negative electrode, an aqueous alkaline solution referred to as alkaline EO water is produced. Acidic EO water in addition to its low pH has a high oxidizing potential, contains hypochlorous acid (HOCL) and has been reported to have a strong bactericidal activity (Kim et al., 2000b; Len et al., 2000; Venkitanarayanan et al., 1999) on various pathogenic and nonpathogenic microorganisms. Alkaline EO water on the other hand has a high reducing potential which plays a significant role in the reduction of free radicals in biological systems.

Electrolyzed water, in its acidic, alkaline and neutral forms has been referred to by as many names as the number of researchers who have worked with it. Acidic EO water has been referred to as, electrolyzed strong acid aqueous solution (ESAAS) (Tanaka et al., 2000; Hayashi et al., 1997), acid oxidizing water (AOW) (Shimada et al., 2000), acidic oxidative potential water (AOPW) (Matsumoto, 2002), strongly acidic electrolyzed water (SAEW) (Sakurai et al., 2002), and acidic electrolyzed water (AcEW) (Koseki et al, 2001). When pH of acidic EO water has been neutralized to about (5-6.5), it has been referred to as neutral oxidizing water (NtOW)(Shimada et al., 2000), neutral electrolyzed water (NEW)(Deza, et. al., 2003) and electrochemically activated solution (EAS) (Yang et al., 1999).

Principle of generation of EO water

EO water is generated by introducing a dilute solution of salt into an electrolytic cell possessing positive and negative inert electrodes that are separated by a semi permeable membrane. When the electrodes are subjected to a DC voltage (8-10 volts), the ions in the dilute salt solution cell migrate through the semi permeable membrane to respective electrodes. Ions, which exist in the salt solution include, Na^+, Cl^-, H^+, OH^-, and several combinations of these. During electrolysis, Na^+ and H^+, ions migrate to the cathode while Cl^- and OH^- ions migrate to the anode. Several reactions of these ions result in the production of different molecules and ions on the two sides of the cell, which consequently become responsible for the properties of the acidic and alkaline EO water produced at the anode and cathode, respectively. The primary chemical reactions that take place in the electrochemical cell are complicated (Gordon et al., 1998) and involve at a minimum, some of the following reactions.

At the anode:

$2Cl^- - 2e^- \rightarrow 2Cl \rightarrow Cl_2$

$Cl_2 + H_2O \leftrightarrow HOCl + HCl$

$HOCl \leftrightarrow H^+ + ClO^-$

$2H_2O \rightarrow 4H^+ + O_2$

The following reactions seem to occur at the cathode:

$H_2O \rightarrow H^+ + OH^-$

$4OH^- - 4e^- \rightarrow H_2O + O_2$

$H_2O + Na \rightarrow NaOH + H^+$

At the anode, chloride ions release electrons (e^-) to form chloride radicals which when combined forms chlorine gas. The Chlorine gas formed combines with water to form hypochlorous acid

(HOCl) and hydrochloric acid (HCl). Oxygen gas is also produced through the reactions that occur at the anode. Sodium ions which migrate to the cathode accept electrons and react violently with water (H_2O) to produce NaOH and hydrogen gas (H_2).

In the operation of the EO water generator, the polarity of the electrodes are periodically changed to prevent the deposition of a scale which can eventually cause clogging of the semi permeable membrane which separates the electrodes, reducing electrolytic efficiency (Anon, 1997). The scale, which contains calcium and magnesium salts and silicate from the tap water and salt used in the EO water generation, deposits on the cathode but is easily dissolved by acid. Treatment of the tap water being introduced into the generator as well as the use of purified salt also helps in preventing problems resulting from the deposition of scale.

Properties of EO water

The properties of EO water which are generally monitored are; pH, Oxidation-reduction potential (ORP) and chlorine concentration. In some cases, the amount of dissolved oxygen is also monitored. The pH, ORP and chlorine concentration have been observed to play very important roles in the inactivation of bacteria (Kim et al., 2000a, Len et al., 2000). Acidic EO water which is produced on the anode side of the EO water generator has a low pH of about 2.5, while the alkaline EO water produced on the cathode side of the generator has a high pH of about 11. Bacteria generally can survive between a pH of 4 – 9, however most of them grow best at a pH around 7.0 (6.6-7.5) (Jay 2000). The pH of acidic EO water falls outside the range most bacteria can survive, and as a result, it has been exploited for their inactivation.

The ORP of a solution is generally defined as the ease with which it loses or gains electrons. When a compound loses electrons, it is said to be oxidized, whereas a substrate that

gains electrons becomes reduced. When electrons are transferred from one compound to another, a potential difference is created between the two compounds and this difference is what is appropriately measured as the ORP and expressed in mV. A highly oxidized substance has a more positive potential while a highly reduced substance has a more negative potential. Acidic EO water has a strong oxidizing potential (ORP~1100mV) while alkaline EO water has a strong reducing potential (ORP~-800mV). Generally aerobic bacteria require a positive potential for growth while anaerobes require reduced conditions for growth. In addition to its low pH and high ORP, acidic EO water contains chlorine. Available chlorine measured in acidic EO water may be in three forms, hypochlorous acid (HOCL), hypochlorous ion (OCL^-) and gaseous chlorine (Cl_2). All these three forms may exist in various ratios in EO water depending on its pH and these easily convert to each other through reversible reactions. Of these three, HOCl has the most bactericidal effect (Len et. al., 2000; White, 1999).

The concentration of chlorine in EO water is dependent on the salt concentration, the flow rate and the amperage at which it is generated. Increasing salt concentration and amperage and decreasing flow rate results in an increase in the chlorine concentration (Hsu, 2005; Ezeike and Hung, 2004). In their research on the pH effect on free chlorine species in EO water, Len et. al., 2000, reported that increasing the pH of EO water resulted in a decrease in the concentration of HOCl and an increase in the concentration of OCl^- as a result of the dissociation of HOCl into H^+ and OCL^-. They also found that the maximum concentration of HOCl in acidic EO water was obtained around pH 4, at which point they also recorded the highest log reduction in *Bacillus cereus* treated with EO water. According to White1999, the bactericidal effectiveness of HOCl is 80 times higher than that of OCl^-. This high bactericidal activity may be attributed to the neutral charge on HOCL, allowing it to easily penetrate the bacterial cell walls to interfere

with key metabolic activities. On the other hand, OCL^- needs a high activation energy to be able to penetrate the bacterial cell wall due to its negative charge. The presence of free radical steady state residuals has also been mentioned as a potential source of disinfection in electrolysis solutions (Gordon et al., 1998). These intermediates however, can be difficult to isolate and identify because of their rapid reactions and interactions.

One of the most important advantages of using acidic EO water is the fact that it can be generated on site simply with NaCl and prevents the handling of large amounts and high concentrations of dangerous chemicals that are used in the production of other chlorinated solutions. It has also been found to be reasonably stable. Under open storage conditions, the chlorine (~ 60ppm) in acidic EO water was completely lost after 30 h when agitated and 100 h when stored without agitation (Len et. al., 2002). Under these conditions, the chlorine loss was not affected by storage lighting. Under closed conditions, Len et al., 2002, reported that approximately 60% of the chlorine was lost after 1400 h, in the presence of diffused light, whereas about 40% of chlorine was lost under dark conditions. According to this same research, the pH of acidic EO water remained unchanged during storage and though the ORP decreased during storage under open conditions as a result of the loss of oxidative chlorine, it only decreased slightly under closed conditions regardless of lighting or agitation.

Application of Electrolyzed water

In Medicine

The early explorations of the beneficial use of EO water was in the area of medicine and to date, research utilizing EO water to kill various microorganisms which cause health problems, continues. Significant success has been achieved in the medical field using, acidic, basic and

neutral electrolyzed water in the healing of wounds, sanitization of hospital equipment and floors as well as for washing of hands by medical personnel. Electrolyzed strong acid aqueous solution (ESAAS) was applied in mediastinal irrigation in 4 patients who developed extensive mediastinitis after cardiovascular surgery and the infection was successfully eradicated in all patients with no evidence of adverse effects related to the use of ESAAS (Hayashi et al., 1997). As an additional favorable outcome, satisfactory growth of healthy granulation tissues was seen after starting irrigation with ESAAS. Another widely used solution for irrigation in such infections is povidone-iodine which is an excellent bactericidal and fungicidal agent, however it has been found to impede the growth of granulation tissues because it substantially damages underlying healthy tissues (Hayashi et al., 1997). The effect achieved with ESAAS in such applications therefore provides another alternative.

Electrolyzed strong acid aqueous solution was also used to perform peritoneal lavage in the treatment of 7 patients with peritonitis or intraperitoneal abscesses and the patients from whom microorganisms had been isolated tested negative within 3-7 days after peritoneal lavage was started (Inoue et al., 1997). The outcome of several disease states associated with the presence and management of wounds, eg accidents and surgery, is affected significantly by the presence of wound infections. In the case of major burn injuries, wound infections can easily lead to sepsis. Nakae and Inaba, 2000, investigated the effect of electrolyzed oxidized water (EOW) as a bactericide in burn injury with *Pseudomonas aeruginosa* infection in a rat burn-wound model. They reported that survival rate of rats was significantly higher when the infected wound was irrigated with EOW than when there was no irrigation and when irrigation was done with physiologic saline. Serum endotoxin levels were also significantly lower in the group irrigated with EOW and they concluded that irrigation and disinfection with EOW may become

useful in preventing burn-wound sepsis. Shimada et al., 2000, compared the bactericidal effects of acid and neutral oxidizing water on cariogenic and periodonto-pathogenic bacteria and their cytotoxicities against epithelial cells, and found them to be similarly potent in inhibiting bacteria plaque formation as conventional chemical plaque control agents such as 0.35% povidone-iodine, 0.2% chlorhexidine, Listerine and 70% ethyl alcohol. In an earlier study, Horiba et al., 1999 also demonstrated bacteriostatic and bactericidal action of electrolyzed neutral water action against 15 strains of bacteria isolated from infected root canals.

Electrolyzed water has also been used for hand-washing in hospitals after nursing procedures and its effectiveness compared to other conventional soaps used for washing hands. Takeshita et al., 2002, reported that electrolyzed water (pH 6-6.5) was as effective as washing with medicated liquid soap and though slightly less effective than washing with 7.5% povidone-iodine, it was considered that the same level of effectiveness observed with povidone-iodine could be expected for relatively lightly contaminated hands after daily nursing procedures. With respect to the bacteria isolated from hands after diapering, changing positions and endotracheal aspiration, hand-washing for 10 and 30 s with electrolyzed water lowered the hand bacterial counts to the same level as or lower than that before the procedures (Takahashi et al., 2002). Fujiwara et al., 1996, also reported that acidic EO water was a more effective disinfectant in cleaning and sanitizing dialysis equipment and pipelines than conventional disinfectants such as sodium hypochlorite and acetic acid.

In Agriculture

Some research has been conducted in the field of agriculture using EO water in an attempt to find safer alternatives to replace the conventional methods of combating plant disease

using pesticides and fungicides. Research with EO water in pest control has been largely motivated by the increasing concerns about worker safety, the effects of pesticides in the environment as well as the development of resistant strains of the causative agents of plant diseases.

Buck et al., 2003, evaluated the foliage and flowers of bedding plants for signs of phytotoxicity after the application of acidic electrolyzed water. Apart from small white spots observed on the flowers and slight necrosis observed on the leaf edges of some of the plants, in general, it appeared to be safe to use as foliar spray on a wide variety of bedding plants grown under greenhouse conditions. In other research studies, acidic EO water was able to reduce powdery mildew on gerbera daisies and was also effective when combined with most pesticides (Mueller et al., 2003). The authors concluded that EO water may be used in an integrated management system in the greenhouse to reduce the use of fungicides for the control of powdery mildew on gerbera daisy. Acidic EO water has also been investigated as an alternative to 0.4% NaOCL in the removal of contaminating microorganisms before the detection and enumeration of viable teliospores of *Tilletia indica* in wheat seed or soil and the isolation of pure cultures for identification by polymerase chain reaction. Sodium hypochlorite (0.4%) is effective in removing contaminants in wheat seed or soil, however, treatments cannot go beyond 2 min without a major decrease in germination, hence leaving very little room for error and precluding the simultaneous testing of large numbers of samples (Bonde et al., 1999). Bonde et al., 1999, demonstrated that treating *T. indica* teliospores, by themselves or in wheat extracts, for 15 to 20 min with acidic EO water resulted in higher germination than the standard 2 min treatment with 0.4% NaOCL. In another study, Bonde et al., 2003, found acidic EO water to be very effective

in eliminating bacteria and fungi from soil extracts making acidic EO water a potential alternative to presently used seed disinfectants.

With the increasing awareness of the effect of nutrition and diet on the health of consumers, many more people are opting for and incorporating raw fruits and vegetables in their diet as a means of achieving healthier lifestyles. Post harvest fungal decay of fruits causes substantial economic loss to the fruit industry and with very few acceptable chemical options the use of EO water may provide an alternative strategy for the control and management of post harvest decay. Al-Haq et al., 2001, applied electrolyzed oxidizing water as a fungicide to control the post harvest brown rot of peach caused by *Monilinia fructicola.* Even though EO water did not control the brown rot in wound inoculated fruit, it reduced the disease incidence and severity in non-wound inoculated peach. EO water also delayed the onset of brown rot to 7 days, which is believed to be about the period peaches stay in the market from a packing house before reaching the consumer. In similar research studies utilizing EO water to suppress fruit rot of pear caused by *Botryosphaeria berengeriana,* Al- Haq et al., 2002, found that EO water suppressed the incidence and disease severity of wounded inoculated fruit when they were immersed in EO water before storage at simulated retail conditions. In addition, no chlorine-induced phytotoxicity was observed on the treated fruit.

In Food Technology

Electrolyzed oxidizing water has been investigated and evaluated as an alternative to sanitizers and disinfectants which are currently used in the food industry to sanitize food and non food contact surfaces, processing equipment, as well as reducing the populations of microorganisms on fruits and vegetables and the skin and hides of poultry and cattle,

respectively. Currently, in the U.S., with respect to EO water research, more studies have been conducted in the area of Food Science and Technology than in any other area of research.

Fruits and vegetables can become contaminated with pathogenic microorganisms while growing in fields or orchards, or during harvesting, post harvest handling, processing, and distribution (Beuchat, 1995). Since most fruits and vegetables, receive very little or no heat treatment before consumption, they serve as prime vehicles for the transmission of food borne illnesses. Acidic EO water has been successfully applied to reduce aerobic bacteria, coliforms, *Bacillus cereus*, *Salmonella enteritica* serovar Typhimurium, *Escherichia coli* O157:H7 and *L. monocytogenes* on lettuce (Koseki et al, 2001; Park et al., 2001; Yang et al., 2003), *E. coli* O157:H7, *S. enteritica* serovar Typhimurium and *L. monocytogenes* on tomatoes (Bari et al., 2003a; Deza et al., *2003)* and *Salmonella* spp. (Kim et al., 2003) and *Escherichia coli* O157:H7 (Sharma and Demirci, 2003; Bari et al., 2003b) on alfalfa seeds and sprouts. Acidic EO water has also been used for reducing the levels of aerobic mesophiles, coliform bacteria and fungi on cucumbers and strawberries (Koseki et al., 2004a, Lin et al., 2005). Acidic EO water was also found to be as effective as chlorinated solutions in controlling the growth of aerobic bacteria, molds, yeasts and coliform bacteria on stored carrots and as an added advantage, acidic EO water did not significantly affect the appearance of the carrots (Workneh et al, 2003).

Electrolyzed water has also been investigated for its potential utilization in the egg, poultry and meat industry. Russell, 2003, applied acidic EO water in an electrostatic spraying system on eggs inoculated with *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* and reported significant reductions in the pathogenic bacteria, even when high inoculations were used. Electrolyzed water was evaluated for its effectiveness in sanitation control at a Grade and Packing Center for eggs. An improvement in

sanitation over the period of nine months of its application was achieved and the use of EO water also helped to reduce the smell of rotten eggs inside the facilities (Achiwa and Nishio, 2003).

Significant reductions in *Campylobacter jejuni* (Park et al. 2002a) and *Salmonella typhimurium* (Yang et al., 1999, Fabrizio et al., 2002) has also been achieved with acidic EO water treatment of poultry. Acidic EO water sprayed on fresh pork bellies significantly reduced populations of *Campylobacter coli*, however it was ineffective in inactivating *Salmonella typhymurium* and *Listeria monocytogenes* within the 15 s exposure time (Fabrizio and Cutter, 2004).

In studies aimed at the use of EO water as a sanitizer on various surfaces, acidic EO water produced significant reductions in, *E. coli* O157:H7 and *L. monocytogenes* on kitchen cutting boards (Venkitanarayan et al., 1999), aerobic mesophiles on ceramic tile platforms used for handling fish in traditional and retail fish markets (Huang et al., 2006), and *Enterobacter aerogenes* and *Staphylococcus aureus* on glass, stainless steel, glazed ceramic tile, unglazed ceramic tile and vitreous china (Park et al., 2002b). Kim et al., 2001, subjected *L. monocytogenes* biofilms on stainless steel coupons to acidic EO water treatment and the bacterial cells were reduced to undetectable levels in 5 min. They reported a rapid inactivation of biofilms within 30 s of applying acidic EO water, after which the inactivation rate was significantly reduced. This may be due to the inability of acidic EO water to rapidly penetrate to the center of bacterial biofilms, after inactivating the bacteria on the surface.

In expanding its mode of application, acidic EO water in the form of ice has been evaluated as a means of maintaining low temperature during storage as well as reducing the bacterial load on vegetables. Populations of aerobic bacteria, *L. monocytogenes* and *E. coli*

O157:H7 on lettuce were reduced after being stored in frozen acidic EO water (Koseki et al., 2004b).

Although numerous studies have been conducted with EO water, there is limited information on the potential of its application in food processing; a report from Japan on its use to make bread with a softer texture than bread made with tap water (Onishi et al., 1999) and a report on improving the textural quality of aged rice by cooking with alkaline EO water (Onishi et al, 2001). Weakly electrolyzed water from both anode and cathode have also been used for kneading wheat flour used in the preparation of Japanese wheat noodles. Hara et al., 2003, reported that kneading the noodles with anode water provided a favorable texture to the cooked noodle, however when the noodles were cooked in either anode or cathode water, they showed less springiness compared with those cooked in tap water, making it less suitable for the average Japanese consumer.

Sequential application of acidic and alkaline electrolyzed water in research

Alkaline EO water, which is water obtained from the cathode side of the EO water machine, has a high pH, around 11 and a strong reducing potential of about -800mV. It had been reported to have very little or insignificant bactericidal activity (Ayebah et. al, 2005b). Most of the published research involving the use of EO water in bacterial inactivation has been focused on the acidic fraction of the water. Recently, however, there has been a growing interest among researchers in the utilization of both alkaline and acidic EO water, often applied in sequence, in the inactivation of microorganisms. A recent study carried out in our lab showed that treatment of *L. monocytogenes* biofilms with acidic EO water alone for 2 min resulted in > 5 log CFU/coupon reduction while a sequential treatment involving the treatment of the biofilms with

alkaline EO water followed by acidic EO water produced an additional reduction of 1.2 log CFU/coupon (Ayebah et al, 2005b). The utilization of both the acidic and alkaline fractions of EO water provides for a more efficient use of the EO water technology, especially in machines which produce both fractions of the water simultaneously when ever the EO water equipment is operated.

In a study with shell eggs inoculated with *Salmonella* and *Listeria*, Park et. al., 2005, reported that 1 min treatment with alkaline EO water followed by a 1 min treatment with acidic EO water containing 41 mg/L chlorine, produced a similar reduction as a 1 min treatment with chlorinated water containing 200 mg/L chlorine. Koseki et al., 2001, also reported that washing lettuce in alkaline electrolyzed water for 1min and then treating with acidic electrolyzed water for another minute, produced significant reduction in aerobic bacteria, molds, and yeasts. Cucumbers washed in alkaline electrolyzed water for 5 min and then treated with acidic electrolyzed water (AcEW) for 5 min showed a reduction in aerobic mesophiles that was at least 2 log CFU per cucumber greater than that of other treatments using AcEW or NaOCl alone (Koseki et al., 2004a). Mahmoud et al., 2006a, treated carp fillets with EO water and solutions of essential oils and concluded that the sequential treatment of alkaline EO water and acidic EO water followed by 1% essential oil treatment produced the strongest antimicrobial and antioxidant effects, compared to all other treatments during drying of the fillets. The same sequential treatment applied for the preservation of the fish resulted in extended shelf life of the carp fillets, compared to the control samples, during storage at 5 and 25°C (Mahmoud et al., 2006b). Alkaline and acidic EO water has also been applied sequentially to reduce aerobic mesophiles, Enterobacteriaceae and *E. coli* O157:H7 on the hides of cattle (Bosilevac et al.,

2005). The sequential application of both alkaline and acidic EO water significantly improves the antimicrobial ability of EO water, making the technology even more attractive.

The high antimicrobial activity, low cost, and ease of use and production of acidic EO water makes it a promising sanitizer for the food industry; however, since acids and aqueous forms of chlorine are known to be corrosive (Daufin et al., 1988; Bohner and Bradley, 1991; McCafferty, 2003; Abd El Meguid and Abd El Latif, 2004), the possibility of corrosion of equipment by acidic EO water, as a result of its low pH and residual chlorine content, is a matter of concern. No matter how effective a sanitizer is, its ability to cause corrosion will be a limiting factor for its use. The need for investigating the potential for acidic EO water to cause corrosion of materials, therefore, cannot be overemphasized.

Corrosion

Corrosion may be defined as the deterioration of a material, usually a metal, by reaction with its environment. Corrosion caused by the use of sanitizers is primarily due to chemical reactions of the sanitizer with the contact surface. This effect may be concentrated locally to form a pit, a crack or may proceed uniformly over the entire exposed surface (Fontana, 1986).

1. ***Uniform corrosion***. This is the most common form of corrosion for steel and copper and it is the easiest form of corrosion to measure. It is normally characterized by a chemical or electrochemical reaction that proceeds uniformly over the entire exposed surface. When it occurs, the metal becomes thinner and eventually fails. Uniform corrosion is the only form of corrosion that may be accurately calculated for lifetime before failure. This type of corrosion is expressed by corrosion rate which is usually reported as mpy (mils per year), mm/y (millimeters per year) or ipm (inches per month) (Fontana, 1986). It can be prevented by the proper selection

of materials, addition of appropriate coating and the removal of the agents of corrosion within its environment.

