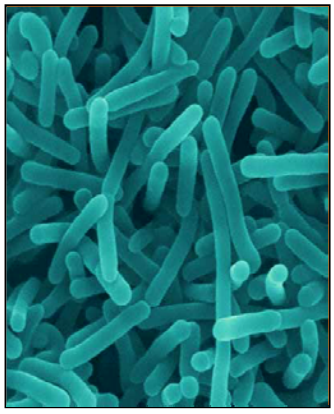
Peter M. Muriana, Ph.D.
Associate Professor
Dept. Animal Sciences &
Oklahoma Food & Ag Products Center
Oklahoma State University
Stillwater, OK 74078-6055
405-744-5563 TEL; 405-744-6313 FAX
peter.muriana@okstate.edu

Oklahoma State University

Electrolyzed Water



Efficacy of electrolyzed water on foodborne pathogens of concern to the meat and poultry processing industry

A report prepared for: SanAquel LLC
502 Industrial Road
Bristow, OK 74010
sales@sanaquel.com

Version: 11/7/05

Efficacy of Electrolyzed Water on Foodborne Pathogens of Concern to the Meat & Poultry Industry

A report prepared for SanAquel LLC, based on research performed by Peter Muriana, Ph.D. (Oklahoma St. Univ.)

1. Effect of EW on *Listeria monocytogenes*, *E. coli* O157:H7, and *S. enteritidis*

L. monocytogenes is the leading pathogen of concern on ready-to-eat (RTE) meat products and has been the focal point of USDA-FSIS with manufacturers of RTE meat and poultry products. *E. coli* O157:H7 has been the main pathogen of concern in raw ground beef, resulting in illnesses and deaths due to the consumption of even a few cells. *Salmonella enteritidis* has been the leading cause of illness associated with egg-related salmonella foodborne illnesses because the ovaries of laying hens can become infected with *S. enteritidis*, resulting in the internal contamination of retail shell eggs.

Each of these significant foodborne pathogens were placed in a solution of electrolyzed water and removed and plated on Tryptic Soy Agar every 2 minutes for up to 8 minutes (2, 4, 6, and 8 min). The same was done with 0.1% buffered peptone water (BPW) which most laboratories use for making dilutions of food samples for plating. A third solution was also used in which the electricity to the cell in the generator unit was turned off (i.e., non-electrolyzed water, or NEW), such that the fluid would be of similar base composition to that which was used for making electrolyzed water, except for the components generated by the electrical current. As you can see with Figures 1, 2, and 3, no organisms were detected from among the 3 different organisms after the first 2 min. From these results, the lethality of electrolyzed water is impressive and may provide extreme interest from within the food processing industry where these microorganisms are routinely problematic to food processors and consumers alike.

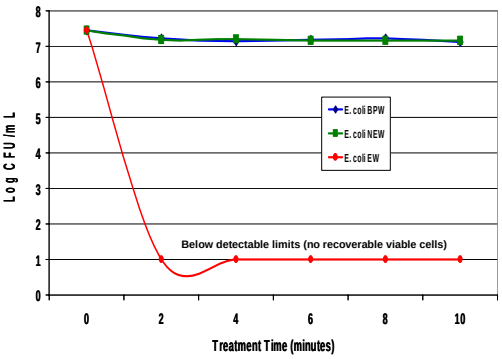


Figure 1. *E. coli* O157:H7 resuspended in buffered peptone water (BPW), non-electrolyzed water (NEW), and electrolyzed water (EW).

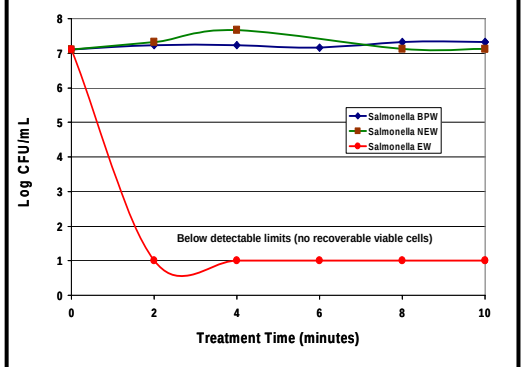


Figure 2. *Salmonella enteritidis* resuspended in buffered peptone water (BPW), non-electrolyzed water (NEW), and electrolyzed water (EW).

