**Effect of acidic electrolyzed water-induced bacterial inhibition and injury
in live clam (*Venerupis philippinarum*) and mussel (*Mytilus edulis*)**

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Running Title: Acidic electrolyzed water-induced bacterial inhibition and injury

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ABSTRACT

The effect of acidic electrolyzed water (AEW) on inactivating *Escherichia coli* O104:H4, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *Campylobacter jejuni* in laboratory contaminated live clam (*Venerupis philippinarum*) and mussel (*Mytilus edulis*) was investigated. The initial levels of bacterial contamination were: in clam 4.9 to 5.7 log₁₀ CFU/g, and in mussel 5.1 to 5.5 log₁₀ CFU/g. Two types of AEW were used for treatment time intervals of 1 and 2 h: strong (SAEW) with an available chlorine concentration (ACC) of 20 mg/L, pH = 3.1, and an oxidation-reduction potential (ORP) of 1150 mV, and weak (WAEW) at ACC of 10 mg/L, pH = 3.55 and ORP of 950 mV. SAEW and WAEW exhibited significant inhibitory activity against inoculated bacteria in both shellfish species with significant differences compared to saline solutions treatments (1-2% NaCl) and untreated controls (0 h). SAEW showed the largest inhibitory activity, the extent of reduction (log₁₀ CFU/g) ranged from 1.4-1.7 for *E. coli* O104:H4; 1.0-1.6 for *L. monocytogenes*; 1.3-1.6 for *A. hydrophila*; 1.0-1.5 for *V. parahaemolyticus*; and 1.5-2.2 for *C. jejuni* in both types of shellfish. In comparison, significantly ($P<0.05$) lower inhibitory effect of WAEW was achieved compared to SAEW, where the extent of reduction (log₁₀ CFU/g) ranged from 0.7-1.1 for *E. coli* O104:H4; 0.6-0.9 for *L. monocytogenes*; 0.6-1.3 for *A. hydrophila*; 0.7-1.3 for *V. parahaemolyticus*; and 0.8-1.9 for *C. jejuni* in both types of shellfish. Among all bacterial strains examined in this study, AEW induced less bacterial injury (~ 0.1-1.0 log₁₀ CFU/g) and more inactivation effect. This study revealed that AEW (10-20 mg/L ACC) could be used to reduce bacterial contamination in live clam and mussel, which may help control possible unhygienic practices during production and processing of shellfish without apparent changes in the quality of the shellfish.

Keywords: Acidic electrolyzed water, Clam, Mussel, Bacterial injury.

1. Introduction

Foodborne bacterial outbreaks due to the consumption of contaminated shellfish may impose significant health and economic threats. There are several sources of contamination by pathogenic bacteria during the production and processing of shellfish; these may include the microbial pollution of the aquatic environment itself and shellfish-growing waters, which is a major common concern in marine coastal areas (Almeida and Soares, 2012; Schulze et al., 2006). Other potential sources of bacterial contamination could be linked to the shellfish indigenous microbiota, the bacteria naturally present in marine and estuarine environments (*e.g.*, *Vibrio* spp., *Listeria monocytogenes* and *Aeromonas hydrophila*); non-indigenous enteric bacteria due to fecal contamination (*e.g.*, *Escherichia coli* and *Campylobacter* spp.); and bacterial pollution due to unhygienic processing and preparation by producers and consumers (Anacleto et al., 2013; Croci et al., 2007; Reilly and Käferstein, 1997). As a result, raw molluscan shellfish is considered a major health risk, in which the main route of bacterial transmission to humans includes the consumption of raw and lightly cooked shellfish (Lees et al., 2010; Newton et al., 2014; Quan et al., 2010).

The clam (*Venerupis philippinarum*) is one of the most commercially important aquaculture shellfish species and has an important socioeconomic aspect because it is easily grown and harvested and has a good nutritional value (Oliveira et al., 2011; Park et al., 2006; Zhang et al., 2015). The blue mussel (*Mytilus edulis*) is a euryhaline and eurythermal shellfish cultivated in intensive aquaculture (Innes and Bates, 1999) and is usually consumed raw, smoked or canned. These two bivalve shellfish feed by filtering large quantities of water and can accumulate bacteria from the aquatic environment (Anacleto et al., 2013; Love et al., 2010; Ovissipour et al., 2013).