2. Crevice corrosion. Crevice corrosion occurs when the corroding metal is in close contact with anything that makes a tight crevice. This type of corrosion is intensive and localized. Crevice corrosion is usually associated with small volumes of stagnant solution caused by holes, gasket surfaces, lap joints, surface deposits (such as sand, dirt and corrosion products) and crevices under bolts and rivet heads (Fontana, 1986). Contact between metal and nonmetallic surfaces can cause crevice corrosion as in the case of a gasket. Crevice corrosion proceeds by the dissolution of the metal present and the reduction of oxygen within the crevice to hydroxide ions. The oxygen present gradually gets depleted, while the metal dissolution continues creating a positive charge which causes the migration of chlorides to the crevice site. There is a resultant increase in dissolution which causes more migration and the result is an autocatalytic process. This type of attack occurs in many solutions, although it is usually most intense in ones containing chloride. Crevice corrosion can be minimized by; (a) closing crevices in existing lap joints by continuous welding and caulking, (b) designing equipment for complete drainage and avoiding sharp corners and stagnant areas, (c) using welded butt joints instead of bolted joints in new equipment, (d) inspecting equipment and removing deposits frequently, and, (e) using solid nonabsorbent gaskets such as teflon, wherever possible.

3. Pitting corrosion. Pitting is a form of extremely localized corrosion that results in holes in the metal. Pits are sometimes isolated or so close together that they look like a rough surface. Pitting is one of the most destructive and insidious forms of corrosion. It causes equipment to fail because of perforation with only a small percent weight loss of the entire structure. It is difficult to measure quantitatively and compare the extent of pitting, because of the varying

depths and numbers of pits that may occur under identical conditions. Sometimes the pits require a long time, several months or a year, to show up in actual service. Acid chloride is the most common cause of pitting of stainless steel. Chloride reacts with chromium to form the very soluble chromium chloride, $CrCl_3$. Thus, chromium is removed from the passive layer leaving only the active iron. As the chromium is dissolved, the electrically driven chlorides bore into the stainless steel creating spherical, smooth wall pits. The residual solution in the pit is ferric chloride, $FeCl_3$, which is very corrosive to stainless steel. Methods that combat crevice corrosion, generally, will also minimize pitting corrosion.

Measurement of corrosion

Numerous corrosion tests are carried out each year with the aim of obtaining reliable prediction data for various purposes such as: the study of corrosion mechanisms in research and corrosion engineering; the selection of materials for construction of equipment for a definite application or a specific environment; evaluation of new or old metals and alloys to determine suitable environments in which they may be used without significant corrosion, and routine tests to investigate the corrosion resistance of a material in use or the aggressiveness of an existing environment. Corrosion testing can be divided into four types of classifications: (1) laboratory tests, including acceptance or qualifying tests; (2) pilot-plant or semi works tests; (3) plant or actual service tests which involves tests in a particular service or a given plant; and (4) field tests which include tests involving exposure to the atmosphere, soils or water bodies at specific geographical locations (Fontana, 1986).

The first steps in corrosion testing, involve the identification and definition of the materials and the environment (water, chemicals or simply the atmosphere in a particular area or

location). Sufficient quantity of these should be obtained in order to have enough specimens for the whole duration of the corrosion tests. If welded construction is involved, then specimens containing welds or weld beads should be tested. Size and shape of specimens vary, and selection is often a matter of convenience. Squares, rectangles, disks and cylinders are often used and flat samples are usually preferred because of easier handling and surface preparation (Fontana, 1986). Specimen for corrosion tests should be carefully and accurately measured to permit calculation of the surface area which is used in the calculation of the corrosion rate. A large surface-to-mass ratio and a small ratio of edge area to total area are desirable (ASTM, 1999). After measuring, the specimen is degreased by washing in a suitable solvent such as acetone, dried and weighed. Change in weight of the specimen is one of the critical and most often used parameter for the calculation of corrosion rate. It is important to weigh as accurately as possible to obtain the correct value for the calculation of the corrosion rate. Proper selection of time of exposure is important and misleading results may be obtained if the duration of the test is not carefully considered. A rough rule for checking results with respect to minimum test time is the formula:

Hours (duration of test) = 2000/[corrosion rate in mpy(mils per year)]

This formula is useful where the anticipated corrosion rates are moderate or low and is based on the general rule that the lower the corrosion rate the longer the test should be run (Fontana, 1986; ASM, 1999). The most common laboratory testing periods are 2 to 7 days (ASTM, 1999). The extent of corrosion of a material is normally expressed in terms of the annual corrosion rate (ACR) and its use normally implies that all mass or weight loss has been due to uniform corrosion and not localized corrosion. The ACR is calculated using the following formula:

$$ACR = KW/A\rho T$$

Where K is the constant for unit of conversion (8.76×10^4), W is the mass loss (g), A is the surface area of the specimen (cm^2), ρ is the density of the specimen (g/cm^3) and T is the total time of exposure (h) (ASTM, 1999; Fontana 1986). The ACR of a material after testing in a given environment is a measure of the relative corrosion resistance of the material to that environment. Every step in the procedures used for corrosion testing is critical, from cleaning of specimens to the determination of weights. When guidelines for corrosion tests are not defined or followed correctly, other uncontrolled factors are introduced leading to wrong interpretation or misleading results. Depending on the metal or alloy and the environment involved, several factors may affect the rate, type and extent of corrosion. A number of these are discussed.

Factors that may influence corrosion

Three important factors influence corrosion; pH, temperature and chloride content. Generally, the higher the temperature and chloride content and the lower the pH, the greater the probability of corrosion. For a given chloride content, a higher temperature and a lower pH will accelerate pitting and conversely, a lower temperature and a higher pH will reduce pitting (Tverberg, 2001, Abd El Meguid and Abd El Latif, 2004). The worst conditions occur with acid chlorides such as low pH waters with sodium chloride or other chlorides. Both hydrogen and chloride ions stimulate the dissolution of most metals and alloys and the entire process accelerates with time.

The rate, as well as the extent of corrosion, is also greatly influenced by the type of material, with some metals being more resistant to corrosion than others. In a recent study on the effect of EO water on different materials, we found stainless steel to be more resistant than

aluminum, which was in turn more resistant than copper and copper was also more resistant than carbon steel (Ayebah and Hung, 2005a). The resistance of a material to any potentially corrosive environment, depends on the components of that material. Molybdenum and chromium when added to alloys, make them more resistant to pitting. Therefore, high molybdenum and high chromium alloys provide the best pitting resistance (Tverberg, 2001). Surface finish often has a marked effect on corrosion resistance. Pitting and localized or crevice corrosion are less likely to occur on polished than on etched or ground surfaces. When metal surfaces are course ground, the surface area of the metal is increased, leading to a corresponding increase in the number of exposed inclusions. These inclusions can act as nucleation sites for the development of pitting corrosion. From a thermodynamic standpoint, coarse surface finishes should possess a higher level of "free energy" due to the mechanical working, therefore the "activation energy" (or energy required to initiate pitting) will be lower and hence the material more susceptible to pitting attack (Daufin et al., 1985).

The susceptibility of stainless steels to crevice corrosion is strongly dependent on surface finish in that, the coarser the surface finish, the easier it is to form a "dead zone" with either a gasket or an abutting metal leading to this type of corrosion. Coarse surface finishes may also lead to easy deposition and retention of scale and the concentration of these, in the presence of potentially corrosive ions such as chloride may lead to crevice corrosion. It should be pointed out that general corrosion is usually regarded independent of surface finish because this type of corrosion occurs when there is a widespread breakdown of the passive oxide film by electrochemical factors induced by the corrosive medium, so surface topography and hence surface finish will have little or no effect (Daufin et al., 1985).

The presence of pits and abrasions is known to reduce cleanability and inactivation of pathogens on food contact surfaces (Frank and Chmielewski, 2000; Holah and Thorpe, 1990). This is because the pits and cracks on the corroded food processing equipment surfaces, allow food material and bacteria to accumulate and form biofilms, which in the end survive the sanitation process and become sources of contamination to food. Corrosion caused by the use of sanitizers in the food industry is therefore highly undesireable.

This section has reviewed the presence of biofilms in food processing plants, the importance of *Listeria monocytogenes* as a food borne pathogen and the various sanitizers commonly used for the control of bacteria in the food industry. Since food processors still have limited sanitation choices for economical biofilm control, further investigation of other promising sanitizers such as electrolyzed water as well as its potential effects on food contact surface materials is important.

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CHAPTER 2

ENHANCING THE BACTERICIDAL EFFECT OF ELECTROLYZED WATER ON *LISTERIA MONOCYTOGENES* BIOFILMS FORMED ON STAINLESS STEEL[1]

[1]Ayebah Beatrice, Yen-Con Hung, and Joseph F. Frank. 2005. Journal of Food Protection. 68:1375-1380. Reprinted here with permission of the publisher.

ABSTRACT

Biofilms are potential sources of contamination to food in processing plants, because they frequently survive sanitizer treatments during cleaning. The objective of this research was to investigate the combined use of alkaline and acidic electrolyzed (EO) water in the inactivation of *Listeria monocytogenes* biofilms on stainless steel surfaces. Biofilms were grown on rectangular stainless steel (type 304, no.4 finish) coupons (2 by 5 cm) in a 1:10 dilution of tryptic soy broth that contained a five-strain mixture of *L. monocytogenes* for 48 h at 25°C. The coupons with biofilms were then treated with acidic EO water or alkaline EO water, or with alkaline EO water followed by acidic EO water produced at 14 and 20 A, for 30, 60, and 120 s. Alkaline EO water alone did not produce significant reductions in *L. monocytogenes* biofilms when compared with the control. Treatment with acidic EO water only for 30 to120 s, on the other hand, reduced the viable bacterial populations in the biofilms by 4.3 to 5.2 log CFU per coupon whereas the combined treatment of alkaline EO water followed by acidic EO water produced an additional 0.3 to 1.2-log CFU per coupon reduction. The population of *L. monocytogenes* reduced by treatments with acidic EO water, increased significantly with increasing time of exposure. However, no significant differences occurred between treatments with EO water produced at 14 and 20 A. Results suggest that alkaline and acidic EO water can be utilized together to achieve a better inactivation of biofilms than when applied individually.

INTRODUCTION

Processing facilities are an important source of pathogens in foods (*2, 25*). Cross-contamination that involves transfer between surfaces to which pathogens have attached or biofilms have formed are one means by which food becomes contaminated.

Listeria monocytogenes has been implicated in many food related outbreaks, and has caused serious illness in certain high risk groups including pregnant women, neonates, immuno-compromised patients and occasionally in persons who have no predisposing underlying condition (36). As a result of recurring outbreaks of listeriosis and the associated high mortality rate among those at risk, the U.S. Department of Agriculture's U.S. Food Safety and Inspection Service (U.S. FSIS) and the U.S. Food and Drug Administration (FDA) established zero tolerance policies for the pathogen in ready-to-eat foods (35). *Listeria monocytogenes* occurs widely in nature (4) and can attach to and form biofilms on a wide range of surfaces used in the food industry (5*, 15, 26*).

Carpentier and Cerf (7) defined biofilms as a community of microbes embedded in an organic polymer matrix, adhering to a surface. Several research efforts on the control of biofilms have shown that bacteria in biofilms are protected from the antimicrobial action of sanitizers and are killed only at concentrations orders of magnitude higher than what is required to kill planktonic cells (13*, 27, 33, 37*). Increased sanitizer resistance of biofilms has been attributed to a) protection of the underlying organisms by the glycocalyx by limiting the penetration of the sanitizer into the biofilm matrix; b) neutralization of the sanitizer inside the matrix; c) genetic induction resulting in modification to the cell wall; d) slow uptake of antimicrobial agents as a result of the significantly slow growth of biofilm-associated cells (*6, 9, 10, 37*). The study of the effects of sanitizers on *L. monocytogenes,* planktonic or biofilm form, is of particular interest due

to the ubiquitous nature of the microorganism, its isolation from processing plants and the continuing recalls of processed foods, as food processors work to comply with the federal 'zero tolerance' policy for the pathogen. Although sanitizing chemicals have been developed that are effective against biofilms (*11, 12*) food processors still have limited sanitation choices for economical biofilm control. Therefore, the evaluation of chemical sanitizers for biofilm control remains an area of active research.

Acidic electrolyzed oxidizing (EO) water has been reported (*17, 24, 39*) to exhibit a strong bactericidal effect on various pathogenic bacteria. It has been successfully used as a disinfectant in agriculture, dentistry and medicine. EO water is produced by subjecting positively and negatively charged electrodes to a DC voltage in the presence of a salt solution. At the anode, acidic EO water that contains chlorine in the form of hypochlorous acid and has a strong oxidizing potential (oxidation reduction potential, [ORP] of approximately 1,100mV) and a low pH (approximately 2.6) is produced. Alkaline EO water, which has a strong reducing potential (ORP of approximately -800mV) and a high pH (approximately 11) is produced at the cathode (*1*). The effect of acidic EO water in reducing microflora on fresh vegetables has been investigated. It has been successfully applied to reduce aerobic bacteria, coliforms, *Bacillus cereus*, *Salmonella enteritica* serovar Typhimurium, *Escherichia coli O157:H7* and *L. monocytogenes* on lettuce (*20, 30, 40*); *E. coli O157:H7*, *S enteritica* serovar Typhimurium and *L. monocytogenes* on tomatoes (*3*) and *Salmonella spp* on alfalfa seeds and sprouts (*19*). Fujiwara *et al*. (*14*) reported that acidic EO water was a more effective disinfectant in cleaning and sanitizing dialysis equipment and pipelines than conventional disinfectants, such as sodium hypochlorite and acetic acid. Acidic EO water also achieved significant reductions in *Campylobacter jejuni* on poultry (*32*). In studies on the potential of EO water as a sanitizer on

various surfaces, acidic EO water produced significant reductions in, *E. coli O157:H7* and *L. monocytogenes* on kitchen cutting boards (*37*) and *Enterobacter aerogenes* and *Staphylococcus aureus* on glass, stainless steel, glazed ceramic tile, unglazed ceramic tile and vitreous china (*33*). Kim *et al*. (*18*) subjected *L. monocytogenes* biofilms on stainless steel coupons to acidic EO water treatment and found that the bacterial cells were reduced to undetectable levels in 5 min. They reported a rapid inactivation of biofilms within 30 s of applying acidic EO water, after which the inactivation rate was significantly reduced. This may be due to the inability of acidic EO water to rapidly penetrate to the center of bacterial biofilms, after inactivating the bacteria on the surface.

Most of the published research involving the use of EO water in bacterial inactivation has been focused on the acidic fraction of the water. Only limited information exists on the potential application of alkaline EO water in food processing: a report from Japan on its use to make bread with a softer texture than bread made with tap water (*28*) and a report on improving the textural quality of aged rice by cooking with alkaline EO water (*29*). Preliminary research in our laboratory showed that alkaline electrolyzed water could produce a 1-log reduction in a pure suspension of *E. coli* 0157:H7 suspension after a 1 -minute exposure. In a study with shell eggs inoculated with *Salmonella* and *Listeria*, Park et. al. (*31*) reported that 1-min treatment with alkaline EO water followed by a 1-min treatment with acidic EO water that contained 41 mg/liter of chlorine, produced a reduction similar to a 1 min treatment with chlorinated water that contained 200 mg/liter chlorine. Koseki *et al*. (*21*) also reported that washing lettuce in alkaline electrolyzed water for 1min and then treating with acidic electrolyzed water for another minute, produced a significant reduction in aerobic bacteria, molds, and yeasts. We hypothesize that alkaline EO water will produce a higher inactivation of adherent bacteria, when applied in

combination with acidic EO water. The objective of this study was to investigate the efficacy of the combined use of alkaline and acidic electrolyzed water in the inactivation of *L. monocytogenes* biofilms on stainless steel.

MATERIALS AND METHODS

Preparation of inocula. Five strains of *L. monocytogenes*, F8027 (celery isolate), F8255 (peach isolate), 101M (beef isolate), H7750 (hot dog isolate) and G3990 (Vacherin Mont d' Or cheese isolate), were used for the study. A loop inoculum of each culture was transferred three times in tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks, MD) and incubated at 37°C at successive 24-h intervals. A 24-h culture of each bacterial strain was then centrifuged two times for 10 min (3,600 X g, 23°C) and the pellet was washed each time with 5 ml of 0.1% peptone water (Difco, Becton Dickinson). Each pellet was resuspended in 5 ml of 0.1% peptone water and the five cultures were combined to form a mixture with a bacteria population of 9 log CFU/ml. Twelve milliliters of the mixture was added to 1.2 L of sterile 1:10 dilution of TSB (3g of dry medium per liter of deionized water) and this inoculated low nutrient medium was used for the preparation of biofilms.

Preparation of stainless steel coupons. New stainless steel (type 304, no. 4 finish) sheets (1 mm thickness) (Stewart Stainless Supply Inc., Suwanee, GA) were cut into rectangular coupons (2 by 5 cm). Coupons were cleaned in acetone using Kim wipes to remove grease, rinsed in deionized water and shaken in a 2% solution of Micro-90 soap (International Products Co., Burlington, NJ) at 120 rpm at 24 ± 2°C for 1 h on a platform shaker (Model C10, New Brunswick Scientific, Edison, NJ). They were then brushed gently with a soft nylon brush,

rinsed thoroughly with deionized water and immersed in 15% phosphoric acid solution for 20 min with shaking at 120 rpm. The coupons were rinsed thoroughly with deionized water, allowed to dry at room temperature and then autoclaved at 121°C for 15 min in a stainless steel pan (53.3 cm by 30.5 cm by 5.1 cm, Delipan, Manning Brothers, Athens, GA).

Preparation of biofilms. The sterile coupons were immersed in the low nutrient medium inoculated with *L. monocytogenes* and incubated at 25°C for 4 h to allow bacterial attachment and then rinsed gently in a circular motion for 10 s with 0.1% peptone water to remove unattached cells. Biofilms were grown by submerging the coupons containing adherent cells in 1.2 liters of sterile low nutrient medium and incubating for 48 h at 25°C to allow further biofilm growth. After incubation, coupons were rinsed gently in a circular motion for 10 s with 0.1% peptone water to remove unattached cells and subjected to EO water treatment.

EO water. EO water produced from a ROX-20TA generator (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at current settings of 14 and 20 A was used for this study. After a stable amperage reading was achieved, alkaline and acidic EO water were collected from the cathode and anode side respectively, into separate sterile 1-liter Nalgene beakers, covered to prevent the loss of chlorine and used within 1 h of production. The ORP and pH of the EO water were measured immediately after preparation with a dual scale pH meter (Accumet ® AR50, Fisher Scientific Co., Fair Lawn, NJ). The residual chlorine content of the acidic EO water was determined by an iodometric method (Hach Co., Ames, IA) with a 0.113 N sodium thiosulfate standard solution.

Treatment of biofilms with EO water. Coupons that contain biofilms were immersed in 150 ml of alkaline EO water, acidic EO water or alkaline EO water followed by acidic EO water for 30, 60 and 120 s at room temperature (24 ± 2°C). To ensure that each treatment went through the same number of rinses, the coupons were treated with sterile 0.1% peptone water when one treatment had to be omitted. After treatment, the coupons were immediately immersed in a neutralizing buffer solution (5.2 g/L, neutralizing buffer; Difco, Becton, Dickinson) for 10 s and then subjected to microbiological analysis.

Microbiological analysis. To enumerate *L. monocytogenes,* the coupons were placed in sterile Nalgene bottles (8 oz) containing 20 ml of sterile 0.1% peptone water and 3 g of acid washed glass beads (425 to 600 μm, Sigma-Aldrich Co., St Louis, MO). The bottles were then shaken for 10 min on an orbital incubator shaker (Model C24, New Brunswick Scientific) at 400 rpm to remove the bacteria from the coupons. Serial dilutions of the peptone water were made after shaking. For the untreated coupons and those treated with alkaline EO water only, the surviving bacteria were enumerated by spread plating 0.1ml of the diluent on tryptic soy agar (TSA; Becton Dickinson) plates. Bacteria in biofilms from treatments that involved acidic EO water were enumerated by pour plating 1ml of the diluent with TSA at 45°C or spread plating 1 ml on four TSA plates (0.25 ml per plate). Plates were incubated at 37°C for 48 h and colonies were counted and recorded as log CFU per coupon. Treated samples that did not show any growth on TSA plates were subjected to enrichment by adding 10 ml of the peptone water used for removing bacteria from the coupons to 10 ml of TSB and incubated at 37°C for 24 h. Tubes that exhibited growth were streaked onto modified Oxford agar (MOX, Oxoid, Basingstoke,

Hampshire, England) plates containing *Listeria* selective supplement (Oxoid) and incubated at 37°C for 24 to 48 h, to confirm the presence of *Listeria*.

Data analysis. Experiments were replicated five times with duplicate treatments in each replication. Data were analyzed using the general linear model procedures of the statistical analysis system (SAS Institute Inc., Cary, NC). Comparisons of means were performed using Duncan's multiple range tests.

RESULTS AND DISCUSSION

Properties of EO water. The properties of acidic and alkaline EO water used in the study are presented in Table 2-1. At 14 and 20 A, the acidic EO water generated had chlorine concentrations of about 47 and 85 mg/liter, respectively. Alkaline EO water did not contain any chlorine. Changing the amperage of the EO water generator did not significantly alter the pH and ORP of the water produced at the electrodes. At both amperages, acidic EO water had an average pH and ORP of about 2.4 and 1,160 mV, respectively, and the alkaline EO water had a pH and ORP of about 11 and -870 mV, respectively (Table 2-1).

Method of enumeration. In spread plating normally 0.1 ml of diluent is plated and in cases where the chemical treatment has been very effective the probability of recovering injured survivors by plating 0.1 ml is low. On the other hand, in pour plating 1 ml of the diluent the probability of recovering bacteria which survived the acidic EO water treatment is increased. However, a concern exists that already injured cells from the acidic EO water treatment may die as a result of the temperature (45°C) of the molten agar used in pour plating. For the current study similar counts were obtained using these two methods ($p>0.05$) (Table 2-2).

Table 2-1. Properties of EO water used for treatment

Water	Amperage(A)	pH	ORP(mV)[a]	Chlorine (mg/liter)
Acidic EO	14	2.40 ± 0.08	1163 ± 7	47.12 ± 2.38
Alkaline EO		11.15 ± 0.10	-868 ± 5	0.00
Acidic EO	20	2.38 ± 0.07	1169 ± 1	84.68 ± 9.41
Alkaline EO		11.26 ± 0.04	-874 ± 7	0.00

[a]ORP, oxidation-reduction potential

The bacteria population that survived after treatment with acidic EO water alone or alkaline EO water followed by acidic EO water decreased as exposure time increased. For any given treatment time, the population of *L. monocytogenes* that survived after the combined treatment was lower than what survived after treatment with acidic EO water alone. Since there were no significant differences between pour plating and spread plating in this study (Table 2-2). Pour plating was chosen as the method of enumeration for subsequent experiments.

Treatment of biofilms with EO water. Recovery of cells from the biofilms, by shaking with glass beads, yielded reproducible results throughout the study. Control coupons, which were treated with deionized water, had an average population of 8 log CFU per coupon (Table 2-3) regardless of the treatment time. Alkaline EO water produced at 14 and 20 A, reduced viable populations of *Listeria* biofilms by 0.04 to 0.30 log CFU per coupon and its effect was independent of treatment time. Overall, the population of *L. monocytogenes* recovered from coupons treated for 30 to120 s with alkaline EO water produced at 14 or 20 A were not significantly different ($p>0.05$) from the control (Table 2-3).