The results indicate that Electrolyzed Water is lethal to exposed cells of various foodborne pathogens, resulting in significant reductions when placed in contact for even 2 minutes. Additional tests will examine shorter contact times and what affect EW has on strong biofilm-forming strains of *Listeria monocytogenes*

> *Electrolyzed Water gave greater than 6-log reduction of E. coli O157, Listeria monocytogenes, and Salmonella enteritidis in solution within 2 min.*

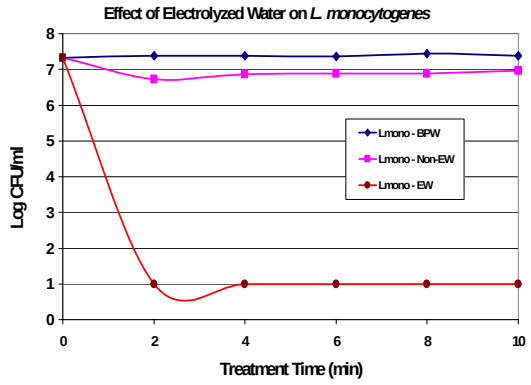


Figure 3. *Listeria monocytogenes* resuspended in buffered peptone water (BPW), non-electrolyzed water (NEW), and electrolyzed water (EW).

2. Variable capacity of strains of *L. monocytogenes* to form biofilms

I have developed a fluorescent biofilm assay for detection of strong (or weakly) attaching strains of *L. monocytogenes* using microtiter plates as an attachment substrate. After several days of incubation of individual strains, the plates are washed and tested for fluorescent signal after addition of a fluorescent substrate. Strains of interest are those that show the greatest levels of fluorescence along with those that show the least (for comparative purposes). Using this assay on individual isolates in microtiter wells, we can distinguish 'strongly' adhering strains from 'weakly' adhering strains based on the fluorescent signals obtained (Fig. 4).

The significance to the food and meat processing industry is that although we attribute equal pathogenicity to all strains of *Listeria monocytogenes*, it is known they have different levels of virulence. Similarly, not all isolated found in meat processing plants are equally capable of lingering around based on the results we have been obtaining. It would be uniquely interesting to find out what relationship, if any, do the 'strongly' attaching strains have with virulence as attachment is one of the first steps in pathogenicity (i.e., attaching to epithelial cells for uptake and intracellular survival by *L. monocytogenes*).

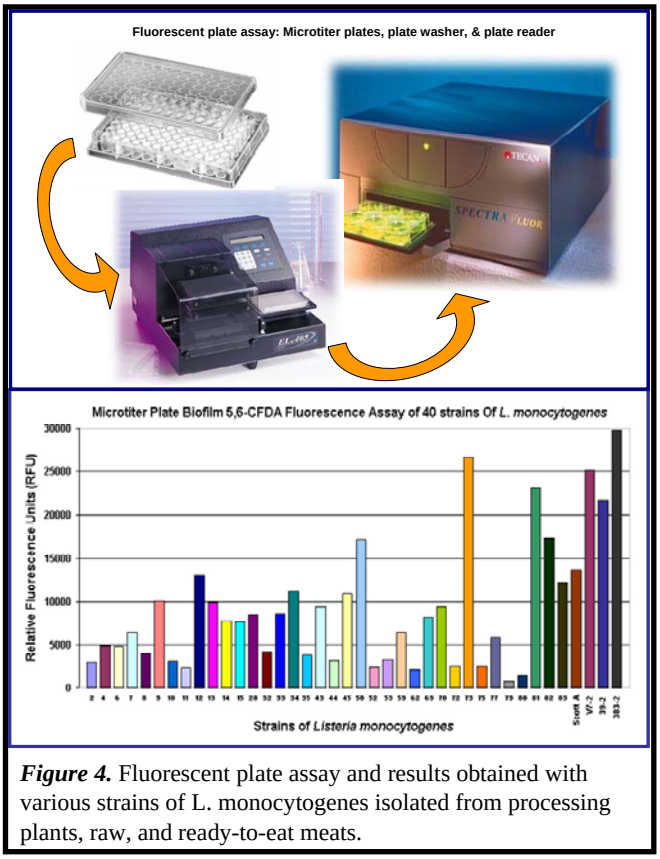


Figure 4. Fluorescent plate assay and results obtained with various strains of L. monocytogenes isolated from processing plants, raw, and ready-to-eat meats.