The worldwide demand for freshly live, whole, high-quality and microbiologically safe shellfish is increasing. Therefore, having effective nontoxic disinfection treatments to control

bacterial contamination and extend the shelf life of perishable shellfish is a major requirement. Technically, the USA National Shellfish Sanitation Program (NSSP) suggests three methods for cleansing and decontamination of shellfish: transfer of shellfish to natural waters for more than 14 days at an appropriate salinity and temperature ranges; depuration in a verified system for more than 44 h; and pasteurization by heat or high pressure (Love et al., 2010; Ovissipour et al., 2013; Ren and Su, 2006). However, most of these treatments require significant personnel training; a considerable initial investment in capital for depuration tanks, etc., and the proposed decontamination treatment is not always effective against microorganisms that colonize the intestinal tract of the shellfish (Ren and Su, 2006). Due to its bactericidal properties, electrolyzed oxidizing water (EO water) could be used to control bacterial contamination in shellfish as a non-thermal post harvest sanitation treatment method. EO water involves electrolysis of deionized water containing sodium chloride in an electrolytic chamber with a diaphragm separating the anode from the cathode. Acidic electrolyzed water (AEW) is produced at the anode with a pH of less than 3.5, an oxidation-reduction potential (ORP) greater than 1000 mV, and available chlorine concentration (ACC) of 10-250 mg/L (Ovissipour et al., 2015). The pH, ORP and ACC of the acidic EO water depend upon the sodium chloride concentration, electrolysis time, and water flow into the electrolysis chamber (Hsu, 2003; Ovissipour et al., 2015; Venkitanarayanan et al., 1999). The use of strong and weak EO water as an antimicrobial solution with high and low ACC has been studied in bacterial cell suspensions (Ovissipour et al., 2015; Quan et al., 2010); to reduce bacterial contamination of *Vibrio* spp. in raw oyster and shrimp (Ren and Su, 2006; Xie et al., 2012) or to study the disinfectant effect in inoculated fish and fish surface (Al-Holy and Rasco, 2015; Huang et al., 2006; Kasai et al., 2000; Shiroodi et al., 2016). However, in most previously reported research, treatment regimes using high chlorine concentrations (>30 mg/L) with prolonged exposure time

(> 2 h) were applied to study the antimicrobial properties of EO water, levels at which significant deterioration in the quality of the fish or shellfish would occur and which may not be legal.

The objective of the current study was to investigate the inhibitory activity and injury of AEW (ACC of 10 and 20 mg/L) against *Escherichia coli* O104:H4, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *Campylobacter jejuni* that had been separately inoculated into the live shellfish through feeding activity.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All bacterial American Type Culture Collection (ATCC) strains were obtained from Microbiologics, Inc. (St. Cloud, MN). *Escherichia coli* O104:H4 ATCC BAA-2326, *Listeria monocytogenes* ATCC 19111, and *Aeromonas hydrophila* ATCC 35654 were cultured and activated by inoculating each specific Kwik-Stik swab (Microbiologics, Inc.) into 100 mL of tryptic soy broth TSB (Difco TM) and then were incubated at 37°C for 24 h. All strains were cultured to yield a cell count of approximately 10⁹ CFU/mL. *E. coli*, *L. monocytogenes* and *A. hydrophila* suspensions were enumerated in duplicate using the spread-plate technique in which a 1-mL aliquot of each dilution was divided into 5 aliquots of 0.2 mL and cultured on tryptic soy agar TSA (Difco TM) (Al-Qadiri et al., 2015; Ovissipour et al., 2015). *Vibrio parahaemolyticus* ATCC 17802 was activated by inoculating the Kwik-Stik swab into 100 mL of TSB (Difco TM) supplemented with 2% NaCl followed by incubation at 37 °C for 24 h (Ovissipour et al., 2015). *Campylobacter jejuni* ATCC 29428 was activated by inoculating the Kwik-Stik swab into 100 mL of Campylobacter enrichment broth consisting of Campylobacter nutrient broth no. 2 (CM0067, Oxoid Ltd.) and supplemented with Campylobacter growth supplement (SR0232E, Oxoid Ltd.). *C. jejuni* broth was

then incubated in an anaerobic jar at 37 °C for 48 h under a microaerophilic atmosphere (~ 6 to 7% O₂) using CampyGen sachets (CN0025, Oxoid Ltd.) (Al-Qadiri et al., 2015). *V. parahaemolyticus* and *C. jejuni* were cultured to obtain a cell count of approximately 10⁹ CFU/mL, thereafter, they were enumerated in duplicate using the spread-plate technique in which a 1-mL aliquot of each dilution was divided into 5 aliquots of 0.2 mL and cultured on TSA (Difco™) supplemented with 2% NaCl and Campylobacter blood-free medium (modified CCDA Preston, CM0739, Oxoid Ltd.), respectively (Al-Qadiri et al., 2015; Astorga and Alonso, 2010; Ovissipour et al., 2015).