Treatment of *L. monocytogenes* biofilms with acidic EO water produced at 14 A for 30 s reduced the bacteria population from 8.0 to 3.7 log CFU/coupon. Extending the treatment times resulted in 4.7 and 5.2 log CFU per coupon reductions after 60 and 120 s, respectively (Table 2-3). Although the surviving population after 30 s exposure to acidic EO water was slightly higher than those exposed for 60 s, the difference between these was not statistically significant ($p>0.05$). Treatment for 120 s with acidic EO water reduced cell populations to significantly ($p\leq0.05$) lower levels than the surviving population after 30- and 60- s treatment.

Table 2-2. Population of *Listeria monocytogenes* recovered from coupons using pour and spread plating

Treatment[a]	Time (s)	Mean Population (Log CFU/coupon)[b] Method of Enumeration	
		Pour Plating	Spread Plating
Acidic EO water	30	3.44 ± 0.54 a	3.56 ± 0.65 a
	60	3.20 ± 0.29 ab	3.35 ± 0.33 ab
	120	2.52 ± 0.40 b	2.67 ± 0.30 b
Combined[c]	30	3.12 ± 0.32 a	3.14 ± 0.34 a
	60	2.48 ± 0.64 b	2.62 ± 0.63 a
	120	1.60 ±0.30 c	1.70 ± 0.37 b

[a]EO water used for treatment was produced at 20 A.

[b]Means followed by the same letters in the same column within each treatment are not significantly ($p>0.05$) different.

[c]Alkaline EO water followed by acidic EO water

This indicates that the amount of time allowed for the acidic EO water to penetrate the biofilm is important in determining its efficiency in inactivating adherent bacteria.

A similar trend was obtained using EO water produced at 20 A, where treatment of adherent *L. monocytogenes* cells for 30, 60 and 120 s achieved 4.6-, 4.8- and 5.1-log CFU per coupon reductions, respectively (Table 2-3).

At both 14 and 20 A, the surviving population after exposure of biofilms to alkaline EO water followed by acidic EO water (combined treatment), were significantly lower ($p \leq 0.05$) than the population, that survived after treatment with acidic EO water alone (Table 2-3). The survival of *L. monocytogenes* after exposure to alkaline EO water followed by acidic EO water was also time dependent, with treatments for 120 s resulting in the highest inactivation.

The combined treatment using EO water produced at 14 A for 30, 60 and 120 s achieved 4.9-, 5.7- and 6.4- log CFU per coupon reductions, respectively. When EO water was produced at 20 A, the combined treatment achieved 4.9-, 5.4- and 6.1- log CFU per coupon reductions in bacteria population after biofilms were exposed for 30, 60 and 120 s, respectively. The combined treatment produced 0.3- to 1.2 log reductions more than the corresponding treatment with acidic EO water alone. For the combined treatment, the surviving population after exposure of biofilms to EO water produced at 14 A for 60 and 120 s were lower than the surviving population after treatment with EO water produced at 20 A. However, these differences between EO water produced at 14 and 20 A were not significant ($p > 0.05$). For any particular set of treatments (ie., alkaline EO water alone or acidic EO water alone or the combined treatments), no significant differences with respect to amperage were observed, even though acidic EO water produced at 20 A contained about twice as much chlorine as that produced at 14 A.

Table 2-3. Survival *of Listeria monocytogenes* biofilms following exposure to electrolyzed water

Amperage (A)	Treatment	Mean population and reduction (Log CFU/coupon) for time of exposure [a]					
		30s		60s		120s	
		Population	Reduction	Population	Reduction	Population	Reduction
14	DW[a]	A8.02 (0.06)A		A8.06 (0.01)A		A7.90 (0.05)A	
	Alkaline EO water	A7.72 (0.07)B	0.30	A8.02 (0.03)A	0.04	A7.68 (0.17)B	0.22
	Acidic EO water	B3.69 (0.02)A	4.33	B3.41 (0.22)A	4.65	B2.69 (0.07) B	5.21
	Combined	C3.17 (0.39)A	4.85	C2.32 (0.01)B	5.74	C1.49 (0.29)C	6.41
20	DW	A8.06 (0.03)A		A7.98 (0.06)A		A7.90 (0.01)A	
	Alkaline EO water	A7.90 (0.00)A	0.16	A7.78 (0.11)A	0.20	A7.75 (0.02)A	0.15
	Acidic EO water	B3.50 (0.15)A	4.56	B3.17 (0.04)AB	4.81	B2.77 (0.03)B	5.13
	Combined	C3.19 (0.10)A	4.87	C2.57 (0.18)B	5.41	C1.80 (0.21)C	6.10

[a]Means preceded by the same letters in the same column within each amperage are not significantly ($p>0.05$) different; Means followed by the same letters in the same row are not significantly ($p>0.05$) different. Initial biofilm population = 8.90 log CFU per coupon. Numbers shown in parentheses are standard deviation values. DW, deionized water (control); combined, alkaline EO water treatment followed by acidic EO water treatment.

Compared with the increasing interest in the use of acidic EO water as an antimicrobial solution, potential uses of alkaline EO water are limited. It has previously been used in combination with acidic EO water or other sanitizers to increase the antimicrobial effect of the treatment (*21, 31, 38*). This study confirms that alkaline EO water, by itself has no significant antimicrobial activity (Table 2-3). Several researchers have demonstrated that *L. monocytogenes* in biofilms on stainless steels exist in the form of clumps, clusters with channels within the biofilm or multilayered microcolonies that may be protected from immediate inactivation by sanitizers (8, *18*, *23*). Longer times of exposure to sanitizer are therefore required to achieve inactivation. Kim *et al.* (*16, 18*) reported that although 10 s exposure of planktonic cells of *L. monocytogenes* to acidic EO water resulted in complete inactivation, 10 s exposure of *L. monocytogenes* biofilms to acidic EO water only reduced bacteria by 5.8 log CFU per coupon. Although they reported a higher reduction for their 10 s acidic EO water treatment than that obtained for the 30 s treatment in the current study, a larger surface area was used for biofilm formation in their case (82.5 versus 21.4 cm^2) and hence the higher initial bacteria population and log reduction.

Increasing the amperage at which the EO water was generated, from 14 to 20 A, increased the chlorine concentration of acidic EO water from 47 to 85 mg/liter. However, no significant differences occurred in the population of bacteria inactivated by these two EO waters, regardless of exposure time (Table 2-3). Lee and Frank (*23*) observed only an approximately 0.25-log CFU/ cm^2 difference for the inactivation of surface-adherent *L. monocytogenes* when treated with sodium hypochlorite with chlorine concentrations of 100 and 150 mg/liter. Similar results were also reported by Rossoni and Gaylarde (*34*) who found no significant differences between counts after treatment of *S. aureus* and *Pseudomonas fluorescens* biofilms with 100 and

200 mg/liter of sodium hypochlorite. From the results of this study and others, there seems to be a threshold chlorine concentration beyond which, a further increase does not result in greater efficacy when applied to biofilms. However increasing time of exposure at this threshold concentration may achieve additional biofilm inactivation. Research by Kim et al. (*18*) on the inactivation of *L. monocytogenes* biofilms for up to 5 min also supports this conclusion.

Treatments of biofilms with alkaline EO water, followed by acidic EO water achieved a significantly ($p \leq 0.05$) higher inactivation of *L. monocytogenes* than when acidic EO water was used alone (Table 2-3). Alkaline EO water by itself is not an effective bactericide; however, it may condition the biofilm to facilitate the antibacterial action of the acidic EO water. Being primarily made of sodium hydroxide which is a saponifier that can react with fats and proteins, alkaline EO water may destabilize or dissolve the extracellular polymeric substances that surround the attached cells, thereby facilitating the penetration of the active components of acidic EO water. Frank et al., (*12*) reported that alkali cleaners can remove *L. monocytogenes* biofilms, although the concentration of alkali used in this study was much greater than the alkaline EO water. Koseki et al. (*21*) also reported a higher efficiency in bacteria inactivation when alkaline and acidic EO water were used in combination. They found that treatment of lettuce with alkaline EO water for 1 min followed by treatment with acidic EO water for 1 min resulted in a 2 log CFU/g reduction in aerobic counts, which was the same reduction obtained when lettuce was treated with acidic EO water alone for 10 min. Recent work that used both fractions of EO water also showed that pre-treatment of lettuce inoculated with *E. coli* O157:H7 and *Salmonella spp* with alkaline EO water and subsequent treatment with acidic EO water resulted in a greater microbial reduction than what was obtained using other pretreatment solutions (*22*).

Since both the acidic and alkaline portions of EO water are always produced together during electrolysis, the additional log reduction achieved when alkaline EO water is applied in combination with acidic EO water, provides for a more efficient use of the EO water equipment, with no additional cost.

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CHAPTER 3

EFFICACY OF ELECTROLYZED WATER IN THE INACTIVATION OF PLANKTONIC AND BIOFILM *LISTERIA MONOCYTOGENES* IN THE PRESENCE OF ORGANIC MATTER[1]

[1]Ayebah Beatrice, Yen-Con Hung, Chyer Kim and Joseph F. Frank. 2006. Journal of Food Protection. 69:2143-2150. Reprinted here with permission of the publisher.

ABSTRACT

The ability of electrolyzed (EO) water to inactivate *Listeria monocytogene*s in suspension and biofilms on stainless steel in the presence of organic matter (sterile filtered chicken serum) was investigated. A five-strain mixture of *L. monocytogenes* was treated with deionized water, alkaline EO water and acidic EO water containing chicken serum (0, 5, 10 ml/liter) for 1 and 5 min. Coupons containing *L. monocytogenes* biofilms were also overlaid with chicken serum (0, 2.5, 5.0, 7.5 ml/liter) and then treated with deionized water, alkaline EO water, acidic EO water, alkaline EO water followed by acidic EO water, and a sodium hypochlorite solution for 30 and 60 s. Chicken serum decreased the oxidation-reduction potential and chlorine concentration of acidic EO water but did not significantly affect its pH. In the absence of serum, acidic EO water containing chlorine at a concentration of 44 mg/liter produced a > 6-log reduction in *L. monocytogenes* in suspension, but its bactericidal activity decreased with increasing serum concentration. Acidic EO water and acidified sodium hypochlorite solution inactivated *L. monocytogenes* biofilms to similar levels and their bactericidal effect decreased with increasing serum concentration and increased with increasing time of exposure. The sequential 30-s treatment of alkaline EO water followed by acidic EO water produced 4- to 5- log reductions in *L. monocytogenes* biofilms, even in the presence of organic matter.

INTRODUCTION

Cleaning and sanitizing are an important part of the processes that occur in a food processing plant and if not properly done, it may result in the contamination of products leading to food borne illnesses, recalls and economic losses. Typical sanitizers that are applied in the food industry include chlorine compounds (hypochlorites, chlorine dioxide), organic acids (peracetic acid), trisodium phosphate, iodophors and quaternary ammonium compounds. Chlorine compounds are often the most effective and least expensive, although they may be more corrosive and irritating than alternatives such as iodine and quaternary ammonium compounds (*8, 14*).

Several factors affect the selection of appropriate sanitizers for food processing plants, and these may include, the composition and amount of soil present, the types of surfaces to be sanitized as well as the types of microorganisms that may be found in the plant. The aim of the cleaning and sanitization step is to remove all food residues present, after processing, as well as to reduce spoilage and pathogenic microorganisms to allowable levels. The cleaning step is important because organic matter reduces the the efficiency of some sanitizers, especially those with chlorine as their active component. Typical sanitation programs involve the use of detergents to remove soil and the application of sanitizers to inactivate bacteria and prevent recontamination (*8*). Several reports have been made on the effect of sanitizers on *L. monocytogenes* biofilms (*5, 7, 18*) and different success rates have been reported.

Recently, considerable research has been focused on the bactericidal effect of acidic electrolyzed (EO) water on various food borne pathogens (*10*, *11*, *17, 21*), with the aim of exploiting its potential for use in the food industry as an alternative chlorine- based sanitizer. Whereas working with other chlorine sanitizers involves the handling of high concentrations of

the stock chemical, acidic EO water is produced from a dilute solution of sodium chloride (NaCl) and can be generated on site, making it safe to handle. It is produced by introducing a dilute salt solution (approximately 0.1%) into an EO water generator that contains an electrolytic cell. By subjecting the positively and negatively charged electrodes to a direct current voltage, two types of water are produced: (i) an acidic electrolyzed water that contains chlorine in the forms of hypochlorous acid, hypochlorite ion and chlorine gas and has a strong oxidizing potential (oxidation-reduction potential [ORP] of ~1,100 mV) and a low pH (~2.6) at the anode side of the cell and (ii) an alkaline EO water, which has a strong reducing potential (ORPof about -800 mV) and a high pH (~11) at the cathode side of the cell (*1*). Acidic EO water has been reported to produce significant reductions in *E. coli O157:H7* and *L. monocytogenes* on kitchen cutting boards (*21*) and *Enterobacter aerogenes* and *Staphylococcus aureus* on glass, stainless steel, glazed ceramic tile, unglazed ceramic tile and vitreous china (*17*). Kim *et al*. (*11*) subjected *L. monocytogenes* biofilms on stainless steel coupons to acidic EO water treatment and found that the bacterial cells were reduced to undetectable levels in 5 min. A recent study showed that treatment of *L. monocytogenes* biofilms with acidic EO water alone for 2 min resulted in a > 5 log CFU per coupon reduction, while a sequential treatment that involved treating the biofilms with alkaline EO water followed by acidic EO water produced an additional reduction of 1.2 log CFU per coupon (*2*).

For the control of biofilms, the presence of organic matter presents an additional challenge as this is an inevitable part of food processing environments. A recent report by Frank *et al.* (6) showed that when cleaning and sanitizing were employed sequentially, using an alkali cleaner and acidified sodium chlorite, *L. monocytogenes* biofilms overlaid with chicken exudates and fat were reduced to nearly undetectable levels, a greater than 7 log reduction. In their

research, when only sanitizers were used, the organic load reduced the efficiency of inactivation. Oomori *et al.* (*16*) determined the effect of nutrient broth, proteose peptone, glycine, glucose, sucrose and corn oil on the properties of acidic EO water. They reported that when nutrient broth and proteose peptone were added to acidic EO water, the free available chorine in these solutions disappeared quickly. Since there are currently no reports on the effect of EO water on the inactivation of *Listeria* in biofilms in the presence of organic matter, the purpose of this study was to determine the effect of organic matter on the properties of EO water and the efficacy of EO water in the inactivation of *L. monocytogenes* (planktonic cells and biofilms) in the presence of organic matter.

MATERIALS AND METHODS

Treatment water. EO water was produced from a ROX-20TA generator (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at current settings of 14 and 20 A. After a stable amperage reading was achieved, alkaline and acidic EO water were collected from the cathode and anode side respectively, into separate sterile 1 liter Nalgene beakers, covered to prevent the loss of chlorine and used within 1 h of production. The ORP and pH of both the alkaline and acidic EO water were measured immediately after preparation with a dual-scale pH meter (Accumet AR50, Fisher Scientific Co., Fair Lawn, NJ). The residual chlorine content of the acidic EO water was determined by an iodometric method (Hach Co., Ames, Iowa) using a 0.113 N sodium thiosulfate standard solution. Sodium hypochlorite solution (chlorine at a concentration of ~ 85 mg/liter) was prepared from a 5 - 6% sodium hypochlorite solution (Fisher) to match the chlorine concentration of acidic EO water produced at 20 A. The pH of the solution was then adjusted

with 5 N HCL (Labchem Inc., Pittsburgh, PA) so that it would be comparable to the pH of the acidic EO water.

Preparation of inocula. Five strains of *Listeria monocytogenes* - F8027 (celery isolate), F8255 (peach isolate), 101M (beef isolate), H7750 (hot dog isolate) and G3990 (Vacherin Mont d'Or cheese isolate) - were used for this study. A loop inoculum of each culture was transferred three times in tryptic soy broth (TSB, Becton Dickinson, Sparks, MD) and incubated at 37°C at successive 24 h intervals. A 24 h culture of each bacteria strain was then centrifuged two times for 10 min (3,600 X g, 23°C) and the pellet was washed each time with 5 ml peptone water (1 g of peptone per liter**;** Becton Dickinson). Each pellet was resuspended in 5 ml of peptone water. The five cultures were combined to form a mixture with a population of approximately 9 log CFU/ml. Ten milliliters of the mixture was added to 1 liter of a sterile 1:10 dilution of TSB (low nutrient medium, with 3 g of dry medium per liter of deionized water) This was used for the preparation of biofilms.

Effect of organic matter on the properties of EO water. Different volumes (0.1 to 1 ml) of sterile filtered chicken serum (Sigma Chemical Co., St Louis, MO) were added to 100 ml of alkaline or acidic EO water in an Erlenmeyer flask. The mixture of EO water and chicken serum were shaken for 5 min at 120 rpm on a platform shaker (Model C10, New Brunswick Scientific, Edison, NJ) and the ORP, pH and chlorine content of the mixture were determined by the previously described methods.

Treatment of planktonic cells of *Listeria monocytogenes* with EO water in the presence of organic matter. One milliliter of the previously described five-strain mixture of *L. monocytogenes* (~ 9 log CFU/ml) was added to 9 ml of deionized, alkaline and acidic EO water containing different concentrations (0, 5 and 10 ml/liter) of sterile filtered chicken serum (Sigma) for 1 and 5 min. Immediately after the exposure time, 1 ml of the bacteria-treatment water mixture was added to 9 ml of a neutralizing buffer solution (neutralizing buffer at 5.2 g/liter; Becton Dickinson). The neutralized mixture was serially diluted and two 0.1-ml aliquots of the diluents were plated onto tryptic soy agar (TSA, Becton Dickinson). The neutralized mixture was enriched for the presence of surviving *L. monocytogenes* by adding 1 ml to 10 ml of TSB and incubating at 37°C for 24 h. Tubes that exhibited growth were streaked onto modified Oxford agar (MOX, Oxoid, Basingstoke, Hampshire, UK) plates containing *Listeria* selective supplement (Oxoid) incubated at 37°C for 24 to 48 h, and presence of typical *L. monocytogenes* colonies was noted.

Preparation of stainless steel coupons. New stainless steel sheets (type 304, no. 4 finish, 1 mm thickness; Stewart Stainless Supply Inc., Suwanee, GA) were cut into 2 by 5 cm (10 cm^2) coupons. They were cleaned in acetone using Kim wipes to remove grease, rinsed in deionized water and shaken in a 2% solution of Micro-90 soap (International Products Co., Burlington, NJ) at 120 rpm and 24 ± 2°C for 1 h on a platform shaker (Model C10, New Brunswick Scientific). They were then brushed gently with a soft nylon brush, rinsed thoroughly with deionized water and immersed in 15% phosphoric acid solution for 20 min at room temperature (24 ± 2°C) with shaking at 120 rpm. The coupons were rinsed thoroughly with deionized water, allowed to dry at room temperature and then autoclaved at 121°C for 15 min.

Preparation of biofilms. The sterile coupons were immersed in the low nutrient medium inoculated with *L. monocytogenes* and incubated at 24 ± 2°C for 4 h to allow bacteria attachment and then rinsed gently in a circular motion for 10 s with peptone water (1 g of peptone per liter) to remove unattached cells. Biofilms were grown by submerging the coupons containing adherent cells in 1 liter of sterile low nutrient medium and incubating for 48 h at 24 ± 2°C to allow further biofilm growth. After the 48 h growth period, the coupons were removed from the spent medium, placed in 1 liter of fresh sterile low nutrient medium, and incubated for another 24 h period to allow for further biofilm growth. After incubation, coupons were rinsed with peptone water (1 g of peptone per liter) to remove unattached cells and allowed to dry at room temperature under a biosafety hood for 30 min.

Soiling of coupons with organic matter. Chicken serum was chosen to represent protein soil that may be found in food processing plants. Various amounts (0, 0.125, 0.25 and 0.375 ml) of the sterile chicken serum were added to the top surface of coupons prepared as previously described; these amounts were selected such that when the coupons with serum were subsequently subjected to treatment with 50 ml treatment solution, the resulting concentration of serum in the water would be 0, 2.5, 5.0 and 7.5 ml/liter respectively. Only one side of the coupons was overlaid with chicken serum to allow precise control of the amount of organic load. All the coupons, including those with no serum, were incubated at 45°C for 30 min, to fix the protein to the coupons and then dried for 1 h under a biosafety hood before being subjected to EO water treatment. Before treatment, two coupons were selected from each serum concentration (i.e. 0, 2.5, 5 and 7.5 ml/iter) and the populations of *L. monocytogenes* recovered from these were used as controls for the respective serum levels.

Treatment of biofilms with EO water. Coupons containing biofilms were immersed in 50 ml of deionized water, alkaline EO water, acidic EO water and alkaline EO water followed by acidic EO water (sequential treatment), and acidified sodium hypochlorite solution for 30 and 60 s at room temperature (24 ± 2°C). For the sequential treatment, the coupons were immersed in alkaline EO water for the specific treatment time, removed and then rinsed for 10 s in 0.1% peptone water to remove any excess alkaline EO water before being immersed in the acidic EO water for the selected treatment time i.e. 30 or 60 s. After treatment, the coupons were immediately immersed in 50 ml neutralizing buffer solution for 40 s and then subjected to microbiological analysis. For treatments with acidic EO water and sodium hypochlorite solution, at the end of the treatment time, 5 ml of the treatment water was added to 5 ml of double-strength neutralizing buffer solution (neutralizing buffer at 10.4 g/liter; Becton Dickinson).

Microbiological analysis. To enumerate *L. monocytogenes,* the coupons (soiled and unsoiled) were placed in sterile Nalgene bottles (8 oz [ca. 237 ml]) containing 20 ml of sterile peptone water (1 g of peptone per liter) and 3 g of acid-washed glass beads (425 to 600 μm, Sigma-Aldrich Co., St Louis, MO) as described by Hassan *et al.* (*9*) with some modifications. The bottles were then shaken for 10 min on an orbital incubator shaker (Model C24, New Brunswick Scientific) at 400 rpm to remove the bacteria from the coupons. Serial dilutions of the peptone water were made after shaking. The surviving bacteria from the control and treated coupons were enumerated by spread plating 0.1 ml of the diluents on TSA. The plates were incubated at 37°C for 48 h, and colonies were counted and recorded as log CFU per coupon. For microbiological analysis of the treatment water, serial dilutions of deionized and alkaline EO water were done without neutralization, enumerated on TSA plates and then incubated at 37°C

for 48 h. For acidic EO water and sodium hypochlorite solution, 1 ml of the double-strength neutralized solution was enumerated by plating 0.25 ml of this water on four TSA plates and incubating at 37°C for 48 h. Treated coupons were subjected to enrichment by adding 10 ml of the peptone water used for removing bacteria from the coupons to 10 ml of TSB and incubated at 37°C for 24 to 48 h. Enrichment of the treatment water was also done by adding 1 ml of the neutralized treatment solution to 10 ml of TSB and incubating at 37°C for 24 h. The presence of *L.* monocytogenes was confirmed as previously described.