3. Visual analysis and quantification of select strains of *L. monocytogenes* in biofilms

Strains of *L. monocytogenes* that had tentatively been identified as 'strongly' adhering strains have were examined by scanning electron microscopy (SEM) in comparison with 'weakly' adherent strains after similar period of attachment using a similar number of cells (Fig. 5)

.

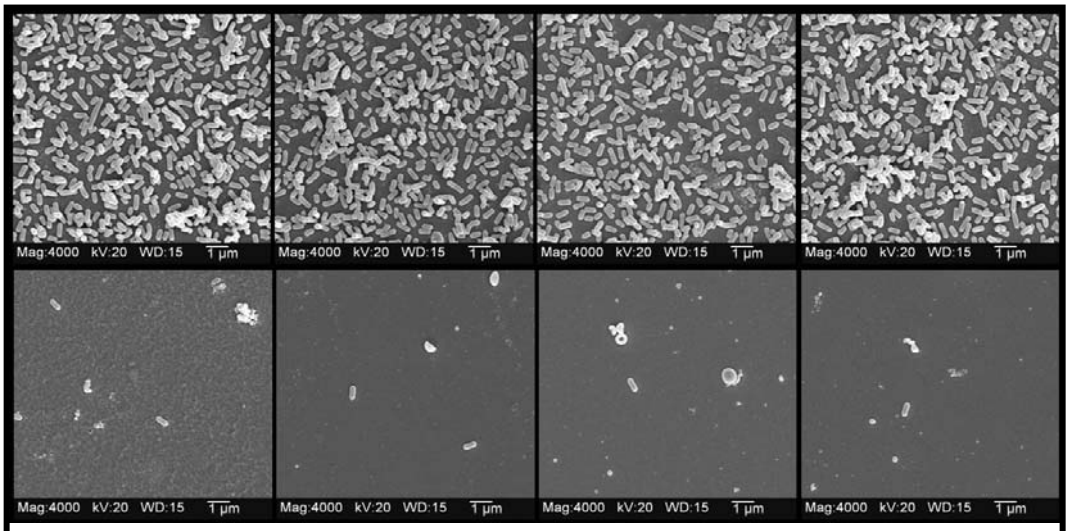


Figure 5. Scanning electron microscopy (SEM) of strains of Listeria monocytogenes differentiated by our fluorescence plate assay into 'strongly' adherent (top row) and 'weakly' adherent strains (bottom row).

In order to better quantify the numbers of cells, we examined a 'protease detachment assay' to remove the attached cells without injury. This allows us to quantify the numbers of CFU (cell-forming-units) during comparisons with strong and weakly attaching strains, or after various treatments (Fig. 6). The difference in attachment capacity, given the same starting level and attachment time, is 100,000-fold greater for the strongly adhering strains than the weak.