2.2. Inoculum preparation

After the appropriate incubation of bacterial cultures, 50 mL broth of each strain was transferred under aseptic conditions to a sterile centrifuge tube and centrifuged for 15 min at 5000 rpm (3380 × g) to harvest bacterial cells (AccuSpin Centrifuge, Thermo Fisher Scientific, Waltham, MA). To eliminate the effect of broth components and bacterial metabolites, the resultant pellets were resuspended in 50 mL of sterile saline solution (0.85 % (wt/vol) NaCl). The tubes were then centrifuged as before, and the resulting pellets of the five strains were then resuspended in 50-mL aliquots and centrifuged for a second time as described above. The supernatant was decanted and the resulting washed pellets were resuspended in sterile 50-mL aliquots, which were then used to inoculate the live shellfish samples. The initial bacterial load for all bacterial types inoculated into clam and mussel ranged between 7.3×10^4 to 5.0×10^5 CFU/g.

2.3. Preparation of acidic electrolyzed water

Acidic electrolyzed water (AEW) was prepared from the anode compartment in the electrolytic cell in which EO water was generated at 9-12 V direct current (DC) for 15 min using a two-compartment batch scale electrolysis apparatus (Super Oxseed Labo, Electrolyzed Water Generator, Aoi Electronic Corp., Kannami, Shizuoka, Japan) with the anode and cathode electrodes

of the chamber divided by an ion exchange diaphragm. The ORP and pH were measured using a pocket sized redox meter (HI 98201, HANNA[®] Instruments, Ann Arbor, Michigan, USA) and a pH meter (FE20, Mettler-Toledo, Columbus, OH, USA), respectively. The available chlorine concentration of the generated AEW was measured using the DPD assay method (Colorimeter[™] Analysis System, Hach Co., Loveland, CO, USA) according to the manufacturer instructions. Two types of AEW were prepared (Ovissipour et al., 2015): strong (SAEW: 200 mg/L NaCl, pH = 3.1, ORP = 1150 mV, and ACC = 20 mg/L) and weak (WAEW: 40 mg/L NaCl, pH = 3.55, ORP = 950 mV, and ACC = 10 mg/L).

2.4. Sample preparation and inoculation process

Live clam (*Venerupis philippinarum*) and mussel (*Mytilus edulis*) were purchased from a local retailer in Pullman, WA, and transferred to the laboratory under cold conditions within 30 min. The purchased shellfish were examined for free residual chlorine (DPD assay method) and found to be in the range of 2-4 mg/L. Clams and mussel samples were treated separately, first by soaking for 2 h in freshly prepared sterile sodium thiosulfate solution (2% (wt/vol) Na₂S₂O₃) to neutralize any residual chlorine at ambient temperature (22 °C). This was done to preclude any external inhibitory effect besides the treatments applied in the current study. During the dechlorination process, any dead clams and mussels were separated from the live ones with the presumption made that any animals with an open shell were dead. After 2 h, sodium thiosulfate solution was decanted and the shellfish were soaked for 1 h in a freshly prepared sterile saline solution (1% (wt/vol) NaCl) at ambient temperature. Following this and under aseptic conditions, the live shellfish were placed into a freshly prepared sterile saline solution (1% (wt/vol) NaCl) for all microbes with the exception of *V. parahaemolyticus* in which case 2% NaCl was used with a ratio of 1 animal per 100 mL solution. For each treatment, 25 live clams or 30 live mussels were

inoculated separately with each of the bacterial strains to be tested. The 50-mL aliquot of the bacterial culture previously described was separately added to the solution containing the live shellfish with the approximate bacterial concentration being 10^7 CFU/mL. The shellfish samples were held in the inoculated saline for 6 h at ambient temperature to let bacteria adhere to the exposed surfaces and to allow the shellfish to filter the water so that the bacteria would become a component of the digestive tract. At the end of the incubation process, the inoculated shellfish were aseptically recovered and placed into containers of freshly prepared SAEW (ACC = 20 mg/L) or WAEW (ACC = 10 mg/L) with a ratio of 1 animal per 100 mL solution and for depuration times of 1 and 2 h. The used AEW contained 1% (wt/vol) NaCl (2% in case of *V. parahaemolyticus*) to minimize bacterial osmotic stresses and to promote filtration activity of the shellfish. Inoculated shellfish not treated with SAEW or WAEW served as controls (0 h). Second control treatments of inoculated shellfish were held in 1% (wt/vol) NaCl (2% in case of *V. parahaemolyticus*) instead of AEW, samples were treated as above for depuration times of 1 and 2 h. All tests were carried out in triplicate.