Data analysis. Experiments were replicated three times with duplicate treatments in each replication. Data were analyzed by the general linear model procedure of the Statistical Analysis System (SAS Institute, Cary, NC). Comparisons of means were calculated with Tukey-Kramer multiple range tests.

RESULTS AND DISCUSSION

Effect of organic matter on the properties of EO Water. The addition of chicken serum of up to 10 ml/liter in treatment water did not significantly affect the pH of alkaline and acidic EO water produced at both 14 and 20 A. In other words, the pH which was originally 11.03 and 11.23 for alkaline EO water and 2.50 and 2.37 for acidic EO water produced at 14 and 20 A, respectively, remained essentially the same at all levels (1 to 10 ml/L) of serum concentration (data not shown).

Data on the effect of serum concentration on the ORP of EO water are presented in Figure 3-1. When chicken serum was added to achieve a concentration of 1 ml/liter, the ORP of

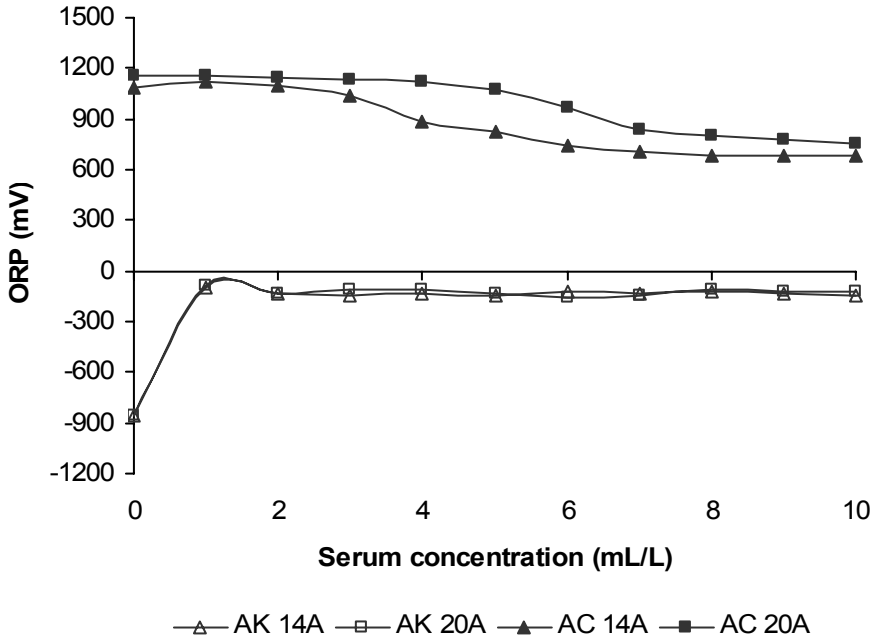

Figure 3-1. Oxidation-reduction potential of electrolyzed water after treating with different concentrations of chicken serum for 5 min.

AK, alkaline EO water; AC, acidic EO water

alkaline EO water increased sharply from -861 mV to -102 mV and then remained about the same with the addition of serum up to 10 ml/liter (Figure 3-1). Alkaline EO water produced at 20 A followed the same trend as that for 14 A, and there were no significant differences between the two. The ORP of acidic EO water produced at 14 A was 1,084 mV, and with the addition of chicken serum to achieve a concentration of 1 to 3 ml/liter, the ORP remained the same, then decreased gradually between 4 and 7 ml/liter to about 700 mV. Beyond this point any further addition of chicken serum to achieve a higher concentration of up to 10 ml/liter did not significantly change the ORP. A similar trend was also observed for acidic EO water produced at 20 A (Figure 3-1). The changes observed in the ORP of acidic and alkaline EO water show that the organic matter had reduced their oxidizing and reducing properties, respectively.

The addition of chicken serum to acidic EO water caused a decrease in the chlorine content (Figure 3-2). As the serum concentration increased, the chlorine concentration decreased steadily from 43 to 3 mg/liter and from 82 to 11 mg/liter for acidic EO water produced at 14 and 20 A, respectively (Figure 3-2). White (*22*) reported that proteins react with chlorine to form organochloramines, which could modify the properties of EO water. The reduction in the oxidizing property of acidic EO water could be due to a reduction in hypochlorous acid. The neutralization of chlorine in acidic EO water and other sanitizers by organic matter has also been demonstrated by other studies. As was observed in this study, El-Kest and Marth (*4*) also reported that the higher the amount of organic matter present, the greater the decrease in concentration of available chlorine.

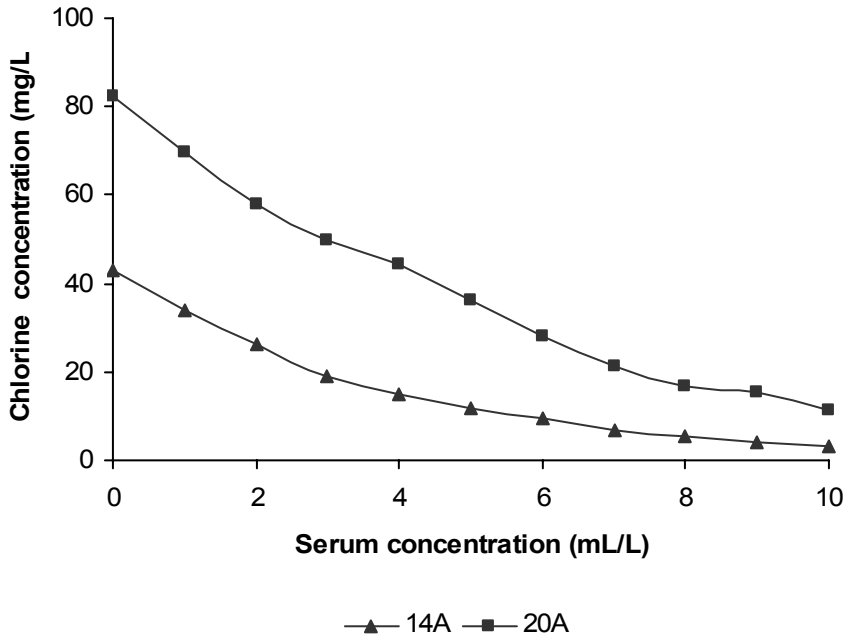

Figure 3-2. Available chlorine concentration of acidic electrolyzed water after treating with different concentrations of chicken serum for 5 min.

Treatment of planktonic cells of *Listeria monocytogenes* with EO water in the presence of organic matter. The properties of the EO water used for this study are presented in Table 1. Even though the pH of the alkaline EO water produced at 14 and 20 A was high (10.94 and 11.23 respectively), *L. monocytogenes* cells in suspension survived this treatment and the populations recovered were not significantly ($p>0.05$) different from the control, irrespective of the serum concentration and time of exposure (Table 3-2). Taormina and Beuchat (*19*) also observed that *L. monocytogenes* survived a 30-min exposure to alkaline cleaners with a pH range of 10.4 to 11.6.

In the absence of serum, acidic EO water generated at 14 and 20 A produced a > 6 log CFU/ml reduction in *L. monocytogenes*, after a 1-min treatment and no cells were recovered upon enrichment (Table 3-2). Our earlier study indicated that 1 min of EO water treatment reduced the initial population (8.90 log CFU per coupon) of *Listeria* biofilms by approximately 5 log CFU per coupon (*2*). With serum at a concentration of 5ml/liter, however, a 1-min treatment with acidic EO water produced at 14 A reduced the population of *L. monocytogenes* by only 0.33 log. As the serum concentration increased, the survival of *L. monocytogenes* after treatment with acidic EO water generally increased (Table 3-2).

Contrary to what was observed with acidic EO water produced at 14 A, in the presence of serum at a concentration of 5 ml/liter, acidic EO water produced at 20 A setting achieved a > 6 log CFU/ml reduction in *L. monocytogenes,* after 1-and 5-min exposures (Table 3-2). The higher reduction observed for acidic EO water produced at 20 A is attributed to the higher concentration of chlorine at that serum level. In the presence of organic matter, the chlorine concentration measured by the iodometric titration method represents the total available chlorine present, and this is made up of both free available and combined forms of chlorine.

Table 3-1. Properties of electrolyzed water used for the treatment of planktonic cells and biofilms of *Listeria monocytogenes*

Amperage (A)	Nature of Organism	Treatment water	pH	ORP (mV)	Total chlorine (mg/L)
14	Planktonic cells	Acidic	2.33	1166	44
		Alkaline	10.94	-864	0
20		Acidic	2.40	1169	94
		Alkaline	11.23	-882	0
20	Biofilms	Acidic	2.29	1163	85
		Alkaline	11.20	-885	0
		NaOCl	2.54	1138	86

Table 3-2. Survival of *Listeria monocytogenes* following exposure to electrolyzed water in the presence of organic matter

Amperage (A)	Time (min)	Treatment[a]	Population (log CFU/ml)[b] Serum concentration (ml/L) in treatment water 0	En[c]	5	En[c]	10
14	1	DI Water	a 7.69 ± 0.18 a		a 7.73 ± 0.22 a		a 7.67 ± 0.14 a
		Alkaline EO	a 7.71 ± 0.15 a		a 7.77 ± 0.15 a		a 7.73 ± 0.23 a
		Acidic EO	b < 1.00 b	0/6	a 7.40 ± 0.34 a		a 7.65 ± 0.25 a
	5	DI Water	a 7.75 ± 0.15 a		a 7.72 ± 0.15 a		a 7.71 ± 0.25 a
		Alkaline EO	a 7.69 ± 0.17 a		a 7.73 ± 0.17 a		a 7.69 ± 0.14 a
		Acidic EO	b < 1.00 c	0/6	b 7.26 ± 0.18 b		a 7.60 ± 0.17 a
20	1	DI Water	a 7.76 ± 0.06 a		a 7.70 ± 0.07 a		a 7.78 ± 0.11 a
		Alkaline EO	a 7.76 ± 0.11 a		a 7.76 ± 0.10 a		a 7.79 ± 0.11 a
		Acidic EO	b < 1.00 b	0/6	b < 1.00 b	3/6	a 7.56 ± 0.26 a
	5	DI Water	a 7.84 ± 0.15 a		a 7.71 ± 0.13 a		a 7.69 ± 0.07 a
		Alkaline EO	a 7.76 ± 0.12 a		a 7.77 ± 0.06 a		a 7.78 ± 0.11 a
		Acidic EO	b < 1.00 b	0/6	b < 1.00 b	2/6	b 6.55 ± 0.90 a

[a]DI water = Deionized water, Alkaline EO = Alkaline electrolyzed water, Acidic EO = Acidic electrolyzed water.

[b] Means preceded by the same letters in the same column within each amperage, time of exposure and serum concentration are not significantly ($p>0.05$) different. Means followed by the same letters in the same row within each treatment, time of exposure and amperage are not significantly ($p>0.05$) different. The detection limit was 10 CFU/ml.

[c] Treatments that were positive for *L. monocytogenes*, out of number of treatments analyzed by enrichment. Initial bacteria population used for treatments was approximately 8.90 log CFU/ml.

The reduced ability of acidic EO water produced at 14 A with serum (5 ml/liter) in inactivating *L. monocytogenes* may be because most of the chlorine present is in the combined unavailable chlorine form. Oomori *et al.* (*16*) reported a decrease in the available chlorine concentration of acidic EO water after the addition of nutrient broth, proteose peptone, glycine, corn oil, cow's milk and minced meat. In another study, Park et al. (*17*) reported that 1 mg/liter of free chlorine in acidic EO water was sufficient to completely inactivate the initial population (8 log CFU/ml) of *L. monocytogenes* within 30 s of treatment. To predict the concentration of available chlorine in the presence of serum, a regression model was developed to fit the experimental data to the general equation (equation 1) by a stepwise regression procedure of the Statistical Analysis System, (release 8.02, SAS Institute).

$$Y = -9.33 + 1.02 * X_1 - 0.085 * X_1 * X_2 \ (R^2 = 0.95) \qquad (1)$$

Where Y is the available chlorine concentration (in mg/liter) after being subjected to organic matter (serum), X_1 is the initial chlorine concentration (in mg/liter) and X_2 is the concentration of organic matter (in milliliters per liter).

The population of *L. monocytogenes* recovered after a 1-min exposure to acidic EO water produced at 20 A, containing serum at 10 ml/liter, was about the same as that from the deionized water treatment (Table 3-2). When the exposure time was extended to 5 min a significantly lower population (6.55 log CFU/ml reduction) was recovered. Therefore, longer exposure times may compensate for the presence of limited amounts of chlorine to achieve a bactericidal effect on *Listeria*. It is interesting to note that acidic EO water produced at 14 A containing serum at a concentration of 5 ml/liter also had a chlorine concentration of 11 mg/liter (Figure 3-2), but did not produce the ~1-log CFU/ml reduction in *Listeria* observed with acidic EO water produced at 20 A containing serum ar a concentration of 10 ml/liter, when the time of exposure was

increased from 1 to 5 min (Table 3-2). These differences in the ability to inactivate *L. monocytogenes* could be due to differences in the proportions of free and combined chlorine in the chlorine concentrations (11 mg/liter) measured i.e., for acidic EO water produced at 14 A containing serum at 5 ml/liter and for acidic EO water produced at 20 A containing serum at 10 ml/liter, however, this is not clear.

The survival of *L. monocytogenes* after treatments with acidic EO water containing chicken serum is a result of the lower bactericidal activity of combined chlorine as opposed to free available chlorine, since in the absence of serum, acidic EO water completely inactivated 8.9 log CFU of *L. monocytogenes* per ml (Table 3-2). In their study on disinfection by acidic EO water in the presence of organic materials, Oomori *et al*. (*16*) showed that the bactericidal activity of acidic EO water against *E. coli* K-12 was reduced when peptone, glycine and nutrient broth were added to the treatment water. Van de Weyer *et al*. (*20*) also tested the efficacy of several disinfectants on *Listeria* in the presence of organic matter and reported that the bactericidal activity of the chlorine containing disinfectant was diminished in the presence of proteins.

Treatment of *Listeria monocytogenes* biofilms with EO water in the presence of organic matter. Higher numbers of *L. monocytogenes* were recovered from control coupons and water-rinsed coupons with added serum than from those without serum (Table 3-3). This may be because of *Listeria* growth after the addition of the serum. A lower initial population (5.98 log CFU per coupon) of *Listeria* in the biofilms was also recovered from control coupons than in our earlier study (8.90 log CFU/coupon, *2*) (Table 3-3). This difference can be attributed to the death of cells as a result of desiccation of the biofilm during the 30-min incubation at 45ºC

Table 3-3. Survival of *Listeria monocytogenes* in biofilms after treatment with electrolyzed water in the presence of organic matter

Treatment water[a]	Serum concn in treatment water (ml/liter)	Populations recovered from coupons (log CFU/coupon) and time of exposure [b]			
		30 s	En[c] (no.positive/no. analyzed)	60 s	En[c] (no.positive/no. analyzed)
Control	0	5.98 ± 0.56		5.92 ± 0.69	
	2.5	6.47 ± 1.16		6.66 ± 0.34	
	5.0	6.77 ± 0.76		7.07 ± 0.49	
	7.5	7.17 ± 0.60		7.30 ± 0.50	
DI Water	0	b 5.19 ± 0.63 a A		b 5.24 ± 0.59 a A	
	2.5	ab 5.61 ± 0.72 a A		ab 5.98 ± 0.38 a A	
	5.0	ab 5.73 ± 0.83 a A		ab 5.66 ± 0.44 a A	
	7.5	a 6.41 ± 0.52 a A		a 6.13 ± 0.51 a A	
Alkaline EO	0	b 4.91 ± 0.57 a A		a 4.73 ± 0.62 a A	
	2.5	ab 5.27 ± 0.66 a A		a 4.88 ± 0.32 a B	
	5.0	ab 5.36 ± 0.78 a AB		a 5.43 ± 0.25 a A	
	7.5	a 6.10 ± 0.49 a AB		a 5.23 ± 0.53 b A	
Acidic EO	0	b 1.45 ± 0.29 a B	4/6	a 1.55 ± 0.53 a B	4/6
	2.5	b 2.71 ± 0.91 a B		a 2.31 ± 1.31 a C	4/6
	5.0	a 4.05 ± 0.97 a BC		a 1.85 ± 0.64 b B	
	7.5	a 5.13 ± 0.85 a B		a 2.97 ± 1.37 b B	
Sequential	0	a 1.51 ± 0.43 a B	3/6	a <1.30 a B	4/6
	2.5	a 1.51 ± 0.50 a C	2/6	a <1.30 a C	2/6
	5.0	a 1.55 ± 0.43 a D	4/6	a 1.35 ± 0.12 a B	1/6
	7.5	a 2.00 ± 1.17 a C	5/6	a 1.36 ± 0.14 a C	2/6
NaOCl	0	c 1.55 ± 0.36 a B	4/6	a 1.51 ± 0.50 a B	4/6
	2.5	bc 2.58 ± 0.45 a BC		a 1.62 ± 0.35 b C	5/6
	5.0	b 3.42 ± 1.14 a C		a 2.31 ± 1.08 a B	
	7.5	a 5.40 ± 0.49 a AB		a 2.70 ± 0.87 b BC	5/6

[a]DI water, deionized water; alkaline EO, alkaline electrolyzed water; acidic EO, acidic electrolyzed water, sequential, alkaline electrolyzed water followed by acidic electrolyzed water.

[b]Means preceded by the same lowercase letters in the same column within each treatment and time of exposure are not significantly ($p > 0.05$) different; means followed by the same lowercase letters in the same row within each treatment are not significantly ($p > 0.05$) different; means followed by the same uppercase letters in the same column within each serum concentration and time of exposure are not ($p > 0.05$) significantly different; Detection limit = 1.3 log CFU/coupon.

[c]Number of treated coupons positive for *L. monocytogenes*, as detected by enrichment, of the number of coupons analyzed by enrichment.

as previously described. Recovery of *L. monocytogenes* from biofilms treated with alkaline EO water was similar to the recovery when treatment was with deionized water (Table 3-3). In both cases a higher population was recovered as the concentration of serum increased. No significant ($p > 0.05$) differences in survival were detected with respect to time of exposure to alkaline EO water.

Fewer survivors were recovered after treatment of biofilms with acidic EO water than with alkaline EO water and deionized water. The survival of *L. monocytogenes* biofilms overlaid with serum, however, depended on the amount of serum applied. The population of *L. monocytogenes* recovered from coupons overlaid with serum at 7.5 and 5.0 ml/liter and exposed to acidic EO water for 30 s was significantly higher ($p \leq 0.05$) than those with serum at 2.5 and 0 ml/liter in that order (Table 3-3). For coupons with serum applied at 0 and 2.5 ml/liter, a > 3-log reduction in *L. monocytogenes* biofilms was achieved in 30 s of exposure to acidic EO water; however higher serum concentrations (5.0 and 7.5 ml/liter) resulted in a < 3-log reduction (Table 3-3).

As the time of exposure was increased from 30 to 60 s for coupons overlaid with chicken serum at 5.0 and 7.5 ml/liter, a significantly ($p < 0.05$) lower number of survivors was also recovered. In the presence of organic matter, a longer time of exposure (> 30 s) may therefore be required for acidic EO water to achieve significant inactivation of pathogenic bacteria. A sanitizer must reduce a microbial population in suspension by 5 log cycles after a 30 s exposure, and an attached or biofilm population by 3 log units or more to be considered effective (*3, 13, 15, 18, 20*). Acidic EO water reduced *L. monocytogenes* in suspension by > 6 log and biofilms by > 4 log, in the absence of serum and can therefore be considered an effective sanitizer. As

demonstrated by other studies (*6, 13, 15*) our results showed that biofilms are more resistant to sanitizers than are planktonic cells.

Irrespective of organic loads, the sequential treatment of biofilms with alkaline followed by acidic EO water was effective, producing a greater than 5-log reduction in *Listeria* biofilm populations (Table 3-3). Statistical analysis of the data indicated that at each level of added serum, significantly ($p < 0.05$) lower numbers of survivors were recovered from the sequential treatment than from the treatment with acidic EO water alone (Table 3-3). In their research on the removal of *L. monocytogenes* biofilms using chemical cleaning and sanitizing agents, Frank et al. (*6*) observed that the presence of organic load reduced microbial inactivation when sanitizers were used without previous cleaning. For practical purposes and effective sanitization, gross amounts of organic soil in food processing facilities should be removed before application of alkaline and acidic EO water. In the application of EO water as a sanitizer for the food industry, the sequential treatment should be used if organic matter may be present as the alkaline EO water may remove food residues and possibly modify the biofilm structure, making the adherent bacteria more susceptible to the acidic EO water (*2*).

Taormina and Beuchat (*19*) observed that *L. monocytogenes* exposed to alkaline cleaners for 30 min became sensitive to subsequent chlorine exposure. Frank et al. (*6*) reported that when cleaning and sanitizing were employed sequentially with an alkali cleaner (10 min exposure) and acidified sodium chlorite (30 min), adherent *L. monocytogenes* were reduced to nearly undetectable levels with a > 7 log reduction. Somers and Wong (*18*) also reported that treatment of *L. monocytogenes* biofilms on various surface materials with a solvated-alkaline product (10 min) followed by a hypochlorite sanitizer (1 min) achieved their target 3-log reduction. In the presence of meat and fat residue however, the target 3-log reduction was achieved only 77% of

the time. Recent studies utilizing both fractions of EO water have also showed that pretreatment of lettuce inoculated with *E. coli O157:H7* and *Salmonella spp* with alkaline EO water followed by acidic EO water resulted in a greater microbial reduction than what was obtained with other pretreatment solutions (*12*).

The results from the treatment of *L. monocytogenes* biofilms with acidified sodium hypochlorite followed a similar trend to that for treatment with acidic EO water (Table 3-3). In the absence of serum and at a serum concentration of less than 5 ml/liter, acidified sodium hypochlorite produced a greater than 3-log reduction in 30 s. However, at a serum concentration of 7.5 ml/liter, a less than 2-log reduction was achieved (Table 3-3). Best et al. (*3*) also reported that in the presence of human serum, sodium hypochlorite at 60 μg/ml achieved a < 1-log reduction of *L. monocytogenes* on stainless steel disks after a 1 min exposure.