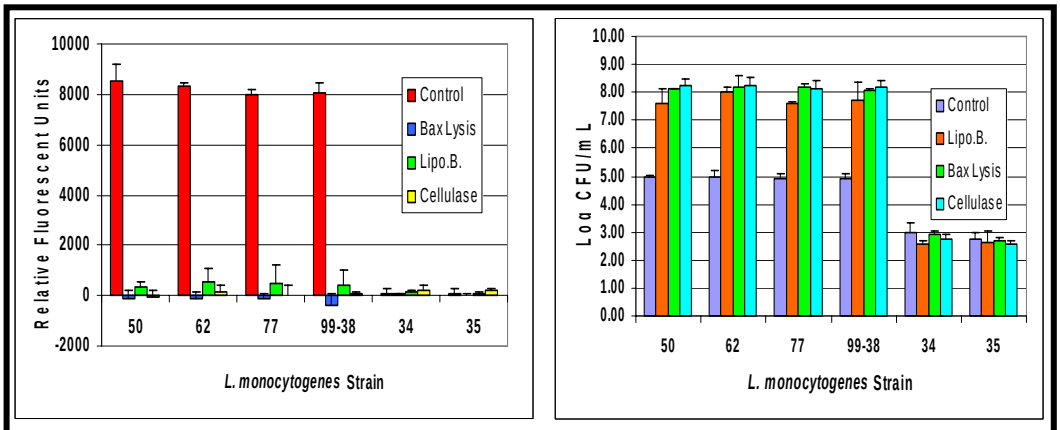


Figure 6. Fluorescence assay of microplate wells containing 4 strong and 2 weakly-adherent strains of *L. monocytogenes*, before and after protease detachment (left). Plate counts of control buffer washes of attached cells and proteolytically detached cells from microplate attachment assays. With strongly adherent *L. monocytogenes*, only 0.1% of attached cells are detected in the buffer wash, whereas with weakly adhering strains, as many cells are obtained with buffer washes as are obtained after protease detachment, indicating a weaker attachment.

4. Inactivation of strongly adhering strains of *Listeria monocytogenes* using electrolyzed water

We have also shown that the attached bacteria can be 'detached' by a short protease treatment, thereby releasing the cells for quantitative enumeration by plate count. When protease release was performed after treatment of cells with either buffer (no EW treatment) or for various timed intervals of EW treatment (15-, 30-, 60-, 120-sec), no detectable *Listeria* were recovered after treatment for even 15 seconds (Fig. 7). These strongly attaching strains of *Listeria monocytogenes* represent the most potent strains for attachment in food processing facilities (approximately 100,000-fold greater than weakly attaching *Listeria*) and are problematic to the food processing industry. An antimicrobial treatment that would inactivate these microorganisms would be immensely beneficial to the food processing industry.

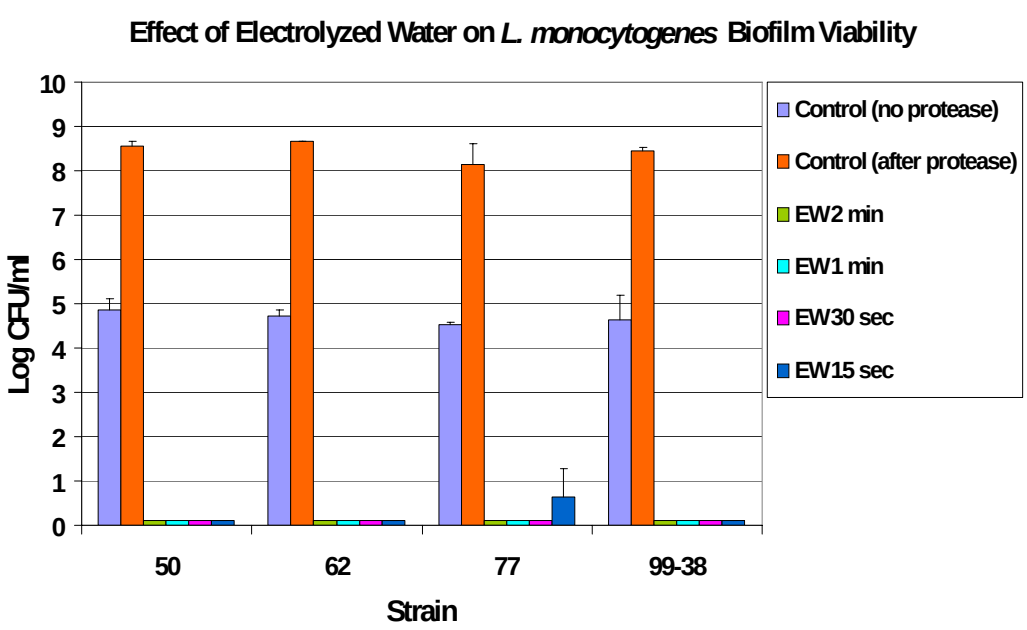


Figure 7. Treatment of strong biofilm-forming strains of L. monocytogenes with electrolyzed water. Four strongly adhering strains of L. monocytogenes were allowed to attach according to out attachment assay. All wells were washed with buffer and then incubated with buffer (controls) or electrolyzed water (test wells) for 2 min, 1 min, 30 sec, or 15 sec before washing with buffer and followed by protease detachment and microbial plating. All data is presented as the mean of triplicate replications with standard deviations of the mean.