2.5. Recovery of bacteria and culture media

To recover the surviving bacteria, shellfish were shucked under aseptic conditions, weighed and examined in duplicate using selective and nonselective culture media. The shellfish flesh was placed in sterile strainer-filter bags and shaken vigorously in 200 ml of sterile D/E Neutralizing Broth (Difco™) using a Stomacher 400 circulator (Seward Ltd., London, UK). One milliliter of the stomached suspension was then serially diluted (dilution range, 10^0 to 10^{-5}) in 9 mL of sterile 0.1% peptone water (Difco™). Samples were examined in duplicate using the spread-plate technique. A 1-mL aliquot of each dilution was divided into 5 aliquots of 0.2 mL, which then they were evenly spread on the following selective culture media for enumeration of *E. coli* O104:H4, *L.*

monocytogenes, *A. hydrophila*, and *V. parahaemolyticus*, respectively: m-Endo agar LES (Difco™) (Al-Qadiri et al., 2011), PALCAM agar supplemented with required antibiotics (CM0877, Oxoid Ltd.) (Liu and Busse, 2010), Aeromonas medium (RYAN) (CM0833, Oxoid Ltd) (Warburton et al., 1994) and Cholera medium TCBS (CM0333, Oxoid Ltd.) (Hara-Kudo et al., 2001). Plates were then incubated at 37 °C for 24-48 h, and the number of viable cells was determined as CFU/g. Selective Campylobacter blood-free medium (modified CCDA Preston), which was prepared with the addition of CCDA selective supplement (SR0155, Oxoid Ltd.), was used to enumerate surviving *C. jejuni* (Astorga and Alonso, 2010). Plates were then incubated at 37 °C for 48 h under microaerophilic conditions. TSA (Difco™) was used as a nonselective culture medium with the addition of 2% NaCl in case of *V. parahaemolyticus* to enumerate total mesophilic bacteria.

2.6. Statistical analysis

The experiment consisted of three independently replicated trials ($n = 3$) and each reported value is the mean viable count $\pm$ standard deviation (SD) of the results of three replicate treatments per experimental trial. An analysis of variance, using the mixed-effects procedure for bacterial counts, was conducted with SAS software (SAS Institute, 2011). Clam or mussel, strong or weak AEW, depuration time and selectivity of culture media were treated as fixed effects. Subjects were random samples from the target population and, therefore, were treated as random effects in the model. The interaction among fixed-effect variables was analyzed. The Kenward and Roger method was used to evaluate the denominator degrees of freedom (Kenward-Roger = DDFM). In order to adjust the estimated standard deviations for fixed effects and interaction effects (Littell et al., 2006), the level of significance was set at a P value of < 0.05 . Post hoc multiple pairwise comparisons of treatment group means were performed with the Tukey-Kramer adjustment (Tukey's honestly significant difference test) to control the type I error rate.

3. Results and discussion

To our knowledge, this is the first study on the antimicrobial activity of AEW against five different bacterial species that were separately inoculated into live clam and mussel and for depuration times of only 1 and 2 h. Table 1 shows the viable counts of *E. coli* O104:H4 in live clam and mussel after being treated with 1% NaCl, SAEW or WAEW for 1 or 2 h. It was observed that holding inoculated shellfish in sterile saline solutions (1% NaCl) for 2 h did not cause a significant reduction in the level of inoculated bacteria compared to the AEW treatments. SAEW (20 mg/L ACC) resulted in approximately 1.4 log₁₀ CFU/g reduction in the count after treatment for 1 h and about 1.6 log₁₀ CFU/g reduction after treatment for 2 h in clam. Although the increase in reduction by extending the treatment time from 1 h to 2 h was only 0.2 log₁₀ CFU/g, it was statistically significant ($P < 0.05$). In comparison, WAEW (10 mg/L ACC) inflicted reductions in *E. coli* counts of 0.7 and 1.0 log₁₀ CFU/g after treatment for 1 and 2 h, respectively. Nonetheless, this reduction was significantly less compared to SAEW treatment. The extent of bacterial injury as measured by the difference in viable counts of the organism on nonselective and selective culture media was approximately 0.2 log₁₀ CFU/g. The same pattern of inhibition was observed for *E. coli* after treatment of mussel with SAEW and WAEW for 1 or 2 h. About 1.6 and 1.7 log₁₀ CFU/g reduction in the organism was observed after treatment with SAEW for 1 and 2 h, respectively. However, WAEW resulted in larger reduction in *E. coli* counts (0.9-1.1 log₁₀ CFU/g) compared to the level of reduction in clam (0.7-1.0) after 1 and 2 h treatment times, respectively. Also, it is worthwhile to mention that WAEW resulted in significantly ($P < 0.05$) less inactivation, yet more cells were injured (0.3-0.6 log₁₀ CFU/g) compared to SAEW.