Survival of *Listeria monocytogenes* in treatment water. After exposure of the biofilm-containing coupons to the various treatments, the water used for the treatments was tested for the presence of *L. monocytogenes* to determine the cross-contamination potential of the treatment solution. The population of *L. monocytogenes,* recovered from the deionized water and alkaline EO water after treatment of coupons without serum were significantly lower than those recovered after treatment of coupons which had been overlaid with a serum concentration of more than 5.0 ml/liter (Table 3-4). The recovery of *L. monocytogenes* from the deionized and alkaline EO water used for treatment confirm their lack of bactericidal activity and their potential for recontamination of equipment (Table 3-4). No *L. monocytogenes* were recovered from the acidic EO water and acidified sodium hypochlorite

Table 3-4. Populations of *Listeria monocytogenes* recovered from treatment water after exposure of biofilms contained on stainless steel coupons to electrolyzed water in the presence of organic matter

Treatment water[a]	Serum concentration in treatment water (ml/L)	Populations recovered from treatment water (Log CFU/ml) and time of exposure[b]			
		30 s	En[c]	60 s	En[c]
DI Water	0	b 3.18 ± 0.90		b 3.38 ± 0.57	
	2.5	ab 4.08 ± 1.02		a 4.71 ± 0.24	
	5.0	a 5.17 ± 0.65		a 4.86 ± 0.62	
	7.5	a 5.23 ± 0.69		a 4.87 ± 0.74	
Alkaline EO	0	c 2.99 ± 0.72		b 3.80 ± 0.54	
	2.5	bc 3.71 ± 0.74		ab 4.01 ± 0.28	
	5.0	ab 4.72 ± 0.61		a 4.77 ± 0.45	
	7.5	a 5.04 ± 0.76		a 4.72 ± 0.71	
Acidic EO	0	ND	0/6	ND	0/6
	2.5	ND	0/6	ND	0/6
	5.0	ND	0/6	ND	0/6
	7.5	ND	0/6	ND	0/6
Sequential	0	ND	0/6	ND	0/6
	2.5	ND	0/6	ND	0/6
	5.0	ND	0/6	ND	0/6
	7.5	ND	0/6	ND	0/6
NaOCl	0	ND	0/6	ND	0/6
	2.5	ND	0/6	ND	0/6
	5.0	ND	0/6	ND	0/6
	7.5	ND	0/6	ND	0/6

[a]DI water, deionized water; alkaline EO, alkaline electrolyzed water; acidic EO, acidic electrolyzed water; Sequential, alkaline electrolyzed water followed by acidic electrolyzed water.

[b]Means preceded by the same letters in the same column within each treatment and time of exposure are not significantly ($p > 0.05$) different; ND, not detectable on direct plate count and negative on enrichment.

[c]Number of treated coupons positive for *L. monocytogenes*, as detected by enrichment, of the number of coupons analyzed by enrichment.

post-treatment waters, and all enrichments were negative for *L. monocytogenes* (Table 3-4). Therefore, rinsing equipment with these solutions is unlikely to result in recontamination.

In conclusion, acidic EO water is an effective sanitizer that may have application in food processing facilities. The sequential use of alkaline followed by acidic EO water achieved a > 4 log reduction in *L. monocytogenes* biofilms even with the presence of organic matter up to 7.5 ml/liter.

ACKNOWLEDGEMENTS

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CHAPTER 4

ELECTROLYZED WATER AND ITS CORROSIVENESS ON VARIOUS SURFACE MATERIALS COMMONLY FOUND IN FOOD PROCESSING FACILITIES[1]

[1] Ayebah Beatrice and Yen-Con Hung. 2005. Journal of Food Process Engineering. 28:247-264. Reprinted here with permission of the publisher.

ABSTRACT

ASTM A-36 medium carbon steel, 110 copper, 3003-H14 aluminum, polyvinylchloride (PVC) type 1, and 304 stainless steel coupons, were immersed in electrolyzed (EO) water, chlorine water, modified EO water and deionized water for a period of 8 days and the properties of these types of water, weights and surface roughness of the coupons were monitored.

EO water significantly increased ($p < 0.05$) the surface roughness of carbon steel, aluminum and copper with time; however, chlorine water, modified EO water and deionized water produced minimal changes on these materials. Regardless of the treatment water used, the surface roughness of stainless steel and PVC essentially remained the same. Carbon steel, copper, aluminum, and stainless steel had a fair, good, good and outstanding corrosion resistance in EO water, respectively. Chlorine and modified EO water had a much less corrosive effect than EO water on all the materials tested.

INTRODUCTION

The use of sanitizers in the food industry is aimed at reducing microbial numbers to safe levels; however, these sanitizers may have other side effects such as corrosion of food contact surfaces due to their reactivity. Corrosion caused by the use of sanitizers is primarily because of chemical reactions of a sanitizer with the contact surface. This effect may be concentrated locally to form a pit or a crack, or may proceed uniformly over the entire exposed surface (Fontana, 1986). The presence of pits and abrasions is known to reduce cleanability and inactivation of pathogens on food contact surfaces (Frank and Chmielewski, 2000; Holah and Thorpe, 1990). This is because the pits and cracks on the corroded food processing equipment surfaces allow food material and bacteria to accumulate and form biofilms, which in the end survive the sanitation process. These bacteria will eventually cause cross-contamination of food during processing and may lead to spoilage or food borne outbreaks, defeating the sanitation process and the use of that sanitizer in the first place. Selection and evaluation of the reactivity of sanitizers with respect to the equipment to which they will be applied is therefore very important, if not critical.

Electrolyzed (EO) water production and its use as a sanitizer, have recently received a lot of attention. EO water is produced by passing a dilute salt solution through an electrolytic cell, within which the anode and cathode are separated by a diaphragm. By subjecting the cell to DC voltage, two types of water are produced. The electrolyzed acidic solution produced at the anode side, which has a strong oxidizing potential (oxidation-reduction potential [ORP] ~1100mV), a low pH (~2.6), contains hypochlorous acid (HOCl), and hence has a strong bactericidal effect (Kim *et al*., 2000). The electrolyzed basic solution which is produced at the cathode side, has a strong reducing potential (ORP~-800mV) and a high pH (~11). Recent research, by Ezeike and

Hung (2004) showed that the properties of EO water can be further controlled and optimized by monitoring the processing parameters. They reported that increasing the voltage and NaCl concentration resulted in a lower pH, higher ORP and residual chlorine of the acidic EO water, and increasing the electrolyte flow rate caused a reversal of these trends due to shorter residence time in the electrolytic cell. Several reports have demonstrated the bactericidal effect of EO water on pathogenic bacteria (Kim *et al*. 2000; Kiura *et al*. 2002) and *Listeria* biofilms (Kim *et al*. 2001). EO water also achieved significant reductions in *Campylobacter jejuni* on poultry (Park *et al*. 2002), *Escherichia coli O157:H7* and *Listeria monocytogenes* on lettuce (Park *et al*. 2001), *Salmonella* on alfalfa seeds and sprouts (Kim *et al*. 2003) and *E. coli O157:H7* and *Listeria monocytogenes* on kitchen cutting boards (Venkitanarayanan *et al*., 1999). The effect of pH, chlorine and mode of application on the chemical properties and bactericidal efficacy of EO water has also been investigated. Park *et al*. 2004 and Hsu *et al*. 2004 reported that increasing pH and spraying decreased the bactericidal efficacy of EO water; however, both reports indicated that with sufficient residual chlorine concentration, EO water can be applied by spraying and within a considerably wide pH range, (2.6 - 7.0) to achieve complete inactivation of *E. coli* O157:H7 and *L. monocytogenes*. EO water has also been successfully applied as a foliar spray to control powdery mildew on gerbera daisies (Mueller *et al*. 2003) and on other bedding plants without serious phytotoxic effects (Buck *et al*. 2003).

The use of EO water as a sanitizer in food processing plants has been suggested due to its high antimicrobial activity, low cost and ease of production and use, among other advantages. Although EO water has demonstrated a strong bactericidal activity, the possibility of corrosion of equipment because of its low pH and residual chlorine content is a matter of concern since acids and aqueous forms of chlorine are known to be corrosive (Daufin et al., 1988a; Bohner and

Bradley, 1991; McCafferty, 2003; Abd El Meguid and Abd El Latif, 2004). Most metals corrode when they come in contact with water, acids, bases, salts, some chemicals and gaseous compounds like acid vapors, sulfur-containing gases and ammonia gas. Stainless steel (types 304 and 316, American Iron and Steel Institute, AISI, Washington, DC) is the most widely used material in the construction of food processing equipment, food contact surfaces and pipes in food processing plants (Bohner and Bradley, 1991). These AISI 300 series stainless steel contain chromium and nickel, which make them resistant in many aqueous environments. Other materials such as aluminum, carbon steel, copper and PVC have also been used in the fabrication of certain parts of equipment or pipes in food processing plants.

Some work has been done in the medical field on the possible corrosive effects of EO water on dialysis equipment and metallic restorations used in the oral cavity. Tanaka et al. (1999) reported no visible significant differences in type 316 stainless steel sheets when soaked in electrolyzed strong acid aqueous solution (ESAAS) or 0.1% sodium hypochlorite (NaOCl). They however reported that stainless steel couplers used in dialysis equipment showed heavy corrosion after soaking in 0.1% NaOCl, while no significant corrosion was evident in ESAAS. EO water holds a lot of promise as an effective sanitizer for the food industry. It is therefore important to investigate its effect of some commonly used materials to aid in its selection as an appropriate sanitizer. The objective of the study was to determine the corrosive effect of EO water on various materials used in the fabrication of food processing equipment.

MATERIALS AND METHODS

Cleaning of Specimen Used for the Study

The following test materials were used: 304 stainless steel, ASTM A-36 medium carbon steel, 3003-H14 Aluminum, 110 copper and PVC type 1. Tests were conducted according to the American Society of Testing and Materials (ASTM) standard G1-90 (1999a) (Standard practice for preparing, cleaning and evaluating corrosion test specimens) and ASTM standard G31-72 (1999b) (Standard practice for laboratory immersion corrosion testing of metals). The specimen of the test materials were cut into 2.5 x 5 cm rectangular pieces referred to as coupons and cleaned by scrubbing with a mild non-bleach cleaner (Versa-Clean, Fisher Scientific Co., Pittsburgh, PA) using a soft nylon bristle brush. After rinsing thoroughly with deionized water, the coupons were dipped in acetone, air-dried and kept in a dessicator until they were used.

Water Used for Corrosion Testing

Four types of water were used for the corrosion tests. EO water, deionized water, chlorine water and modified EO water. Fresh EO water was produced from a ROX-20TA EO water generator (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at a current setting of 14 A. Deionized water, was collected from the deionized water faucet in the lab. Chlorine water (1.2%) was prepared from calcium hypochlorite ($Ca(OCl)_2$; Fisher Scientific Co., Fair Lawn, NJ) to match the total chlorine concentration of EO water. Modified EO water was produced from a laboratory EO water generator at a current setting of 12.3 A to produce EO water with a chlorine concentration similar to that of the regular EO water at a pH value around 6.

Measurement of the Properties of Water

The pH and ORP of all the types of water were measured using a digital pH/ORP meter (Acumet model 15, Fisher scientific Co., Fair Lawn, NJ) and the total chlorine concentration was determined by the iodometric method using a total chlorine test kit (Hach Co., Ames, IA).

Immersion Tests

For each coupon, 600ml (24ml /cm^2 of specimen) of freshly prepared water at room temperature (22°C) was poured into a 2 L glass jar. The coupon was placed on a glass cradle and immersed in the test solution and the jar was tightly covered to prevent loss of chlorine through evaporation. Each day (after 22 h of immersion), the coupon was taken from the water, washed using the cleaning procedure described above and dried. The pH, ORP and chlorine concentrations of the water after immersion were also measured daily. The weight of the dried coupon was determined, using an analytical balance (Voyager, Ohaus Co., Pine Brook, NJ). The surface roughness of each coupon was measured using an automatic surface tester (Hommel tester T1000, Hommel America Inc., New Britain, CT). During measurement, a stylus with a diamond tip attached to a pick-up arm is traversed over the surface to be measured. The pick-up arm is connected to two laminae and two coils in a pick -up housing and each movement of the stylus tip following its traverse over a rough surface causes the inductance of the coils to vary. These changes are picked up and converted into a signal that is proportional to the displacement and then reported as the corresponding surface dimension figure. The following surface roughness parameters, Ra (average surface roughness), Rz(DIN) (average of five largest peak-to-valley heights within one cut-off length) and Rmax (DIN) (the maximum peak-to-valley height within one cut-off length), were calculated based on the surface dimension figure. After

these measurements, the dried coupons were reimmersed into freshly prepared, EO, deionized, chlorine and modified EO water. The procedure was repeated for 8 days.

Annual Corrosion Rate Determination

The average weight lost over the period of immersion was calculated for each coupon and these values were used in determining the annual corrosion rate (ACR) which is a measure of the relative corrosion resistance of the material to the test solution.

$$ACR = KW / A\rho T$$

Where K is the constant for unit of conversion (8.76×10^4), W is the mass loss (g), A is the surface area of the specimen (cm^2), ρ is the density of the specimen (g/cm^3) and T is the total time of exposure (h)

STATISTICAL ANALYSIS

Data were analyzed using the general linear model procedures (SAS, 1995). Comparison of means was performed using the Duncan's multiple range tests.

RESULTS AND DISCUSSION

Change in Water Properties

The initial properties (pH, ORP and chlorine concentration) of all four types of treatment water are presented in Table 4-1. Changes in water properties are reported as the average of changes occurring daily over a period of 8 days.

Change in pH

Slight changes in the pH of the water were recorded. The pH of EO water and chlorine water increased during immersion (Figure 4-1). The change in pH of these two types of water were however not significant ($p < 0.05$). The pH of deionized and modified EO water, which were close to neutrality decreased during immersion (Figure 4-1). There was a significant ($p < 0.05$) interaction between the type of material and water. The magnitude of pH change in deionized water and modified EO water was therefore dependent on the type of material immersed in the water. The changes in pH of modified EO water were not significant for all the test materials except carbon steel which had an average decrease in pH of 2.7 per day (Figure 4-1).

Change in Oxidation-Reduction Potential

The ORP of chlorine and modified EO water increased during immersion (Figure 4-2). The change in ORP of modified EO water was higher than that of chlorine water; however, the magnitude of change was highly dependent on the type of material immersed in the water. The ORP of EO and deionized water decreased during immersion, and the immersion of carbon steel in EO water produced the highest loss in ORP (Figure 4-2). The change in ORP observed for EO water was, however, not significantly ($p < 0.05$) different from the change in chlorine water except for immersion of carbon steel.

Chlorine Loss

The initial chlorine concentration of fresh EO, chlorine water and modified EO water was approximately 50 mg/L (Table 4-1). The chlorine content in the water decreased during the 22 h

TABLE 4-1.

PROPERTIES OF WATER USED FOR CORROSION TESTING

Water	Properties measured		
	pH	ORP (mV)	Chlorine concentration (mg/L)
EO	2.42 ± 0.04	1077 ± 67	48.66 ± 1.06
DI	6.37 ± 0.56	584 ± 5	0
CLW	8.72 ± 0.56	656 ± 90	49.16 ± 0.16
MEO	6.12 ± 0.12	774 ± 3	50.39 ± 0.07

ORP, oxidation-reduction potential; EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified EO water.

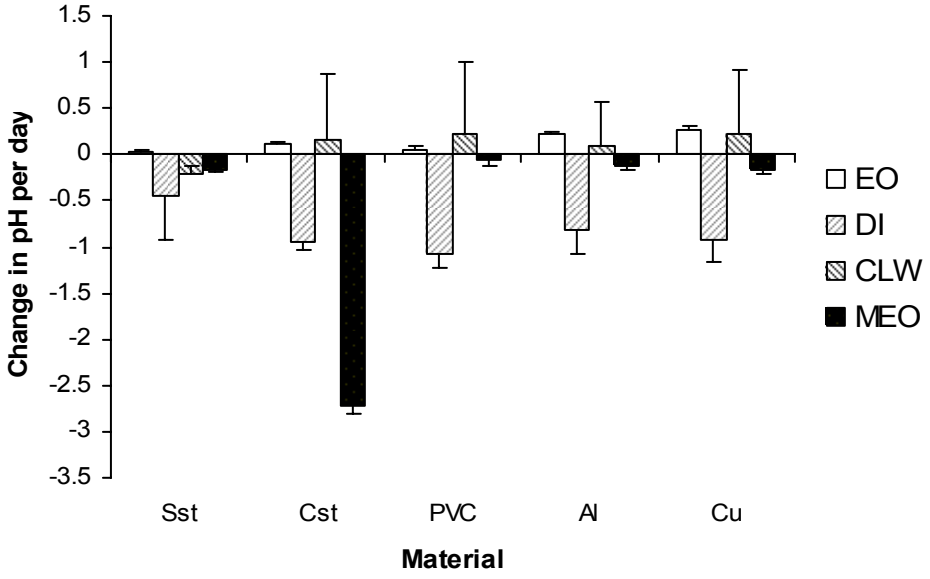

FIGURE 4-1. CHANGE IN pH OF WATER DURING IMMERSION

Sst, 304 stainless steel; Cst, ASTM A-36 medium carbon steel; PVC, polyvinyl chloride Type 1; Al, 3003-H14 aluminum; Cu, 110 Copper. EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.

immersion; however, the magnitude of loss was dependent on the material tested. The most reactive type of water was EO water, which lost about 85% of the chlorine present per day, during the immersion of carbon steel (Figure 4-3). EO water also lost about 69% of its chlorine during testing with copper, followed by aluminum (49%), then stainless steel (24%) and PVC (23%). Chlorine water and modified EO water lost very little chlorine during testing with stainless steel (0.74 and 1.94 mg/L, respectively) and PVC (0.67 and 0.85 mg/L respectively) (Figure 4-3).

When aluminum was immersed in chlorine and modified EO water, chlorine water lost 3.4 mg/L daily and modified EO water lost only half as much (Figure 4-3).
Len *et al*. (2002) reported a minimal change (<3 mg/L) in chlorine concentration when EO water was stored in a closed container for 150 h. This suggests that reduction in the residual chlorine concentration during immersion of coupons, in this study, was largely because of a reaction between the coupons and the reactive chloride ions. The least resistant material in this study, carbon steel, lost about 50% less chlorine in modified EO water (22 mg/L reduction per day) than in EO water (42 mg/L reduction per day) showing that EO water is more aggressive than modified EO water. With more resistant materials such as stainless steel, PVC, aluminum and copper the percentage of chlorine lost during testing with modified EO water was less than 20% of what was lost in EO water (Figure 4-3).

Weight Loss

Weight loss determination is one of the most popular methods of estimating corrosion losses in metals. It is simple and direct, requiring no theoretical assumptions or approximations and applicable to all corrosive environments, irrespective of the type of corrosion occurring.

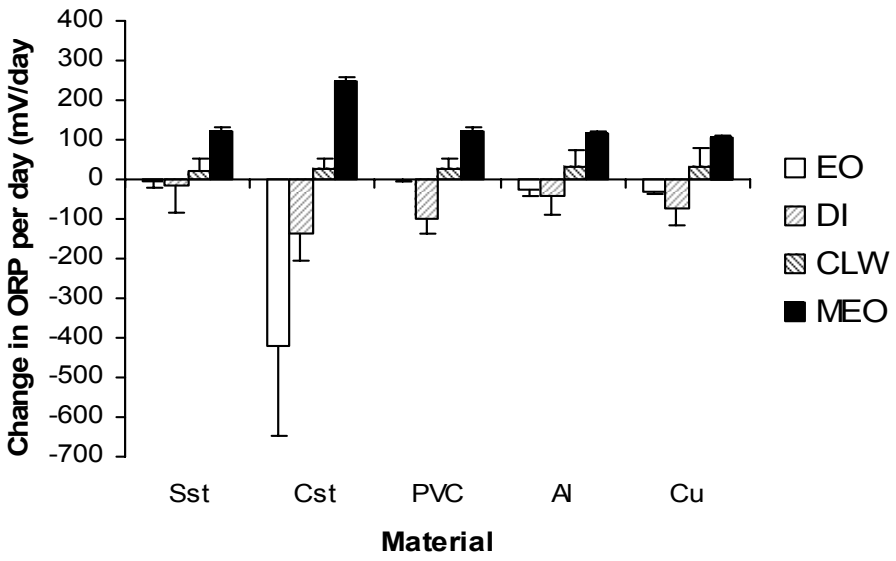

FIGURE 4-2. CHANGE IN OXIDATION REDUCTION POTENTIAL (ORP) OF WATER DURING IMMERSION

Sst, 304 stainless steel; Cst, ASTM A-36 medium carbon steel; PVC, polyvinyl chloride Type 1; Al, 3003-H14 aluminum; Cu, 110 Copper. EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.

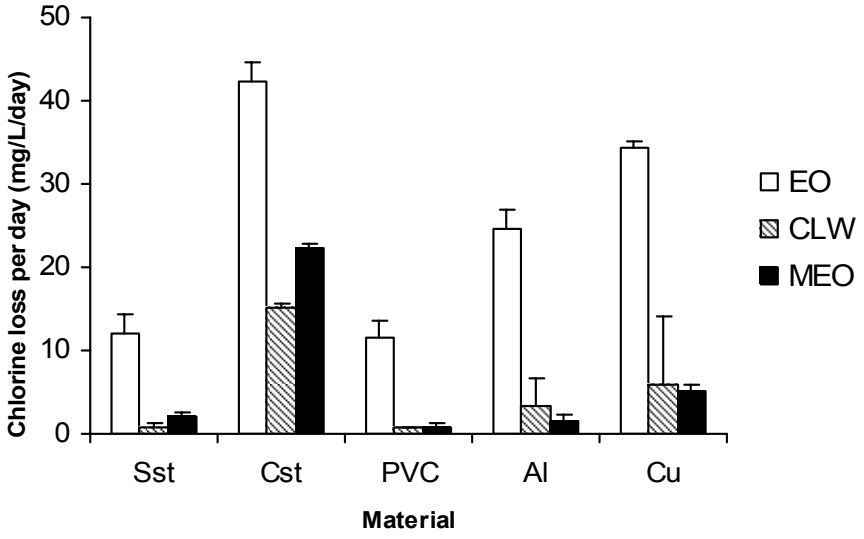

FIGURE 4-3. CHLORINE LOST DURING IMMERSION.

Sst, 304 stainless steel; Cst, ASTM A-36 medium carbon steel; PVC, polyvinyl chloride Type 1; Al, 3003-H14 aluminum; Cu, 110 Copper. EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.

The average weight loss per day was determined from the weight measurements taken. With the exception of PVC, the coupons from all the other materials either maintained their original weight or decreased in weight after immersion. Irrespective of the type of water in which it was immersed, PVC had an increase in weight (Figure 4-4). This was a result of the absorption of water into the coupons. Since PVC is porous to a very small extent, the amount of water absorbed was small with the maximum weight increase per day being 0.0015g for PVC immersed in EO water. Tanaka *et al*., (1999) also reported that vinyl chloride, polypropylene and viton rubber increased in weight when soaked in EO water for 5 weeks.

Carbon steel had the highest weight loss per day (0.04g), followed by copper (0.03g) during the immersion in EO water (Figure 4-4). Aluminum was affected to a lesser extent and stainless steel was virtually not affected by the EO water. The extent of weight loss was dependent on the type of material as well as on the type of water in which it was immersed. Significant interactions ($p < 0.05$) were found between the type of material and water. Stainless steel, aluminum, copper and carbon steel essentially maintained their original weights after immersion tests in deionized water. Chlorine water did not affect stainless steel and aluminum; however, it caused weight losses in copper (0.001g/day) and carbon steel (0.008g/day).