> ***Electrolyzed Water*** *gave greater than an 8-log reduction of the most strongly-adherent strains of Listeria monocytogenes within 15 sec as no detectable Listeria were recovered. Such strains would be the most difficult strains to eliminate from meat and poultry processing facilities.*

5. Effect of dilution of electrolyzed water on *Listeria monocytogenes*

We also examined the effect of dilution of electrolyzed water obtained directly from the generators (considered as 100%), using 10%, 7%, 5%, and 3% solutions. Complete inactivation of *L. monocytogenes* in solution was obtained with as low as 7% EW (made by diluting EW to 7% with distilled water). The results showed that 10% and even 7% provided no recoverable *Listeria* when treated for 2 min (Fig. 8).

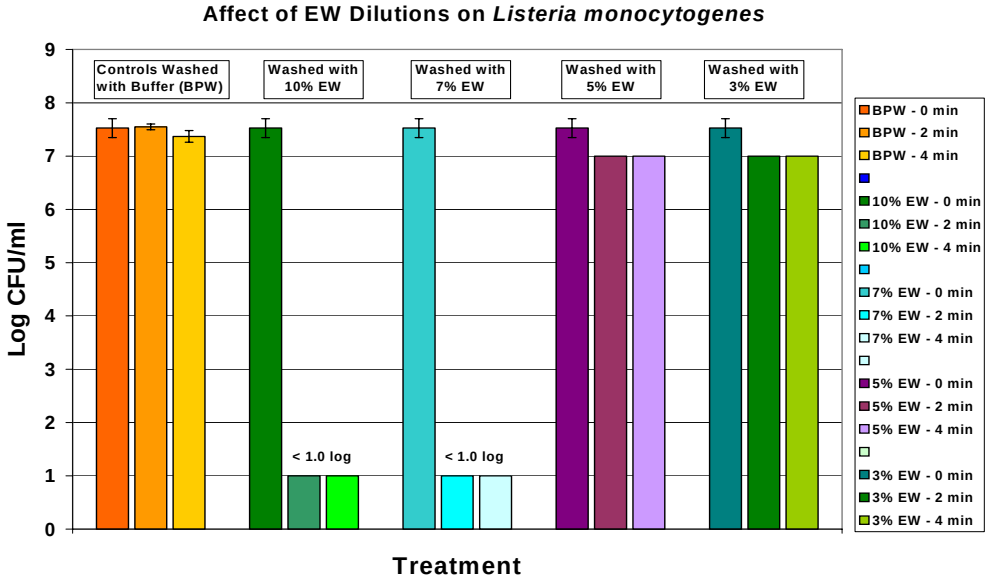


Figure 8. The effect of dilution of electrolyzed water. *Listeria monocytogenes* was inoculated into buffered peptone water or various % solutions of EW and plated after 2- or 4-min.

6. Mode of action of electrolyzed water on *Listeria monocytogenes*

The fluorescent attachment assay is based on the uptake of carboxyfluorescein diacetate (CFDA) by attached cells. Once inside the bacterial cells, CFDA is hydrolyzed to a strongly fluorescing derivative. We examined the level of fluorescence of attached *L. monocytogenes* cells that have taken up CFDA and converted it into the fluorescent derivative after washing the substrate-treated cells with buffer vs. cells substrate-treated cells washed with electrolyzed water (both sets were then washed with buffer to remove residual external substrate). Treatment with EW resulted in loss of 50-80% of cellular fluorescence obtained with control cells that were simply washed with buffer. The decrease in fluorescence obtained after EW treatment suggests that either there is a loss of cells after EW treatment or, that the integrity of the cell wall is compromised leading to intracellular leakage and death of the cells (Fig. 9).