Table 2 shows the viable counts of *L. monocytogenes* in live clam and mussel after treatment with 1% NaCl, SAEW or WAEW for 1 or 2 h. Saline treatment did not elicit a noticeable

reduction in *L. monocytogenes* count compared to the untreated control (0 h). SAEW resulted in approximately 1.0 and 1.3 log₁₀ CFU/g reduction in clam after 1 and 2 h treatment times, respectively. While WAEW resulted in about 0.7 and 0.8 log₁₀ CFU/g reduction in *L. monocytogenes* count after 1 and 2 h treatment times, respectively. Longer treatment time (2 h) resulted in significantly ($P < 0.05$) larger reduction compared to the 1 h treatment time. The extent of *L. monocytogenes* injury in treated clam ranged between 0.1-0.2 log₁₀ CFU/g in both SAEW and WAEW. Likewise, the same pattern of *L. monocytogenes* inhibition was observed in treated mussel. Treatment with 1% NaCl had a marginal inhibitory activity against *L. monocytogenes*. In comparison, SAEW posed significantly ($P < 0.05$) larger inactivation compared with WAEW, where it elicited about 1.3-1.6 log₁₀ CFU/g reduction in the viable count. The level of *L. monocytogenes* injury ranged between 0.2 and 0.3 log₁₀ CFU/g.

Table 3 shows the inhibitory activity and AEW induced bacterial injury against *A. hydrophila* in clam and mussel. SAEW resulted in approximately 1.4-1.6 log₁₀ CFU/g reduction, whereas, WAEW resulted in about 0.7-1.3 log₁₀ CFU/g reduction in *A. hydrophila* viable count in the treated clam, in which SAEW resulted in significantly ($P < 0.05$) higher reduction compared to WAEW. For mussel, SAEW resulted in approximately 1.3-1.5 log₁₀ CFU/g reduction in *A. hydrophila*, which is significantly larger compared to the reduction induced by WAEW (0.6-0.7 log₁₀ CFU/g).

Table 4 shows the viable counts of *V. parahaemolyticus* in live clam and mussel after treatment with 2% NaCl, SAEW or WAEW for 1 or 2 h. Treatment with 2% NaCl did not result in a noticeable reduction in the level of *V. parahaemolyticus* viable count. In comparison, SAEW elicited 1.3 to 1.5 log₁₀ CFU/g reduction, which was significantly ($P < 0.05$) larger compared to the level of inhibition induced by WAEW (1.0-1.3 log₁₀ CFU/g reduction) in the treated clam. In

SAEW treated mussel, about 1.0 to 1.2 log₁₀ CFU/g reduction was obtained. WAEW resulted in significantly ($P < 0.05$) less reduction (0.7-0.9 log₁₀ CFU/g) in *V. parahaemolyticus* count compared to SAEW. Also, it is worthwhile to mention that SAEW and WAEW resulted in remarkably high level (~ 0.4-0.9 log₁₀ CFU/g) of injured *V. parahaemolyticus* cells compared to other bacteria investigated in the current study.