Modified EO water did not produce appreciable weight changes in stainless steel, aluminum and copper but caused 0.018g loss in weight per day in carbon steel (Figure 4-4). Depending on the water used in testing, each of the five materials behaved differently as a result of their completely different properties. Considering the least resistant material, carbon steel, EO water was the most reactive among the four types of water used in testing. Modified EO water produced half as much weight loss in carbon steel as EO water. A similar trend was also observed with chlorine losses where the least resistant material, carbon steel, lost about 50% less

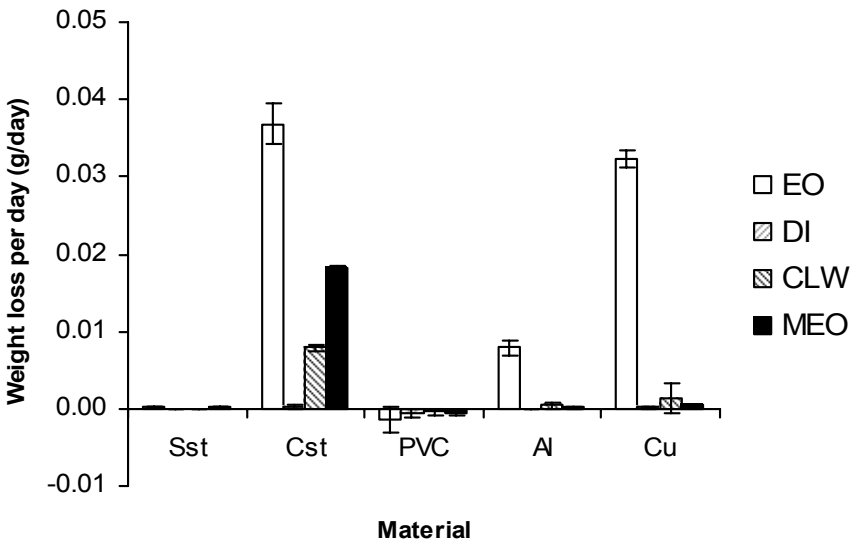

FIGURE 4-4. WEIGHT LOSS IN COUPONS DURING IMMERSION

Sst, 304 stainless steel; Cst, ASTM A-36 medium carbon steel; PVC, polyvinyl chloride Type 1; Al, 3003-H14 aluminum; Cu, 110 Copper. EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.

chlorine in modified EO water than in EO water. From these results, modified EO water had 50% less corrosive effect than EO water. A highly significant correlation (r = 0.91) was found between the concentration of chlorine lost and the average weight loss per day in this study, suggesting that the chloride ions were one of the primary causes of corrosion. Alhough chlorine water and modified EO water had comparable amounts of chlorine as EO water, these two caused significantly less loss in weight loss in all the tested materials, and hence, less corrosion than EO water. It is apparent therefore, that the chloride ions were not the only cause of weight loss due to corrosion, that other properties of the EO water could be involved as well. Daufin *et al.,* (1988a) stated that corrosion of metallic materials in contact with aggressive media involves a whole range of factors, which may act singly or jointly. The higher loss in weight per day during immersion in EO water can be attributed to a joint action of its low pH and high oxidizing power (high ORP) enhancing the corrosive potential of the chloride ions. The presence of high concentrations of free hydrogen ions (H^+), low pH, in EO water could speed up the rate of release of electrons from the metal surface resulting in the higher weight loss observed.

Annual Corrosion Rate

The ACR of all the tested materials, except PVC, was calculated using the total mass lost after 8 days of immersion in the test water. The ACR gives a measure of the resistance of the material to the environment in which it is tested. The higher the ACR, the less resistant the material is to that environment, and vice versa. The highest ACR was produced when the materials were tested for their resistance to EO water. Carbon steel had the highest ACR (0.5730 mm/year) in EO water, followed by copper (0.4590 mm/year), aluminum (0.3438 mm/year) and stainless steel (0.0038 mm/year) (Table 4-2). Relative corrosion resistance rating was assigned

based on the table developed by Fontana (1986) (Table 4-3). From this table, carbon steel, which had the highest ACR, was rated as having a fair corrosion resistance in EO water, while copper and aluminum were rated as having a good corrosion resistance in EO water (Tables 4-2 and 4-3). Carbon steel also had a good resistance in chlorine and modified EO water and its resistance to deionized water was rated as outstanding, showing the dependence of corrosion of materials on the type of environment it is in contact with. The corrosion resistance of carbon steel in modified EO water was 50% more than that in EO water, and copper and aluminum were even more resistant in modified EO water (Table 4-2). The ACR of aluminum was 74% higher in chlorine water than in modified EO water and that of copper was 65% higher in chlorine water than modified EO water, suggesting that chlorine water, which is commonly used as a sanitizer in the food industry, is also more corrosive to these materials than modified EO water. All four materials had an outstanding corrosion resistance in deionized water and except for carbon steel, the other three materials had an outstanding corrosion resistance in modified EO water.

Stainless steel, which is the most commonly used material in the fabrication of equipment in the food industry, had an outstanding corrosion resistance to all the types of water tested (Table 4-2). Similar results were obtained by Tanaka *et al*., (1999) who reported less than 0.01 mm/year loss in stainless steel (SUS316) dialysis couplers soaked in electrolyzed strong acid solution for 36 days.

Corrosion was attributed to a reaction between the metals and the chloride ions in the water and a high correlation ($r = 0.95$) was found between the annual corrosion rate and the concentration of chlorine lost daily, to confirm this relation. Fontana (1986) reported that both hydrogen and chloride ions stimulate the dissolution of metals and alloys, and a similar observation was made from the results of this study.

TABLE 4-2.

ACR OF MATERIALS USED IN CORROSION TESTS*

Material	Type of Water			
	EO	DI	CLW	MEO
Sst	0.0038(0.0010)	0.0000(0.0008)	0.0002(0.0008)	0.0048(0.0003)
Cst	0.5730(0.0393)	0.0021(0.0037)	0.1223(0.0063)	0.2827(0.0052)
Al	0.3438(0.0398)	0.0000(0.0011)	0.0169(0.0191)	0.0044(0.0049)
Cu	0.4590(0.0161)	0.0015(0.0010)	0.0150(0.0265)	0.0053(0.0027)

*Units for Annual Corrosion Rate: mm/year; values in brackets are standard deviations

Sst, 304 stainless steel; Cst, ASTM A-36 medium carbon steel; PVC, polyvinyl chloride Type 1; Al, 3003-H14 aluminum; Cu, 110 Copper. EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.

TABLE 4-3.

RELATIVE CORROSION RESISTANCE RANKINGS (Fontana, 1986)*

Relative corrosion resistance	Annual corrosion rate (mm/yr)
Outstanding	<0.02
Excellent	0.02 - 0.1
Good	0.1 - 0.5
Fair	0.5 - 1.0
Poor	1 - 5.0
Unacceptable	5.0+

* Based on typical ferrous and nickel based alloys

The presence of a higher concentration of hydrogen ions (low pH) in EO water seemed to facilitate the reaction of the chloride ions, resulting in higher corrosion rates in EO water than chlorine and modified EO water. From electrochemical considerations, it is also known that the presence of oxidizing agents greatly enhances the corrosivity of chloride ions (Daufin *et al*., 1988b). The corrosion of carbon steel, aluminum and copper in EO water was therefore caused by the joint action of the low pH (high concentration of (H+)), the high ORP and chloride ions. It is important to note that the extent of reactivity of each type of water, is dependent on the type of material in contact with it and this will also directly influence the extent of corrosion of that material. In essence, though some materials will corrode in some types of water, eg. Carbon steel in EO water, others will not.

Appearance of Materials after Testing

Immersion of carbon steel in EO water over time produced rust colored products, from the reaction of iron present in the carbon steel with chloride, oxygen and water. Modified EO water also caused some minor rusting during immersion of carbon steel; however, it was not as corrosive to carbon steel as EO water (Table 4-2). Rust formation is believed to be the result of a series of complex processes that begin with the oxidation of iron to ferrous (Fe^{2+}) ions and then to ferric (Fe^{3+}) ions. The electrons provided from the EO water (because of the strong oxidation potential) may reduce oxygen that would in turn combine with ferric ions to form ferric oxides such as iron (III) hydroxide ($Fe(OH)_{3)}$, iron oxide (Fe_3O_4) and ferric oxide (Fe_2O_3). Ferric oxides when hydrated in water form rust ($Fe_2O_3.XH_2O$). At the end of the immersion period (8 days), deionized water and chlorine water did not change the color of carbon steel. Modified EO

water and EO water, however, caused bleaching of its dark-gray color with the extent of bleaching being higher in EO water than modified EO water.

During testing of stainless steel with modified EO water and chlorine water, a small amount of yellow colored rust was observed on the cut and exposed edge of the coupon. This tainting, although very small, was not observed with EO water. Stainless steels rely on the stable chromium oxide film to provide corrosion resistance. The rust observed on the cut edge is evidence of some amount of corrosion because of the destruction of the oxide film on that side of the coupon during cutting and exposing iron to the environment. Besides the evidence of some rusting on its exposed sides, stainless steel had an outstanding corrosion resistance to modified EO water (Table 4-2). All the types of water tested also did not alter the shiny appearance of the stainless steel coupons.

The appearance of PVC did not change after immersion, irrespective of the test water used. After immersion in deionized and modified EO water, the appearance of aluminum did not change. On the other hand, chlorine water changed the silvery luster of the aluminum coupons to a dull tarnished color and EO water bleached the coupons white.

Deionized water did not change the shiny appearance or color of copper, however, chlorine water and modified EO water changed its appearance to a dull rusty red color. Black rust streaks that started from the edge of the coupons and grew progressive over its surface with time, were observed on copper coupons immersed in chlorine and modified EO water. Coupons immersed in chlorine water had more of these black rust streaks than coupons immersed in modified EO water. EO water caused pitting on copper and also made its color pale.

Besides deionized water, modified EO water had the least effect on the tested materials. In the case of stainless steel, in so far as the surface is not broken resulting in the destruction of

the protective oxide layer, no problems will be encountered and modified EO water can be safely used. Modified EO water also had no effect on the appearance of aluminum and PVC. However, the application of modified EO water in an environment containing copper or carbon steel may not be desirable.

Surface Roughness

Corrosion causes changes in the surface properties of the metal as a result of the formation of pits, crevices or cracks and these changes can be monitored to obtain additional information on the extent and type of corrosion.

In this study the effect of the treatment water on the surface roughness of the coupons was highly dependent ($p<0.05$) on the type of material. The average surface roughness (Ra) profile of stainless steel and PVC remained about the same during immersion regardless of the treatment water (Figure 4-5A, C). EO water significantly ($P < 0.05$) increased the Ra of carbon steel, aluminum and copper than the other types of water tested. Multiple comparison tests showed that deionized water, chlorine water and modified EO water had the same effect on the Ra of carbon steel, aluminum and copper (Figure 4-5B, D, E).

The increase in Ra of the carbon steel, aluminum and copper after being immersed in EO water can be attributed to the dissolution of these metals and the formation of pits as a result of corrosion. Ra measures the average of the surface roughness and hence it is not sensitive to individual peaks and valleys such as Rz and Rmax (Mummery, 1992). Rz is a measure of the average depth of pits formed on the materials as a result of corrosion during immersion in the treatment water and Rmax is a measure of the depth of the largest pit formed within the assessed profile of the coupons.

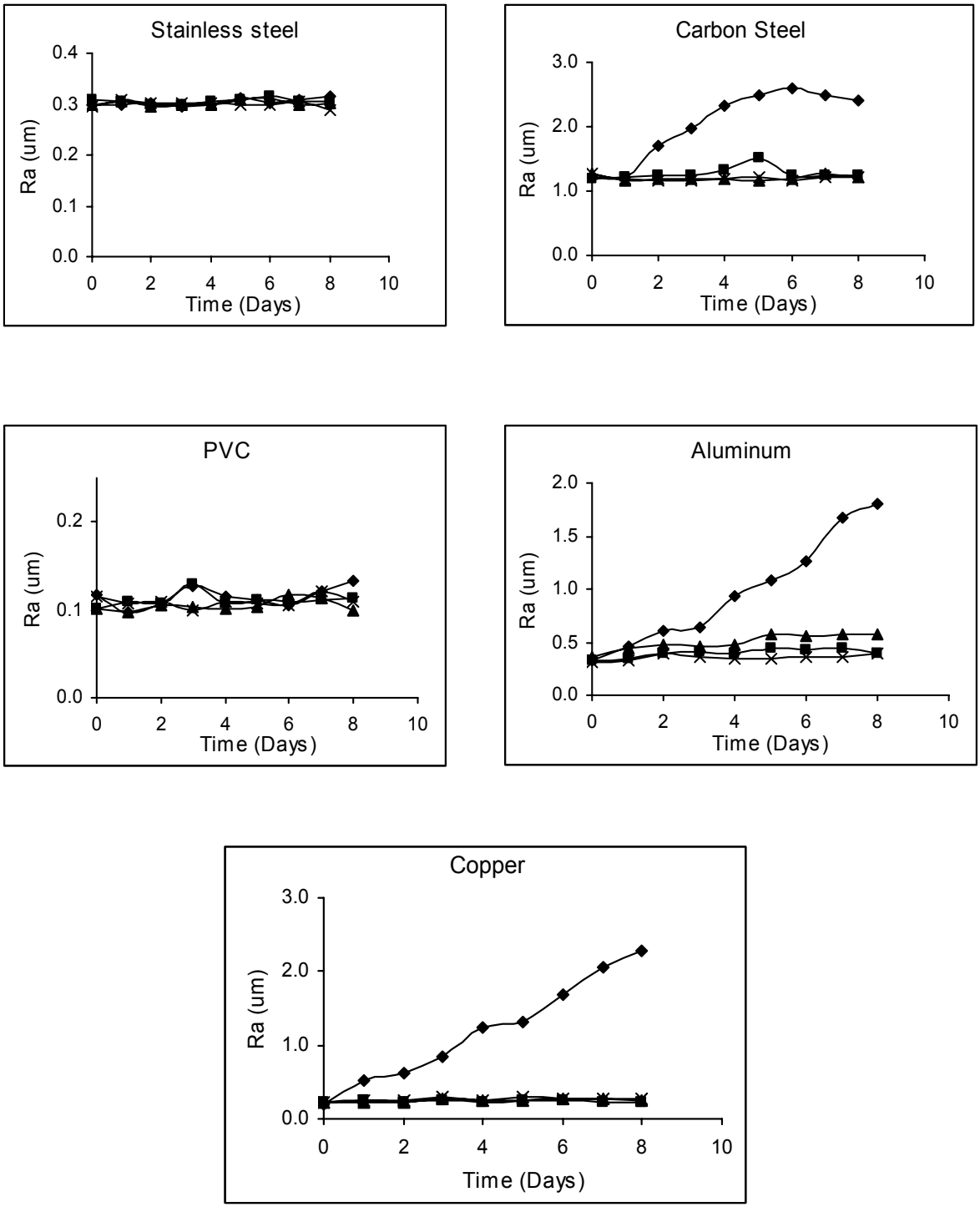

FIGURE 4-5. CHANGE IN AVERAGE SURFACE ROUGHNESS OF COUPONS WITH TIME

(◆) Electrolyzed water, (■) De-ionized water, (▲) Chlorine water and (X) Modified electrolyzed water.

Graphs showing the change in Rz and Rmax of the materials with time, followed the same trend as that of Ra with time (data not shown). The only difference was that for every material, at any point in time, Rmax was greater than Rz which was in turn greater than Ra. For example, the initial surface roughness measurement ie., day 0, Ra for stainless steel was 0.307µm while Rz was 2.455µm and Rmax was 3.122µm. These differences in magnitude are a result of what each parameter is measuring with Rmax accounting for the largest pit while Rz accounts for the average of the 5 largest pits. As a result of the similar trends, graphs of Rz and Rmax with time, are not shown.

The Rz and Rmax of stainless steel and PVC also remained about the same throughout the period of immersion in all four types of water and statistical analysis showed that the treatment water did not significantly alter the Rz and Rmax of these materials. This indicates that the treatment water did not affect the surface roughness of these materials.

The multiple comparison tests showed that the effect of deionized water, chlorine water and modified EO water on the Rz and the Rmax of carbon steel, aluminum and copper were the same and changes occurring in the original surface profile of these, during immersion were minimal. As was observed with Ra measurements, EO water was the only test water that caused a significant increase ($P < 0.05$) in Rz and Rmax (evidence of pitting corrosion) on carbon steel, aluminum and copper. Significant changes in Rz and Rmax of carbon steel were observed only after the 2nd day of immersion, after the 3rd day for copper and after the 4th day for aluminum. It was thought that, the shorter the initiation period for pitting, the less resistant the material may be in that environment. This was confirmed by the annual corrosion rates of these materials in EO water (Table 4-2).

Because EO water contained about the same amount of residual chlorine as chlorine water and modified EO water, the aggressive nature of the EO water environment, causing significant changes in surface roughness, was attributed to a joint action of its low pH and high oxidizing power (high ORP) enhancing the corrosive potential of the chloride ions present.

CONCLUSIONS

The results of this study showed that corrosion of metals is dependent on the type of environment and the resistance of the material in question. Carbon steel was the least resistant of all the materials tested. Although carbon steel had a fair, good and good corrosion resistance in EO water, chlorine water and modified EO water respectively, its use in such environments is not recommended. Stainless steel, which is the most commonly used material in food contact surface and equipment fabrication in the food industry, had an outstanding corrosion resistance to all the types of water tested. Because EO water did not have any adverse effect on stainless steel, it can still be safely used as a sanitizer to inactivate bacteria on food contact surfaces made from stainless steel, in food processing plants. Among the four types of water tested, EO water was the most aggressive causing uniform and pitting corrosion of carbon steel, copper and aluminum. When EO water was modified to a higher pH, it ceased to be as aggressive and in most cases only had the same effect as deionized water. These results demonstrate that EO water and modified EO water are promising sanitizers for the food industry.

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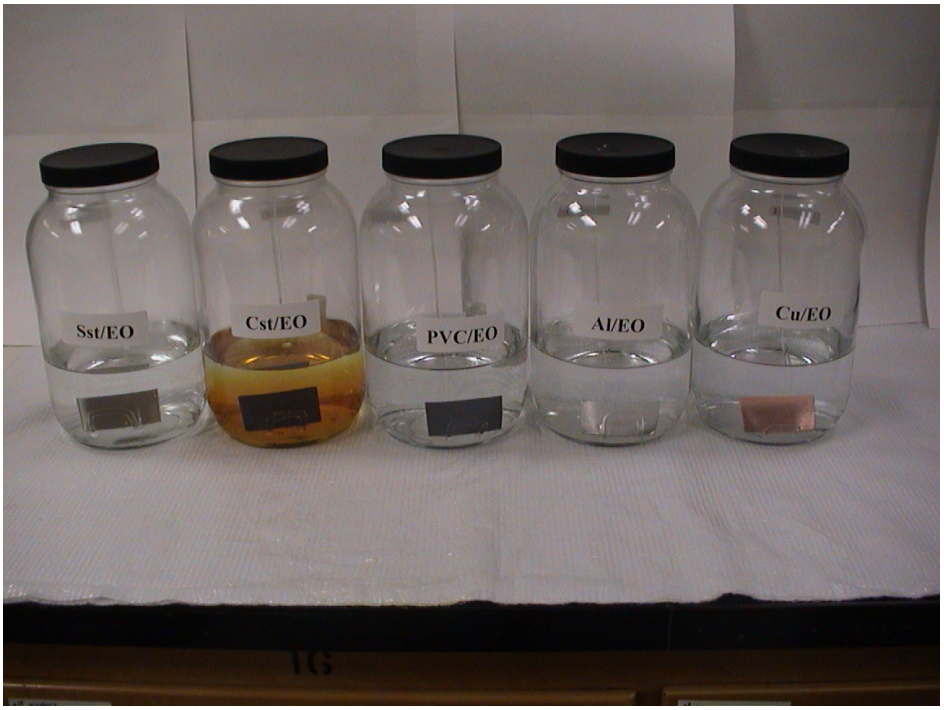
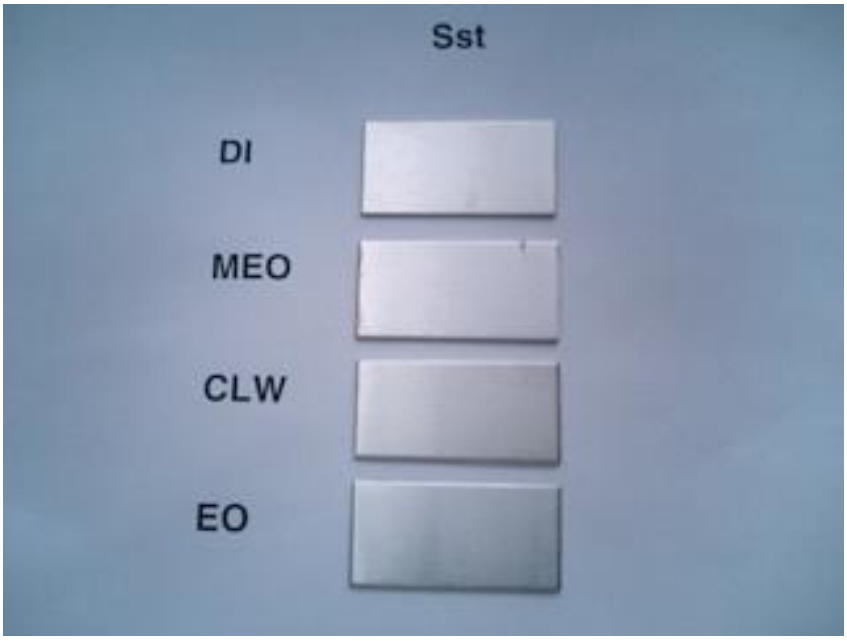
APPENDIX



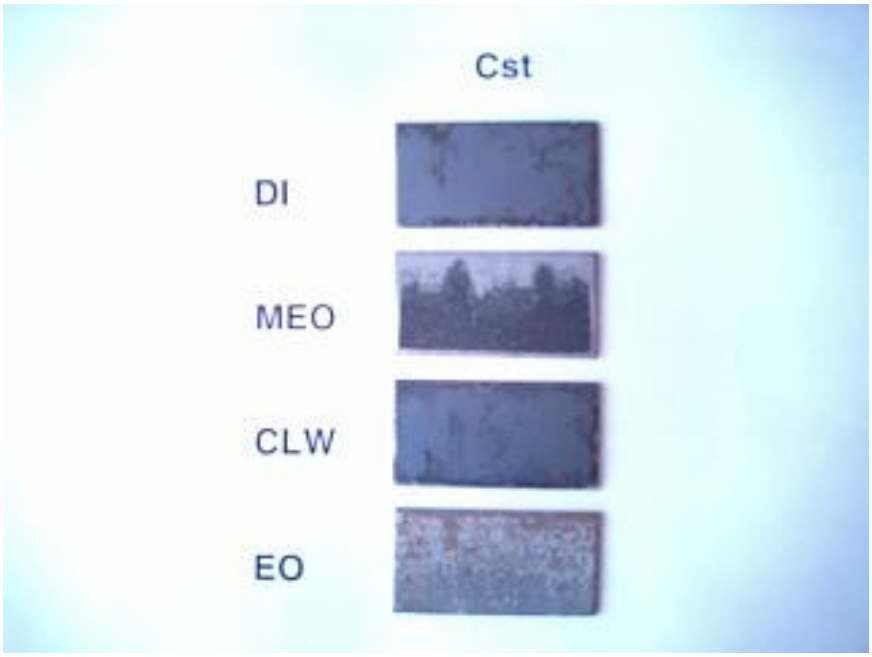
APPENDIX 4-1. IMMERSION OF COUPONS IN ELECTROLYZED WATER

Sst, 304 stainless steel; Cst, ASTM A-36 medium carbon steel; PVC, polyvinyl chloride Type 1; Al, 3003-H14 aluminum; Cu, 110 Copper. EO, electrolyzed water



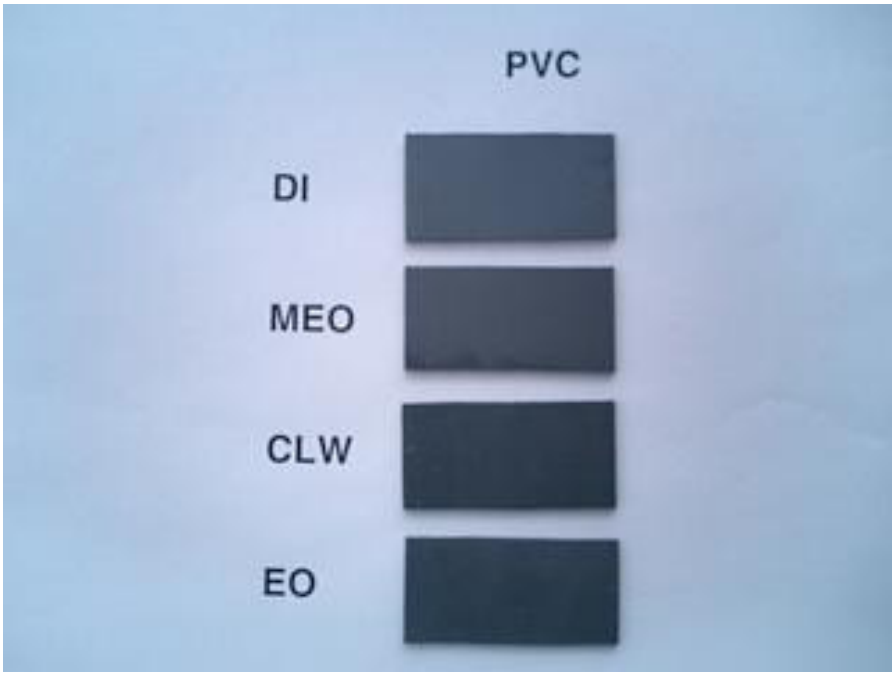
APPENDIX 4-2. STAINLESS STEEL COUPONS EXPOSED TO DIFFERENT TYPES OF WATER FOR 8 DAYS

EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.