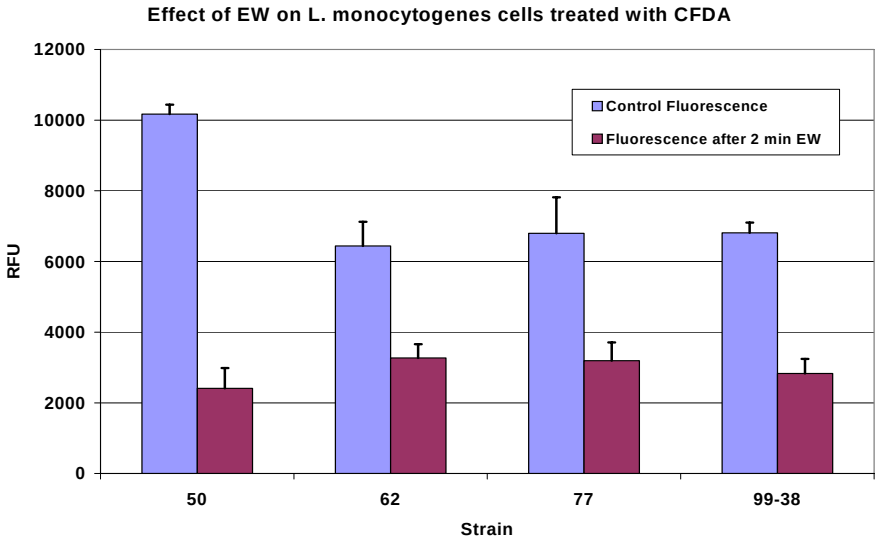


Figure 9. Four strongly adhering strains of Listeria monocytogenes were allowed to attach via our biofilm attachment assay, incubated with fluorescence substrate, and then washed with either buffer or electrolyzed water for 2 min and washed again with buffer before fluorescence assay. Treatment with EW resulted in loss of greater than 50% of intracellular fluorescein that is retained by the buffer treated cells.

7. Effect of electrolyzed water on *Listeria monocytogenes* cell morphology

The strong adhering strains of *Listeria monocytogenes*, i.e. strains 50, 62, 77, and 99-38 were allowed to attach to glass chips as per our biofilm attachment assay. Pairs of chips inoculated with the same attachment strain of *L. monocytogenes* were then washed 5x with BPW and then split up so that one would be further treated with BPW (control) and the other with electrolyzed water (treatment) for 2 min. After the 2 min treatment, all glass chips were then washed again with BPW and submitted for scanning electron microscopy. Our study shows a dramatic change in the appearance, number, and distribution of cells in the various SEM photo's (Fig. 10), suggesting that rinsing with EW results in reduced cell numbers, even when the most stringently adherent cells are used. Furthermore, the data suggests that cell death possibly occurs by disruption as determined by the appearance of cellular debris compared to companion assays washed with buffer (Fig. 10).