Table 5 shows the viable counts of *C. jejuni* in live clam and mussel after treatment with 1% NaCl, SAEW or WAEW for 1 or 2 h. Treatment with 1% NaCL exhibited a significant ($P < 0.05$) reduction (0.7-0.8 log₁₀ CFU/g) in the *C. jejuni* count compared to the untreated control (0 h). SAEW resulted in about 1.5 to 1.7 log₁₀ CFU/g reduction, which was significantly ($P < 0.05$) larger compared to the inhibitory effect of WAEW that resulted in only 0.8-1.0 log₁₀ CFU/g reduction in the treated clam. Similarly, SAEW treated mussel resulted in a remarkable reduction level that ranged between 2.1-2.2 log₁₀ CFU/g in the *C. jejuni* count. In comparison, WAEW resulted in significantly ($P < 0.05$) less inhibitory effect (1.8-1.9 log₁₀ CFU/g reduction) against *C. jejuni* compared to SAEW. Notwithstanding, the level of AEW induced *C. jejuni* injury ranged between 0.2-0.5 log₁₀ CFU/g in both types of shellfish.

In the current study treating shellfish with 1 or 2 % NaCl resulted only in a slight reduction of all pathogens inoculated in clam and mussel. This marginal reduction is possibly due to the physical removal of the pathogens off the shellfish. AEW usually exert antibacterial activity due to its content of free chlorine and low pH. Furthermore, AEW possesses high ORP (+ 950 - +1150 mV), which is associated with greater oxidizing power and hence poses inhibitory activity particularly against strict anaerobic and facultative anaerobes (Jay et al., 2005). In addition, AEW inhibits bacteria by increasing the permeability of the cell membrane and therefore causing leakage of intracellular constituents (Zeng et al., 2010).

Generally, AEW is more effective as an antimicrobial treatment in pure microbial systems compared to food systems. The inhibitory effect of AEW was smaller against bacteria inoculated into shellfish as revealed in the current study compared to artificial broth media. A complete inhibitory effect of acidic and alkaline EO water against the same bacterial strains grown in artificial bacteriological broth (more than 7 log₁₀ CFU/mL reduction) within 6 min of AEW at 20°C was achieved (Ovissipour et al., 2015). The findings in the current study are consistent with previous studies reporting that EO water was highly effective against pathogens in suspension culture compared to food (Huang et al., 2006; Kim et al., 2000; Oomori et al., 2000; Park et al., 2002; Quan et al., 2010; Ren and Su, 2006; Venkitanarayanan et al., 1999). The Limited antibacterial activity is likely due to the presence of organic materials, primarily proteins and lipids that react with the free available chlorine forming combined chlorine that has much lower bactericidal activity than the free form. In this experiment, the bactericidal activity of AEW could have been minimized because of interactions between the AEW and organic components during filter feeding, which may result in reducing the activity of the AEW before the bacterial cells accumulated inside the digestive tract of the animals could be reached. Additionally, sanitizers usually exert stronger antimicrobial activity against pathogens existing on smoother surfaces. Hence, the irregular surfaces found in shellfish may provide a safe shelter for contaminating pathogens (Al-Holy et al., 2015).

In the current study, it is possible that the limiting factor for bactericidal activity might have been the ACC rather than treatment time. Huang et al. (2006) and Quan et al. (2010) reported that no further antibacterial activity was achieved by extending treatment time to kill *E. coli* and *V. parahaemolyticus* in cell suspension and inoculated seafood. However, the increase in chlorine concentration may cause deterioration and loss of quality of the perishable fish and shellfish,

particularly in the situation tested here in which live shellfish were used. Ren and Su (2006) indicated that treating inoculated raw oysters with EO water significantly reduced the level of *V. parahaemolyticus* and *V. vulnificus* by 1.1 log₁₀ MPN/g. However, they demonstrated that the treatment process should be limited to a chlorine concentration of less than 30 mg/L and a processing time of 4-6 h or less to avoid death of oysters. On the other hand, another study reported that depuration with clean artificial seawater was not effective in reducing bacterial contamination in treated oysters (Eyles and Davey, 1984; Ren and Su, 2006). Longer depuration period of oysters (16 days) was required to elicit complete elimination of *V. vulnificus* spiked into oysters (Kelly and Dinuzzo, 1985). In the current study, the used AEW was less effective against *L. monocytogenes* compared to other pathogenic bacteria inoculated into shellfish. This is possibly because Gram negative are generally more sensitive than Gram positive bacteria to AEW disinfection due to the difference in cell wall structure (Jay et al., 2005).