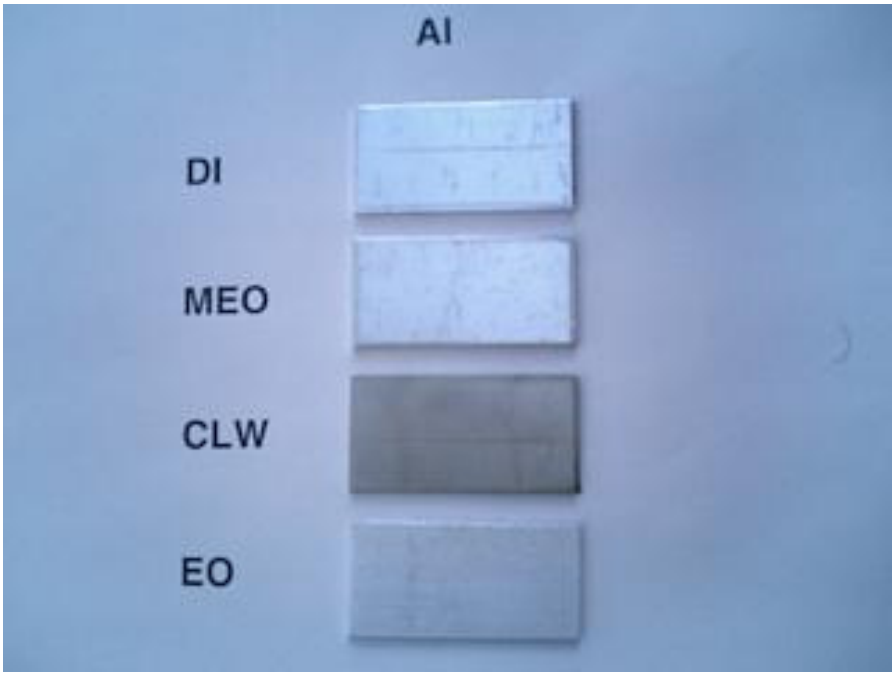
APPENDIX 4-3. CARBON STEEL COUPONS EXPOSED TO DIFFERENT TYPES OF WATER FOR 8 DAYS

EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.



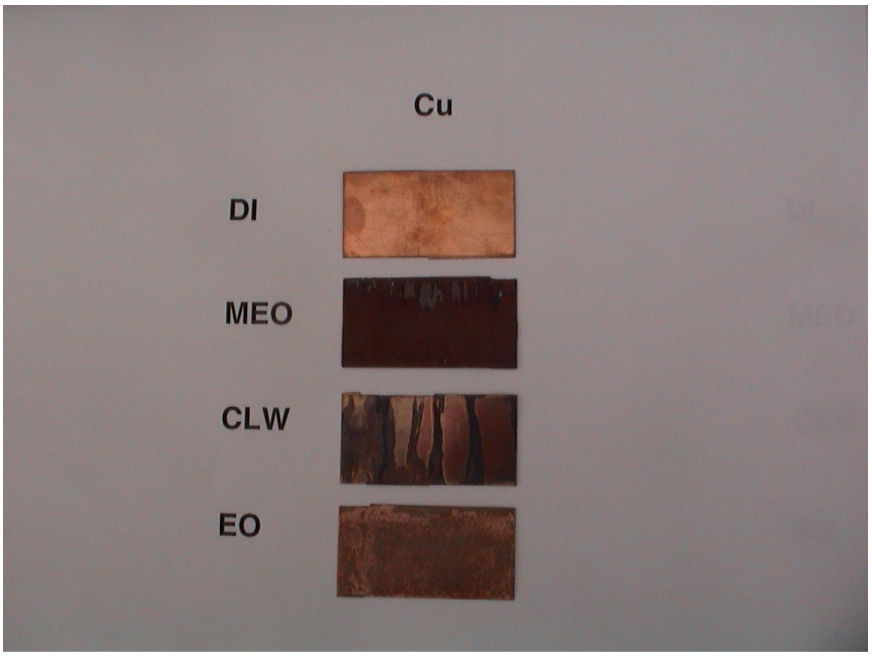
APPENDIX 4-4. PVC COUPONS EXPOSED TO DIFFERENT TYPES OF WATER FOR 8 DAYS

EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.



APPENDIX 4-5. ALUMINUM COUPONS EXPOSED TO DIFFERENT TYPES OF WATER FOR 8 DAYS

EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.



APPENDIX 4-6. COPPER COUPONS EXPOSED TO DIFFERENT TYPES OF WATER FOR 8 DAYS

EO, electrolyzed water; DI, deionized water; CLW, chlorine water; MEO, modified electrolyzed water.

CHAPTER 5

EVALUATION OF A LABORATORY SCALE CONVEYOR SYSTEM FOR THE APPLICATION OF ELECTROLYZED WATER IN SANITIZING SURFACES CONTAINING *LISTERIA MONOCYTOGENES* BIOFILMS[1]

[1]**Ayebah, Beatrice, Yen-Con Hung, and Glen D. Farrell. To be submitted to Applied and Environmental Microbiology.**

ABSTRACT

Biofilm formation by *L. monocytogenes* and its subsequent inactivation by sanitizers may be influenced by several factors including, the composition of the surface material, the presence or absence of other bacteria, the age of the biofilm and the time of exposure to sanitizers. This study was carried out to determine the effectiveness of acidic electrolyzed oxidizing (EO) water for inactivation of *L. monocytogenes* in monospecies and mixed biofilms, with *Pseudomonas putida,* on stainless steel and teflon surfaces. A laboratory scale conveyor system was fabricated and evaluated for its use in the application of electrolyzed water for inactivating *L. monocytogenes* biofilms. *L. monocytogenes* biofilms either alone or with preexisting *P. putida* biofilms were allowed to grow on stainless steel or teflon at 25°C for 2 or 7 days and then subjected to EO water treatments. *L. monocytogenes* alone or with *P. putida* biofilms on stainless steel were more susceptible to inactivation with acidic EO water, producing significantly higher ($p \leq 0.05$) log reductions than biofilms on teflon. Seven day biofilms were generally more susceptible to inactivation by acidic EO water than 2 day biofilms. The incorporation of a 2 min residual time after the immersion treatment of biofilms resulted in additional inactivation of up to 0.47 and 0.82 log CFU per coupon in *L. monocytogenes* biofilms on stainless steel and teflon, respectively. After the 5^{th} reuse of acidic EO water, *L. monocytogenes* was reduced by 4.03 and 1.74 log CFU per coupon on stainless steel and teflon coupons, respectively, and the difference in the population inactivated after the 1^{st} use and 5^{th} reuse was < 1 CFU/coupon, on both materials. This research demonstrates that acidic EO water can be reused several times during immersion cleaning without significant reduction in its bactericidal efficacy or the probability of recontamination from the used water.

INTRODUCTION

In nature, most bacteria typically do not exist as pure cultures but rather as complex sessile communities, usually referred to as biofilms (7, 8). The food processing environment particularly presents an excellent opportunity for the formation of biofilms because of the existence of favorable conditions, such as the presence of microorganisms in raw material and the environment, food and non-food contact surfaces to which microorganisms can attach, the availability of water and ample amounts of food or its residues which serve as nutrients for microbial growth and metabolism.

One of the important foodborne pathogens frequently found in food processing environments is *Listeria monocytogenes*. It has been isolated from the environment (floors and floor drains), equipment (whipping filling, molding, dicing and packaging machines), raw material and finished products from meat, poultry, seafood and ice cream plants (21, 25, 29, 30). In most of these processing plants, *L. monocytogenes* persisted for long periods of time (several months to years) and it is possible that this pathogen was able to survive and persist in these environments by adhering to and forming biofilms on available surfaces.

Pseudomonas spp. are the most common spoilage organisms (13) and as such are responsible for significant economic losses in the food industry. When they are isolated from processing plants and food, *Pseudomonas* spp. are usually found in association with other bacteria such as Enterobacteriaceae, Aeromonadaceae, *Shewanella* spp and *Lactobacillus* spp. (9, 11, 17). Several investigations have been carried out to determine the nature and effect of interactions of *Pseudomonas* spp. biofilms with biofilms of other spoilage and pathogenic bacteria (4, 12, 14). In mixed biofilms, *Pseudomonas* spp. may enhance (12, 20), reduce (4, 26) or have no effect on the growth of other bacteria. Additionally it has been reported that

Pseudomonas spp. in multispecies biofilms with *L. monocytogenes* may make the pathogen more susceptible (20) or resistant (1) to sanitizers. The extent of biofilm formation and the resistance or susceptibility of biofilm inhabitants to sanitizers may also be influenced by the age of the biofilm and the type of surfaces to which they are attached.

The objectives of this research were to (i) determine the effectiveness of acidic electrolyzed water in the inactivation of *L. monocytogenes* in mixed biofilms with a food processing isolate of *Pseudomonas putida* on stainless steel and teflon surfaces; (ii) evaluate a laboratory scale conveyor system in the application of acidic electrolyzed water for inactivating *L. monocytogenes* biofilms.

MATERIALS AND METHODS

Treatment water. EO water was produced from a ROX-20TA generator (Hoshizaki Electric Inc., Toyoake, Aichi, Japan) at a current setting of 14 A. After a stable amperage reading was achieved, alkaline and acidic EO water were collected from the cathode and anode side respectively, into separate sterile Nalgene™ carboy containers and used within 1 h of production. The Oxidation Reduction Potential (ORP) and pH of both the alkaline and acidic EO water were measured immediately after preparation with a dual scale pH meter (Accumet ® AR50, Fisher Scientific Co., Fair Lawn, NJ). The total chlorine content of the acidic EO water was determined by an iodometric method (Hach Co., Ames, IA) using a 0.113 N sodium thiosulfate standard solution.

Preparation of inocula. Five strains of *Listeria monocytogenes* F8027 (celery isolate), F8255 (peach isolate), 101M (beef isolate), H7750 (hot dog isolate) and G3990 (Vacherin Mont d'Or cheese isolate) and *Pseudomonas putida* (food processing isolate) were used for this study. A loop inoculum of each culture was transferred three times in tryptic soy broth (TSB, Becton, Dickinson & Co., Sparks, MD) and incubated at 37°C (*L. monocytogenes*) or 30°C (*P. putida*) at successive 24 h intervals. A 24 h culture of each bacterial strain was then centrifuged two times for 10 min (3,600 x g, 23°C) and the pellet was washed each time with 5 ml peptone water (1 g peptone/L**,** Becton Dickinson & Co). Each pellet was resuspended in 5 ml of peptone water and the five *L. monocytogenes* cultures were combined to form a mixture with a population of approximately 9 log CFU/ml. *P. putida* cells were resuspended in peptone water to obtain 8 log CFU/ml. These cultures were used for preparing inoculum for biofilm formation.

Preparation of stainless steel and teflon coupons. New stainless steel sheets (type 304, no. 4 finish; 1-mm thickness; Stewart Stainless Supply Inc., Suwanee, GA) and teflon (1-mm thickness) were cut into 2 by 5 cm (10 cm^2) coupons. The coupons were degreased in acetone, rinsed in deionized water and shaken in a 2% solution of Micro-90 soap (International Products Co., Burlington, NJ) at 120 rpm and 24 ± 2°C for 1 h on a platform shaker (Model C10, New Brunswick Scientific, Edison, NJ). They were then brushed gently with a soft nylon brush, rinsed thoroughly with deionized water and immersed in 15% phosphoric acid solution for 20 min at room temperature (24 ± 2°C) with shaking at 120 rpm. The coupons were rinsed thoroughly with deionized water, allowed to dry at room temperature and then autoclaved at 121°C for 15 min.

Preparation of mono and duospecies biofilms. To prepare biofilms containing both *Pseudomonas* and *L. monocytogenes*, ten ml of the *P. putida* culture was added to 1 L of sterile 1:10 dilution of TSB (referred to as dilute TSB, dTSB, 3 g of dry medium/L of deionized water) to produce an initial inoculum of 6 logCFU/ml for the preparation of biofilms. The inoculated dTSB was then dispensed (30 ml) into sterile test tubes holding sterile stainless steel or teflon coupons and incubated at 25°C for 4 h to allow the *P. putida* cells to attach to the surfaces. After the 4 h attachment period, the coupons were rinsed gently in a circular motion for 10 s with peptone water (1 g peptone/L) to remove unattached cells and re-immersed in sterile dTSB contained in sterile test tubes and incubated at 25°C for 48 h to allow biofilm formation. After the 48 h incubation period, the coupons containing *P. putida* biofilms were removed from the spent medium, gently rinsed in sterile peptone water (1 g peptone/L) and placed in sterile test tubes. An inoculum of *L. monocytogenes* was prepared by adding 10 ml of the mixed cocktail (5 strains of *L. monocytogenes*) to 1 L of sterile dTSB to produce an inoculum with 7 log CFU/ml of *Listeria*. This inoculum was dispensed into the tubes containing the coupons with *P. putida* biofilms and incubated at 25°C for 4 h to allow the *L. monocytogenes* cells to attach to the *Pseudomonas* biofilms. The coupons were removed, gently rinsed in peptone water to remove unattached cells and placed in test tubes containing fresh sterile dTSB. Incubation at 25°C was continued for 48 h (2 days) and 7 days to allow the incorporation of *Listeria* into the *Pseudomonas* biofilm. *L. monocytogenes* biofilms (monospecies) were prepared by immersing sterile coupons into test tubes containing 30 ml of dTSB inoculated with a five-strain cocktail of *L. monocytogenes*, incubated at 25°C for 4 h to allow attachment of cells to the surfaces. After attachment the coupons were gently rinsed as previously described and further incubated in fresh sterile dTSB for 48 h at 25°C for biofilm formation. Before subjecting the coupons to treatment

with acidic EO water or deionized water, the coupons were removed from the medium used for preparing the biofilm and gently rinsed in sterile peptone water (1 g peptone/L) to remove unattached cells.

Treatment of biofilms with EO water. Coupons containing biofilms were immersed in 250 ml of deionized water, acidic EO water and alkaline EO water followed by acidic EO water (sequential treatment) for 30 s at room temperature (24 ± 2°C). For the sequential treatment, the coupons were immersed in alkaline EO water for 30 s, removed and then rinsed for 10 s in 0.1% peptone water to remove any excess alkaline EO water before being immersed in the acidic EO water for 30 s. Immediately after the exposure time the coupons were placed in 100 ml neutralizing buffer solution (neutralizing buffer at 5.2 g/liter; Becton Dickinson), for 30 s, to neutralize the active component of the acidic EO water left on the coupon. After neutralization, the coupons were subjected to microbiological analysis.

Laboratory conveyor system. A laboratory scale conveyor system was fabricated and used to model the application of EO water for sanitizing conveyor systems in the food industry. The design of the laboratory scale conveyor is shown in figure 1. The system consisted of a 3/8 inch sprocket chain conveyor, made of nylon with stainless steel reinforcing links and mounted on a stainless steel frame, with nylon sprockets. A coupon carrier was made from stainless steel with PVC and nylon attachment components, for securing the coupons, to be treated, in place. The chain conveyor was driven by a DC variable speed gearmotor drive unit (Model 4Z726A, Dayton Electric Mfg. Co., Niles, Illinois) controlled by a switch panel. During operation, the coupon carrier was attached to the chain conveyor using stainless steel thumbscrew nuts and

conveyed at a speed of 144cm/min into an immersion tank (made from PVC), containing the appropriate treatment solution. The immersion tank had a drain which was connected to a waste container (Nalgene™ carboy) with a ¾ inch diameter laboratory grade tygon tubing. Drainage of the immersion tank was facilitated by the use of vacuum from a central source, in the lab. At the end of the immersion treatment, the coupon carrier was conveyed out of the immersion tank by reversing the direction of the conveyor using a polarity reversion switch on the switch panel. Treatment solutions (acidic EO water and deionized water) were contained in two separate 2 ½ gallon polyethylene containers with lids, equipped with fountain pumps (Model M60 A, Beckett Corp., Irving, Texas) and a ¾ inch diameter tubing for dispensing the treatment solution into the immersion tank.

Treatment of biofilms using the conveyor system. Treatment of biofilms using the conveyor system involved the use of deionized water (control) and acidic EO water. Prior to being used and in between treatments, the whole conveyor equipment (Figure 1) was thoroughly sprayed with 70% ethanol, to reduce the aerobic bacteria load in the immersion tank, on the coupon carrier and the chain conveyor and also to prevent cross-contamination between the different treatments. The two containers were filled separately with sterile deionized water and freshly prepared acidic EO water. As a means of conditioning the immersion tank, before each treatment, some of the water to be used for the treatment was dispensed into the immersion tank and drained out into the waste container. Following this rinse procedure, the water to be used for the treatment was dispensed into the immersion tank to fill it up to the 1 L mark (level at which coupons on carrier will be totally immersed in the treatment water). The coupons containing the biofilms were then secured with sterile forceps to the coupon carrier which was in turn attached

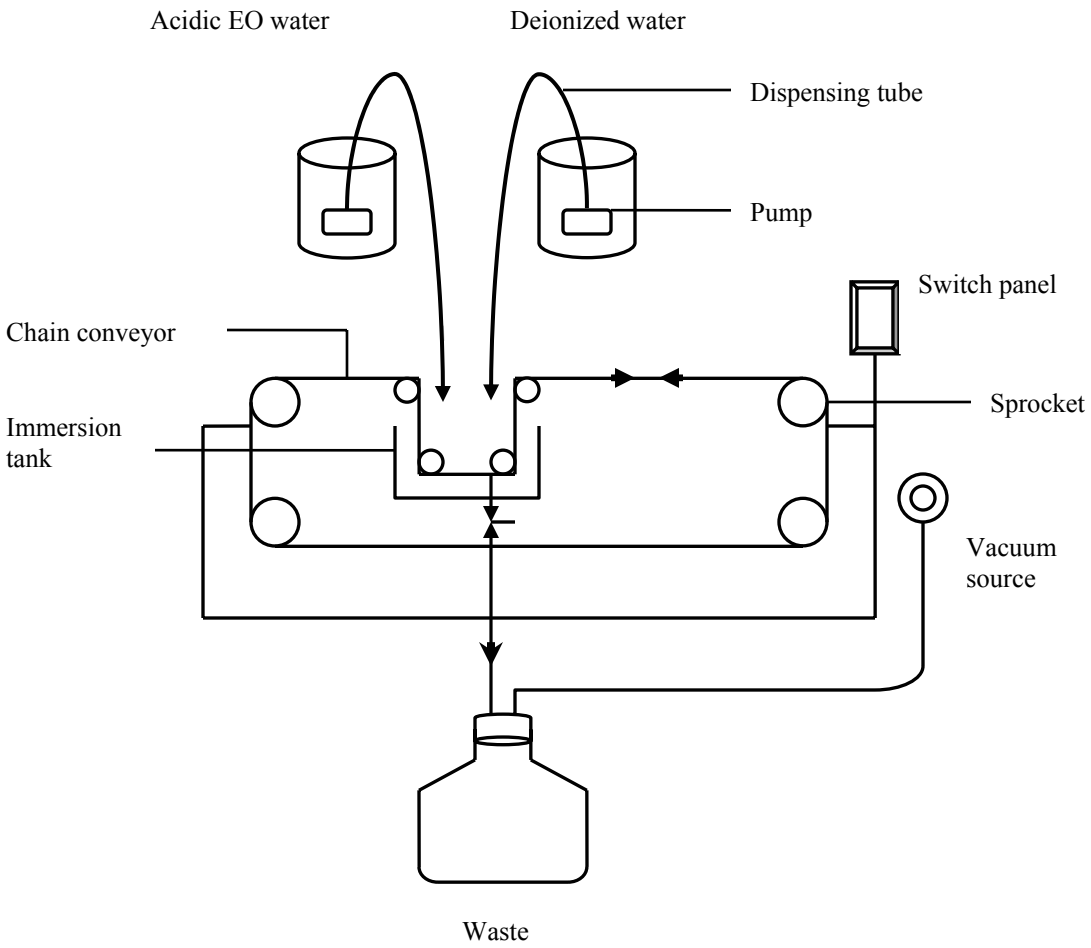


Figure 5-1. Laboratory scale conveyor system used for the application of electrolyzed water.

to the conveyor chain drive and secured with the thumb screw nuts. Using the controls on the switch panel the carrier with the coupons were conveyed into the immersion tank, allowed toimmerse for the specific treatment time eg. 60 s and then conveyed out by reversing the direction of the chain conveyor and detaching the coupon carrier. Coupons going through treatments with no residual time (0 min) were immediately removed from the carrier with sterile forceps and immersed in 100 ml neutralizing buffer solution for at least 1 min before being subjected to microbiological analysis. For treatments with 2 min residual time, the treated coupons were left on the carrier for 2 min before being immersed in neutralizing buffer solution.