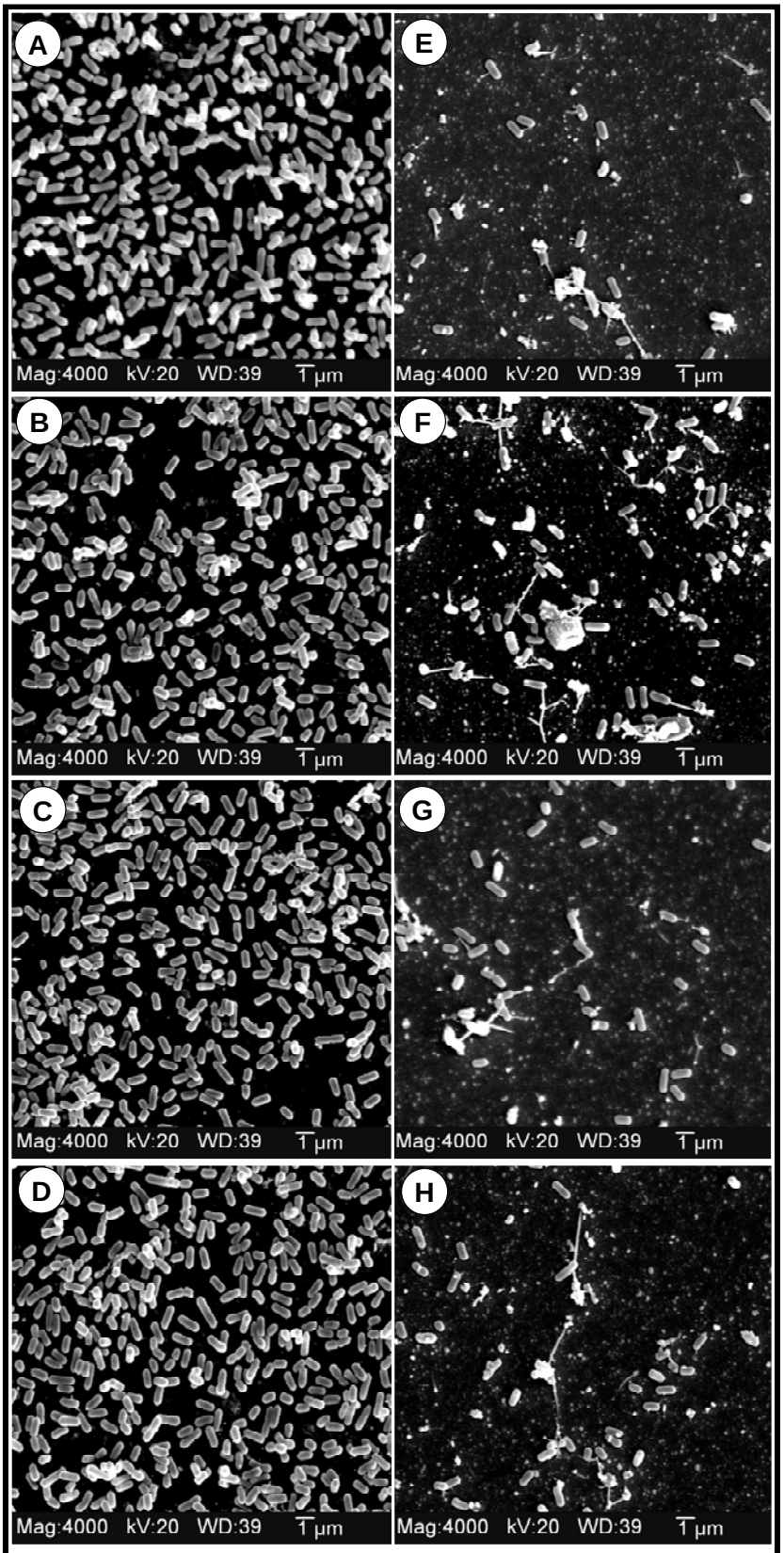


Figure 10. Scanning electron microscopy (SEM) of strong adhering strains of *L. monocytogenes* 50 (panel A), 62 (B), 77 (C), and 99-38 (D). Cells were washed with either buffer (left) or full-strength electrolyzed water (right) for 2 min prior to final washing in preparation for SEM imaging.

8. Effect of Electrolyzed Water on Slicer Blades.

The effect of a 15-second rinse of 10% or 100% Electrolyzed Water was examined on reduction of *L. monocytogenes* inoculated onto 'clean' and 'dirty' stainless steel slicing blades. The reduction was compared to a 15-sec rinse using sterile distilled water. A 2"x2"-inch section on a stainless steel slicing blade was inoculated with 0.2 ml of mixed cultures of 4 strongly-adhering strains of *L. monocytogenes* described earlier. The inoculum was allowed to dry for 20 min before rinse treatments. After a rinse treatment, a sterile 2"x3"-inch sponge pad (used for taking HACCP surface samples), was moistened with buffer and used to recover remaining cells from the blade surface by swiping the surface in 2 directions. The sponge pad was then placed in a sterile stomacher bag to which was added 5 ml of buffer diluent, stomached for 60 sec, and plated by pour plate for enumeration. The 'dirty' slicing blade was obtained by dragging the blade several times through an RTE turkey deli product to acquire a surface film typical of RTE meats. The 15-sec 'rinse' treatments were applied as light shower of spray mist from a pressurized canister purchased at a local hardware store. Each sampling was performed in triplicate replications.

The data shows that the sterile water rinse resulted in a reduction of the applied cells by rinsing off some of the loosely held inoculum (Fig. 11). Application of the 10% EW on clean blades resulted in a 3.6-log reduction of *L. monocytogenes* while no recoverable cells were obtained using the 100% EW (>5.66 log). When 'dirty' slicing blades were used, we obtained only a 0.64-log reduction of *L. monocytogenes* with 10% EW but a 3.34-log reduction with 100% EW (Fig. 11).