However, one of the main benefits of using AEW in this study was the low chlorine levels of less than 20 mg/L. The tested residual chlorine content in flesh samples after 2 h of treatment was less than 1.0 ppm. The US Environmental Protection Agency (EPA) has approved the use of AEW in the food industry which makes the application of AEW in aquaculture is possible. Using chlorine solutions at levels of 20 mg/L or less would be recognized as a safe usage level (GRAS). Codex permits up to 20 mg/L chlorine in water that comes in contact with fish and fish products and up to 100 mg/L in water used in cleaning and sanitation of equipment and facilities, however, when chlorinated water used in the seafood industry; the residual chlorine level should not exceed that of potable water (Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), 2000, 2008). Additionally, beside treatment with AEW water, other hurdles (*e.g.*, mild heat and UV treatments) can be applied to effectively control bacterial

contamination in fish and fishery products (Kasai et al., 2011; Shiroodi et al., 2016; Xie et al., 2012).

In conclusion, this study showed that AEW with ACC of 10-20 mg/L could be used to substantially reduce the level of contamination of common bacterial pathogens in live clam and mussel within a depuration time of 2 h. Although both of SAEW and WAEW were effective in reducing the level of pathogens inoculated in clam and mussel, SAEW inflicted larger inhibitory effect. Therefore, the use of AEW may help control possible unhygienic practices during production and processing of shellfish without apparent deterioration in the quality.

Acknowledgments

This research was supported by Agricultural Research Center at Washington State University, the University of Jordan and USDA-NIFA 2011-68003-20096.

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Table 1
Mean viable counts of *E. coli* O104:H4 (log₁₀ CFU/g)^{1,2} inoculated into live clam and mussel

Treatment	Treatment time (h)			
	1		2	
	Nonselective media	Selective media	Nonselective media	Selective media
<i>Clam</i> ³				
(1% NaCl)	5.6 ± 0.1 ^a (0.1)	5.6 ± 0.1 ^a (0.1)	5.6 ± 0.1 ^a (0.1)	5.6 ± 0.0 ^a (0.1)
SAEW	4.3 ± 0.0 ^e (1.4)	4.2 ± 0.1 ^f (1.5)	4.1 ± 0.1 ^f (1.6)	3.9 ± 0.0 ^g (1.8)
WAEW	5.0 ± 0.1 ^b (0.7)	4.8 ± 0.1 ^c (0.9)	4.7 ± 0.0 ^c (1.0)	4.5 ± 0.0 ^d (1.2)
<i>Mussel</i> ⁴				
(1% NaCl)	5.4 ± 0.0 ^a (0.0)	5.4 ± 0.0 ^a (0.0)	5.3 ± 0.1 ^a (0.1)	5.3 ± 0.1 ^a (0.1)
SAEW	3.8 ± 0.1 ^{ef} (1.6)	3.6 ± 0.1 ^{gh} (1.8)	3.7 ± 0.0 ^{fg} (1.7)	3.5 ± 0.1 ^h (1.9)
WAEW	4.5 ± 0.0 ^b (0.9)	3.9 ± 0.1 ^{de} (1.5)	4.3 ± 0.0 ^c (1.1)	4.0 ± 0.1 ^d (1.4)

¹Values are the means of three independent replicate trials ± standard deviation, with log₁₀ reductions (expressed in CFU/g) presented in parentheses.
² For each studied animal, treatment means without shared superscripts are significantly different (Tukey's HSD test, *P*<0.05).
^{3, 4} Mean viable counts of the inoculated untreated controls (0 h): 5.7 and 5.4 log₁₀ CFU/g for clam and mussel, respectively, and which selected as “a” in statistical analysis.

Table 2

Mean viable counts of *L. monocytogenes* (log₁₀ CFU/g)^{1,2} inoculated into live clam and mussel

Treatment	Treatment time (h)			
	1		2	
	Nonselective media	Selective media	Nonselective media	Selective media
<u>Clam</u> ³				
(1% NaCl)	4.8 ± 0.1 ^b (0.2)	4.7 ± 0.0 ^b (0.3)	4.7 ± 0.1 ^b (0.3)	4.7 ± 0.0 ^b (0.3)
SAEW	4.0 ± 0.0 ^d (1.0)	3.7 ± 0.1 ^e (1.3)	3.7 ± 0.0 ^{ef} (1.3)	3.6 ± 0.1 ^f (1.4)
WAEW	4.2 ± 0.1 ^c (0.8)	4.1 ± 0.0 ^{cd} (0.9)	4.2 ± 0.1 ^c (0.8)	4.0 ± 0.0 ^d (1.0)
<u>Mussel</u> ⁴				
(1% NaCl)	5.4 ± 0.1 ^a (0.1)	5.4 ± 0.0 ^a (0.1)	5.2 ± 0.1 ^b (0.3)	5.1 ± 0.0 ^b (0.4)
SAEW	4.2 ± 0.1 ^f (1.3)	4.0 ± 0.0 ^g (1.5)	3.9 ± 0.0 ^g (1.6)	3.5 ± 0.1 ^h (2.0)
WAEW	4.9 ± 0.0 ^c (0.6)	4.6 ± 0.1 ^d (0.9)	4.6 ± 0.1 ^d (0.9)	4.4 ± 0.0 ^e (1.1)

¹Values are the means of three independent replicate trials ± standard deviation, with log₁₀ reductions (expressed in CFU/g) presented in parentheses.