Treatment of biofilms with continuous use of acidic EO water in the conveyor equipment. For the continuous use of the acidic EO water, after the first set of coupons with biofilms were subjected to immersion treatment with fresh acidic EO water in the conveyor system, the water was not drained but used again for the treatment of the next set of coupons containing biofilms and this treatment was referred to as the 1st reuse. The 2nd reuse involved the use of that same treatment solution as well as the 3rd, 4th and 5th reuse, each time using new untreated coupons with biofilms. The 3rd and 4th reuse coupons were not subjected to further microbial analysis. Before each use of the acidic EO water, 20 ml of the water in the immersion tank was obtained with a sterile pipette and this was used in determining the chlorine content of the water before immersion of the coupons. After each reuse of the water, 10 ml of the acidic EO water in the immersion tank was also obtained with a sterile pipette and this was also added to two tubes containing 5 ml each of double-strength neutralizing buffer (neutralizing buffer at 10.4 g/liter) and these were used to determine the presence of bacteria in the treatment solution.

After each treatment, the coupons were immediately detached with sterile forceps and neutralized in 100 ml neutralizing buffer solution.

Microbiological analysis. To enumerate *L. monocytogenes,* the treated and neutralized coupons were placed in sterile Nalgene™ bottles (8 oz) containing 20 ml of sterile peptone water (1 g peptone/L) and 3 g of acid washed glass beads (425 - 600 microns, Sigma-Aldrich Co., St Louis, MO) as described by Hassan et al. (12) with some modification. The bottles were then shaken for 10 min on an orbital incubator shaker (Model C24, New Brunswick Scientific, Edison, NJ) at 350 rpm to remove the bacteria from the coupons. Serial dilutions of the peptone water were made after shaking. The surviving bacteria from the control and treated coupons were enumerated by spread plating 0.1 ml of the diluents on modified Oxford agar (MOX, Oxoid, Basingstoke, Hampshire, UK) plates containing *Listeria* selective supplement (Oxoid) incubated at 37°C for 48 h. Typical *L. monocytogenes* colonies were counted and recorded as log CFU per coupon. For plates which did not exhibit growth on plates, the peptone water used for recovering the *L. monocytogenes* from the coupons was enriched for the presence of surviving *L. monocytogenes* by adding 1 ml to 10 ml of TSB and incubating at 37°C for 24 h. Tubes which exhibited growth were streaked onto modified Oxford agar (MOX, Oxoid, Basingstoke, Hampshire, UK) plates containing *Listeria* selective supplement (Oxoid) incubated at 37°C for 48 h, and presence of typical *L. monocytogenes* colonies was noted. Enumeration of the bacteria in the treatment water was done by serially diluting the neutralized acidic EO water with peptone water (1 g peptone/liter), spread plating on MOX agar plates and incubating at 37°C for 48 h. Enrichments of the neutralized acidic EO water were also done by adding 1 ml of

the double neutralized mixture to 10 ml TSB and incubating at 37°C for 24- 48 h and checking for growth.

Data analysis. Experiments were replicated three times with duplicate samples in each replication. Data were analyzed using the general linear model (GLM) procedures of the statistical analysis system (SAS Institute, Cary, NC). Comparisons of means were calculated using the Fisher least-significant-difference test. Significant differences are presented at a 95 % confidence level ($p \leq 0.05$).

RESULTS AND DISCUSSION

Treatment of mono and duospecies biofilms with EO water. The highest populations of *L. monocytogenes* were recovered from the control coupons (Table 5-1). Treatment of coupons with acidic EO water for 30 s resulted in significant inactivation of *L. monocytogenes* on both stainless steel and teflon when compared to the controls. The population of *L. monocytogenes* recovered after acidic EO water treatment of biofilms on teflon coupons were however, significantly higher ($p \leq 0.05$) than the population recovered from stainless steel coupons (Table 5-1). Other researchers have also observed the effect of the attachment surface on the resistance or susceptibility of adherent bacteria to sanitizers (10, 19, 6). *L. monocytogenes* biofilms developed on conveyor belting material (with polyester and polyurethane components) were more resistant to cleaning and sanitizing than stainless steel (28). Previous studies by Mafu et al. (22), reported that, lower concentrations of four commercial sanitizers were required for the inactivation of *L. monocytogenes* cells on stainless steel than on polypropylene and rubber surfaces. Midelet and Carpentier (24) determined attachment strength of *L. monocytogenes*

biofilms to stainless steel to be weaker than on polyvinyl chloride and polyurethane. This weaker attachment strength could be responsible for the higher inactivation of biofilms on stainless steel, as opposed to teflon, observed this study. In the interests of food safety, it is important that food processors be aware of materials that may limit sanitizer effectiveness. From these results, acidic EO water may be more appropriate for sanitizing stainless steel surfaces than hydrophobic materials such as teflon.

The sequential treatment which involved the exposure of biofilm containing coupons to alkaline EO water followed by acidic EO water resulted in significantly higher inactivation of *L. monocytogenes* than the treatment of biofilms with acidic EO water alone (Table 5-1). Similar results were reported in previous studies (2, 3). Other researchers have explored the sequential treatment in the inactivation of bacteria on shell eggs (27), lettuce (15), cucumbers (16), carp fillets (23) and hides of cattle (5). In all these inactivation caused by acidic EO water alone was further enhanced in the sequential treatment. Where applicable, the sequential treatment of alkaline EO water followed by acidic EO water provides a more efficient use of EO water and therefore, encouraged.

On both stainless steel and teflon, 7-day biofilms seemed to be less resistant to acidic EO water than the 2-day biofilms. Generally, higher log reductions were achieved after the treatment of 7-day biofilms with acidic EO water and the sequential treatment than after the treatment of 2-day biofilms, however the differences were not significant ($p > 0.05$), except for the acidic EO water treatment of *L. monocytogenes* biofilms in monospecies and duospecies on stainless steel and teflon, respectively. In their work on 2- and 5-day *L. monocytogenes* biofilms, Somers and Wong (28) found, in contrast to our results, that overall, 5-day biofilms were more resistant to cleaning and sanitizing than 2-day biofilms. The higher susceptibility of the 7-day *L.*

monocytogenes biofilms to acidic EO water than the 2-day biofilms, could have been due to a weakening of the bacterial cells as a result of depletion of nutrients in the medium used for biofilm formation. However, this could not be confirmed.

The behavior of *L. monocytogenes* in monospecies and duospecies biofilms were compared to determine the effect of the presence of *P. putida* on *L. monocytogenes*, during treatment with EO water. When the biofilms were formed on stainless steel, *L. monocytogenes* were more susceptible to acidic EO water in duospecies with *P. putida* than in monospecies. The log reductions (5.25 and 6.58 log CFU per coupon) in *L. monocytogenes* achieved after treatment of 2- and 7- day duospecies biofilms with acidic EO water were significantly higher ($p \leq 0.05$) than the reductions achieved (4.03 and 4.96 log CFU per coupon) after treatment of the 2- and 7- day monospecies biofilms, respectively. On the other hand, with biofilms formed on teflon, significantly higher log reductions in *L. monocytogenes* were obtained after treatment of 2 day monospecies biofilms with acidic EO water as compared to duospecies biofilms (Table 5-1). *L. monocytogenes* seemed to be more susceptible to inactivation in duospecies than monospecies when the sequential treatment was applied, however the differences observed between the two were not statistically significant ($p > 0.05$). Studies by other researchers show that the presence of *Pseudomonas* spp. may result in increased susceptibility (20) or increased resistance (1) of the associated bacteria, to sanitizers. The general trend observed in this study shows that *L. monocytogenes* was more resistant in monospecies than in duospecies. However, concrete conclusions on the behaviour of *L. monocytogenes* in mono and duospecies biofilms cannot be drawn from these observations due to the lack of statistical significance in several of the comparison tests.

Table 5-1. Populations of *Listeria monocytogenes* recovered after treatment of biofilms, in monospecies and in association with *Pseudomonas putida* biofilms, with electrolyzed water.

Bacteria[a]	Treatments[b]	Age of biofilm (Days)	Populations of L. monocytogenes (logCFU/coupon)[c]				
			Stainless steel			Teflon	
			Population	Reduction	En[d]	Population	Reduction
Monospecies	Control	2	a7.41±0.71			a7.86±0.89	
	Acidic EO		b3.39±0.47	B4.03aB		b5.33±0.10	A2.53bA
	Sequential		c1.64±1.82	A5.78aA		c4.82±0.37	A3.04bA
Duospecies	Control		a7.35±0.24			a7.07±0.66	
	Acidic EO		b2.11±1.41	A5.25aA		b5.67±0.46	B1.40bB
	Sequential		c0.19±0.47	A7.17aA	2/6	c3.81±0.33	A3.26bA
Monospecies	Control	7	a7.03±0.60			a7.66±0.50	
	Acidic EO		b2.07±0.80	B4.96aA	5/6	b4.74±0.40	A2.92bA
	Sequential		c0.57±1.38	A6.46aA	3/6	c3.98±0.64	A3.68bA
Duospecies	Control		a6.58±0.53			a6.30±0.44	
	Acidic EO		b< 2.30	A6.58aA	3/6	b4.18±0.32	A2.12bA
	Sequential		b< 2.30	A6.58aA	36	c2.21±0.82	A4.09bA

[a]Monospecies, *L. monocytogenes*; Duospecies, *L. monocytogenes* and *Pseudomonas putida*.

[b]Control, treatment with deionized water; Acidic EO, acidic electrolyzed water (pH = 2.46, ORP = 1,165mV, total chlorine = 48 mg/liter); Sequential, alkaline electrolyzed water (pH =11.44, ORP = -836mV) followed by acidic electrolyzed water.

[c]Means preceded by the same lowercase letters in the same column within each species and age of biofilm are not significantly ($p > 0.05$) different; means followed by the same lowercase letters in the same row within each species, treatment and age of biofilm are not significantly ($p > 0.05$) different; means preceded by the same small cap letters in the same column within each age of biofilm and treatment are not significantly ($p > 0.05$) different; means followed by the same small cap letters in the same column within each species and treatment are not significantly ($p > 0.05$) different. Detection limit = 2.3 LogCFU/coupon

[d]Number of treated coupons positive for *L. monocytogenes*, as detected by enrichment, of the number of coupons analyzed by enrichment.

Treatment of biofilms using the conveyor system. The conveyor system (Figure 5-1) was fabricated to carry out experiments in the lab which would model the application of acidic EO water for cleaning conveyor systems, at appropriately determined intervals during processing, to minimize the potential for the microbial contamination of food.

Biofilms formed on stainless steel were exposed to acidic EO water in the conveyor system for 15 and 60 s. When compared to deionized water, acidic EO water reduced *L. monocytogenes* biofilms by 4.76 – 6.60 log CFU per coupon and 2.74 – 3.89 log CFU per coupon on stainless steel and teflon, respectively (Table 5-2). The population of *L. monocytogenes* which survived after 15 s exposure to acidic EO water was higher than the population which survived after 60 s exposure (Table 5-2). Exposure for 60 s to acidic EO water with no residual time (0), resulted in the reduction of a significantly higher ($p \leq 0.05$) population of *L. monocytogenes* than 15 s exposure with no residual time (Table 5-2).

Biofilms formed on teflon were exposed to acidic EO water for 1 and 5 min in the laboratory scale conveyor system. Even after 5 min exposure to acidic EO water, the population of *L. monocytogenes* biofilms inactivated on teflon was more than 1.2 log CFU per coupon lower than the population inactivated on stainless steel coupons exposed to EO water for 15 s. At the respective residual times of 0 and 120 s, the population of *L. monocytogenes* reduced after exposure of biofilms on teflon to acidic EO water for 5 min was slightly higher than the population inactivated after 1 min of exposure, however, this difference was not statistically significant ($p > 0.05$). Most commercial sanitizers applied during cleaning and sanitizing operations in food processing facilities specify minimum required contact times, necessary for efficiency, in the manufacturer's recommendation's for use.

Table 5-2. Populations of *Listeria monocytogenes* recovered after treatment of biofilms with electrolyzed water in a laboratory scale conveyor system.

Material	Treatment[a]	Treatment Time (s)	Residual Time (s)	Log CFU/coupon[b]		En[c]
				Population	Reduction	
Stainless steel	DI water	15	0	6.42 ± 0.43 A		
			120	6.45 ± 0.45 A		
	Acidic EO		0	1.59 ± 1.71 B	b 4.83 a	
			120	1.32 ± 1.06 B	a 5.13 a	
	DI water	60	0	6.60 ± 0.12 A		
			120	6.56 ± 0.12 A		
	Acidic EO		0	< 2.30 B	a 6.60 a	5/6
			120	0.23 ± 0.51 B	a 6.33 a	5/6
Teflon	DI water	60	0	7.39 ± 0.28 A		
			120	7.58 ± 0.27 A		
	Acidic EO		0	4.66 ± 1.81 B	a 2.73 b	
			120	4.20 ± 1.66 B	a 3.38 a	
	DI water	300	0	7.47 ± 0.41 A		
			120	7.53 ± 0.48 A		
	Acidic EO		0	4.40 ± 0.98 B	a 3.07 b	
			120	3.65 ± 1.13 B	a 3.88 a	

[a]DI water, deionized water; Acidic EO, acidic electrolyzed water (pH = 2.31, ORP = 1169mV, total chlorine = 48 mg/liter).

[b]Means followed by the same small cap letters in the same column within each material, treatment time and residual time are not significantly ($p > 0.05$) different; means preceded by the same lowercase letters in the same column within each material, treatment and residual time are not significantly ($p > 0.05$) different; means followed by the same lowercase letters in the same column within each material, treatment and treatment time are not significantly ($p > 0.05$) different.

[c]Number of treated coupons positive for *L. monocytogenes*, as detected by enrichment, of the number of coupons analyzed by enrichment.

Since the experiment with the conveyor system was to simulate realistic applications, the effect of residual time (time allowed for coupons to sit after treatment before neutralization), was also investigated.

When *L. monocytogenes* biofilms on teflon were exposed to acidic EO water for 1 min using the conveyor system, a reduction of 2.74 log CFU per coupon was achieved and this increased to 3.38 log CFU per coupon when a residual time of 2 min was allowed. A significantly higher ($p \leq 0.05$), log reduction was also achieved when a residual time of 2 min was allowed after treatment of biofilms on teflon for 5 min (Table 5-2). On stainless steel, the difference in log reduction achieved after treatment with acidic EO water, with and without a residual time of 2 min was not statistically significant ($p>0.05$) (Table 5-2). When the stainless steel coupons were treated with acidic EO water for 60 s, no growth was detected on direct plate count except for one of the treatment from which one colony was observed on the MOX plates. The lack of significance between 0 and 2 min residual time for this treatment was a result of the high inactivation in *L. monocytogenes* biofilms, achieved by acidic EO water. The reduction of *L. monocytogenes* biofilms on teflon after treatment for 60 s with 2 min residual time with acidic EO water, was higher (3.38 log CFU/coupon) than the 5 min treatment with no residual time (3.07 log CFU/coupon). Where only small contact times can be permitted, acidic EO water can be applied in a sanitation regime with short contact times and longer residual times to achieve effective disinfection. The short exposure-long residual time combination can also be applied in sanitation regimes utilizing sprays to cut down on the volume of acidic EO water used.

Treatment of biofilms with continuous use of acidic EO water in the conveyor equipment. Treatment of industrial effluents before its discharge into surface water is expensive and as such most industries put in much effort to prevent the excessive use of water, especially during cleaning and sanitation. In the application of immersion cleaning in the food industry, it is more practical that a given volume of sanitizer will be used and reused to clean a number of pieces of equipments or several parts of a piece of equipment before being discarded. In this study, the effect of using acidic EO water for continuous cleaning of a conveyor system and its bactericidal efficacy, was evaluated. The total chlorine concentration of the acidic EO water was determined immediately before its use or reuse in the conveyor system.

Although the chlorine concentration of the freshly prepared acidic EO water was 50 mg/liter, after being dispensed into the immersion tank of the conveyor equipment its chlorine content had dropped to an average of 30 mg/liter (Table 5-3). The chlorine concentration of acidic EO water decreased further after each reuse, however, sufficient residual remained to continue bacterial inactivation, even after being reused for the 5^{th} time over a 50 min period (Table 5-3). Lee and Frank (18) also monitored the residual chlorine levels after treatment of adherent *L. monocytogenes* with hypochlorite and reported the dissipation of chlorine during treatments. The observed reduction in total chlorine with each reuse of EO water in this study can be attributed to a reaction of the organic component of the biofilms with the free chlorine species in the acidic EO water. As observed in previous results (Table 5-2), *L. monocytogenes* biofilms on teflon were more resistant to inactivation with acidic EO water than biofilms formed on stainless steel (Table 5-3). On teflon, the highest reduction (2.54 log CFU per coupon) in populations of *L. monocytogenes* biofilms was achieved after immersion of coupons into the fresh EO water and the lowest reduction (1.74 log CFU per coupon) was achieved after the

Table 5-3. Effectiveness of the continuous use of electrolyzed water in the inactivation of *Listeria monocytogenes* biofilms, using a laboratory scale conveyor system.

Material	Treatment[a]	Exposure time (min)	Log CFU/coupon[b]		Treatment water		
			Population	Reduction	Total Chlorine (mg/liter)	Population[c] LogCFU/coupon	En[d]
Stainless steel	Control	1	6.67 ± 0.10				
	Fresh EO		1.95 ± 1.07	ab4.72 a	28	ND	0/6
	1st Reuse		2.26 ± 1.50	b4.41 a	26	ND	0/6
	2nd Reuse		1.21 ± 1.89	a5.46 a	19	ND	0/6
	5th Reuse		2.64 ± 1.41	b4.03 a	16	ND	0/6
Teflon	Control	5	7.98 ± 0.42				
	Fresh EO		5.45 ± 1.00	a2.53 b	32	ND	0/6
	1st Reuse		5.97 ± 0.49	b2.01 b	26	ND	0/6
	2nd Reuse		5.78 ± 0.63	ab2.20 b	21	ND	0/6
	5th Reuse		6.25 ± 0.57	b1.73 b	16	ND	0/6

[a] Control, deionized water treatment. Fresh EO, acidic EO water used for the first time after its preparation; 1st reuse, 1st time acidic electrolyzed water is reused after the initial use of the freshly prepared solution; 2nd Reuse, 2nd time acidic electrolyzed water is reused after the initial use of the freshly prepared solution; 5th reuse, 5th time acidic electrolyzed is reused after the initial use of the freshly prepared solution. Freshly prepared acidic electrolyzed water had pH = 2.37, ORP = 1,161mV, total chlorine = 50 mg/liter.

[b]Means preceded by the same lower case letters in each column within each material and exposure time are not significantly ($p > 0.05$) different; means followed by the same lowercase letters in each column within each treatment are not significantly ($p > 0.05$) different.

[c]ND, not detectable on direct plate count.

[d]Number of treated coupons positive for *L. monocytogenes*, as detected by enrichment, of the number of coupons analyzed by enrichment.

acidic EO water was reused for the 5th time (Table 5-3). On stainless steel, the highest reduction was 5.46 log CFU per coupon while the lowest reduction was 4.03 log CFU per coupon. The difference between the reduction achieved after immersion of the biofilm containing stainless steel coupons in the fresh acidic EO water (4.74 log CFU per coupon) and that achieved after the 5th reuse of the acidic EO water (4.03 log CFU per coupon) was only 0.69 log CFU per coupon. The high reduction obtained after the 2nd reuse of acidic EO water for the treatment of biofilms formed on stainless steel, was not expected and this result could have been due to an unusual susceptibility of the *L. monocytogenes* biofilms formed on a few of the coupons subjected to that treatment.

As opposed to the exponential decrease in total chlorine content of acidic EO water, after each reuse, the differences between the populations of *L. monocytogenes* biofilms inactivated after each reuse were not large. The level of inactivation achieved with chlorinated compounds depends on the concentration of chlorine, its pH, the temperature of use, the organic load as well as the time of exposure. The small decrease in bactericidal efficacy of acidic EO water after being reused 5 times shows that, after the selection of the appropriate levels and combinations of chlorine concentration, time of exposure, temperature, pH, etc., to achieve the highest efficiency of disinfection, acidic EO water can be used and reused several times to obtain effective sanitization, before being discarded. In immersion applications of acidic EO water requiring limited use of water, a portion of the spent acidic EO water can be drained out after several uses and the remaining reseeded with fresh EO water to increase the chlorine content for effective sanitization. No bacteria were isolated from the acidic EO water after being used several times, even upon enrichment (Table 5-3).

Similar results were observed in studies on the application of EO water for the inactivation of *L. monocytogenes* biofilms in the presence of organic matter (3). Acidic EO water can therefore be safely reused without cross contamination from the used EO water.

CONCLUSIONS

Stainless steels are the most commonly used materials for the fabrication of food contact surfaces in industry, and from the scope of our results, it remains the best material for food contact since bacteria on it were much more easily inactivated. Equipment or sites in the food processing plant facbricated with hydrophobic materials, such as teflon, will require more attention during sanitation in order to effectively inactivate any adherent bacteria present. *L. monocytogenes* biofilms in monoculture or in association with *P. putida* may behave differently in the presence of sanitizers. Higher inactivation can be achieved by incorporating residual time in a sanitizing regime utilizing acidic EO water. We also conclude that acidic EO water can be reused several times during immersion cleaning or treatment without significant reduction in its bactericidal efficacy or the probability of recontamination from the used water.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Acidic EO water is an effective bactericide. The sequential treatment of alkaline EO water treatment followed by acidic EO water treatment produces significantly higher inactivation of bacteria than acidic EO water alone, even in the presence of organic matter. The application of both alkaline and acidic EO water, provides for a more efficient use of the EO water Technology. Organic matter reduces the chlorine concentration of acidic EO water and ultimately reduces its bactericidal efficiency. The extent of influence of organic matter on acidic EO water depends on the organic load. Modified EO water is less aggressive than acidic EO water, and therefore, it is safer to use with materials that are affected by acidic EO water. Stainless steel, the most commonly used food contact surface in the food industry, has an outstanding corrosion resistance to acidic EO water and remains the best material for EO water applications. Acidic EO water is much more effective in the inactivation of *L. monocytogenes* biofilms on stainless steel than on teflon. Continuous use of acidic EO water for the inactivation of biofilms results in a decrease in its chlorine content, however, enough residual remains to continue bacterial inactivation. During sanitization of surfaces containing *L. monocytogenes* biofilms, bacteria do not survive in the treatment solution. Acidic EO water can therefore be recycled during cleaning without the risk of recontamination from the treatment solution.