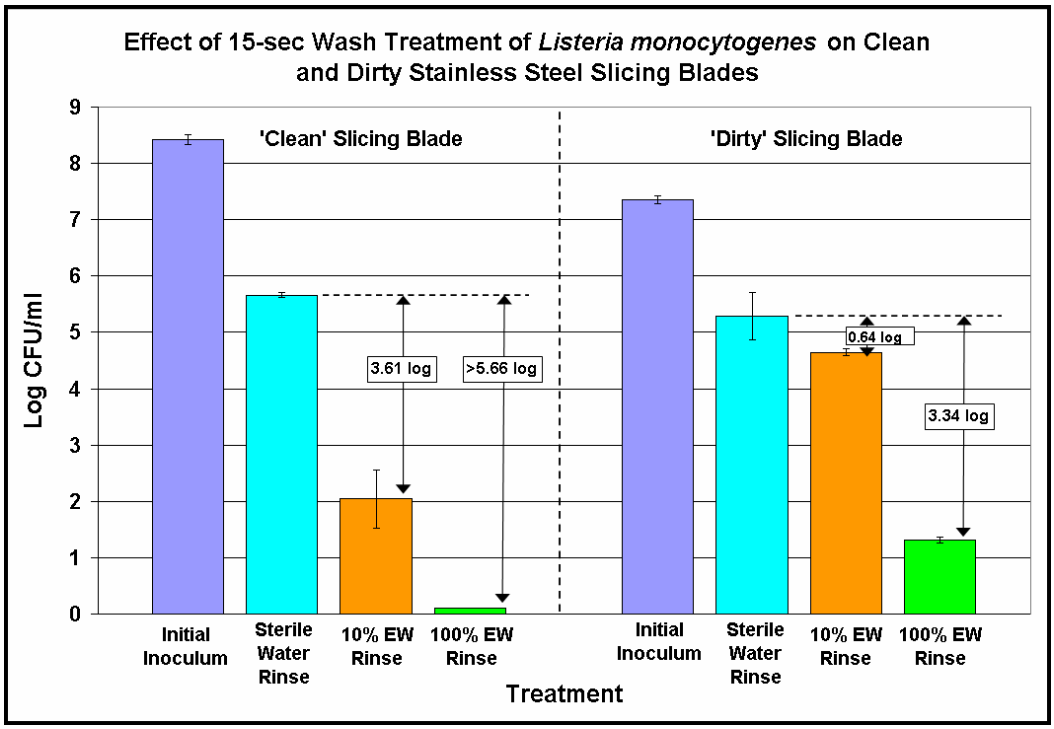


Figure 11. Clean (left) or dirty (right) slicing blades were inoculated with *Listeria monocytogenes* and rinsed for 15-sec with sterile water, 10% EW, or 100% EW (~825 ORP, pH 7.0). The slicing blades were used to make several cuts through RTE deli turkey breast to condition the blade as 'dirty' with an organic load.

The results indicate that EW works extremely well on clean surfaces to eliminate residual *L. monocytogenes* that may have escaped sanitation regimens. The data implicates the degree to which EW may work when organic film is removed via a detergent rinse and

followed by EW. Although the effect on the 'dirty' blades may look somewhat reduced, the 100% EW was still very effective for a short, 15-sec rinse (as compared with some sanitizers that require a 2-min contact time for effective eradication of *Listeria*). Since the 100% EW contains approximately 200 ppm free chlorine, it will need to be followed by a water rinse. It should be noted that although the reduction obtained with the 10% EW on dirty blades was not exemplary (0.64-log reduction), one must consider the compounded effect of frequent periodic rinses with such a microbial intervention that can be freely sprayed directly on food and food contact surfaces, that does not require a post-application rinse, and that the solution applied (~pH 7.0) was not the most effective EW treatment. We hope to follow this work shortly with similar treatments using EW at various lower pH levels whereby we may readily obtain a 1-log, or greater, reduction on dirty surfaces (pH 3.0, 4.0, 5.0, and 6.0). The merit in this treatment will be that it can be applied to food and food contact surfaces with no subsequent rinse treatment, and therefore, periodic re-application of such innocuous solutions may merit from pathogen reduction from repeated application, even during processing operations.

9. Conclusion.

Having used the most tenaciously adherent strains that we could identify using our biofilm attachment assay, treatment with Electrolyzed Water results in a quantitative reduction of cell counts as determined from proteinase release assays (Fig. 7), fluorescence assay (Fig. 9), and scanning electron microscopy (Fig. 10). The data suggests that application of EW in similar fashion to food processing facilities could significantly reduce, or eliminate, *Listeria monocytogenes* as an environmental surface contaminant on both clean or dirty surfaces (Fig. 11).