² For each studied animal, treatment means without shared superscripts are significantly different (Tukey's HSD test, *P*<0.05).

^{3, 4} Mean viable counts of the inoculated untreated controls (0 h): 5.0 and 5.5 log₁₀ CFU/g for clam and mussel, respectively, and which selected as “a” in statistical analysis.

Table 3

Mean viable counts of *A. hydrophila* ($\log_{10}$ CFU/g)^{1,2} inoculated into live clam and mussel

Treatment	Treatment time (h)			
	1		2	
	Nonselective media	Selective media	Nonselective media	Selective media
<u>Clam</u> ³				
(1% NaCl)	5.4 ± 0.1 ^b (0.3)	5.4 ± 0.0 ^b (0.3)	5.3 ± 0.1 ^b (0.4)	5.3 ± 0.0 ^b (0.4)
SAEW	4.3 ± 0.1 ^{ef} (1.4)	4.1 ± 0.1 ^f (1.6)	4.1 ± 0.1 ^f (1.6)	3.8 ± 0.0 ^g (1.9)
WAEW	5.0 ± 0.0 ^c (0.7)	4.4 ± 0.1 ^{de} (1.3)	4.4 ± 0.3 ^d (1.3)	4.3 ± 0.1 ^{ef} (1.4)
<u>Mussel</u> ⁴				
(1% NaCl)	5.4 ± 0.1 ^a (0.0)	5.3 ± 0.1 ^a (0.1)	5.3 ± 0.1 ^a (0.1)	5.2 ± 0.1 ^a (0.2)
SAEW	4.1 ± 0.1 ^e (1.3)	3.8 ± 0.1 ^f (1.6)	3.9 ± 0.0 ^f (1.5)	3.5 ± 0.0 ^g (1.9)
WAEW	4.8 ± 0.0 ^b (0.6)	4.5 ± 0.1 ^c (0.9)	4.7 ± 0.1 ^b (0.7)	4.3 ± 0.0 ^d (1.1)

¹Values are the means of three independent replicate trials ± standard deviation, with $\log_{10}$ reductions (expressed in CFU/g) presented in parentheses.

² For each studied animal, treatment means without shared superscripts are significantly different (Tukey's HSD test, $P < 0.05$).

^{3,4} Mean viable counts of the inoculated untreated controls (0 h): 5.7 and 5.4 $\log_{10}$ CFU/g for clam and mussel, respectively, and which selected as "a" in statistical analysis.

Table 4

Mean viable counts of *V. parahaemolyticus* (log₁₀ CFU/g)^{1,2} inoculated into live clam and mussel

Treatment	Treatment time (h)			
	1		2	
	Nonselective media	Selective media	Nonselective media	Selective media
<u>Clam</u> ³				
(2% NaCl)	5.2 ± 0.1 ^{ab} (0.1)	5.1 ± 0.1 ^{ab} (0.2)	5.1 ± 0.1 ^b (0.2)	5.1 ± 0.0 ^b (0.2)
SAEW	4.0 ± 0.0 ^d (1.3)	3.9 ± 0.0 ^{ef} (1.4)	3.8 ± 0.1 ^f (1.5)	3.6 ± 0.1 ^g (1.7)
WAEW	4.3 ± 0.1 ^c (1.0)	4.1 ± 0.0 ^d (1.2)	4.0 ± 0.1 ^{de} (1.3)	3.9 ± 0.0 ^{de} (1.4)
<u>Mussel</u> ⁴				
(2% NaCl)	5.0 ± 0.1 ^a (0.1)	5.0 ± 0.1 ^a (0.1)	5.0 ± 0.0 ^a (0.1)	4.9 ± 0.0 ^a (0.2)
SAEW	4.1 ± 0.1 ^{cd} (1.0)	3.2 ± 0.1 ^g (1.9)	3.9 ± 0.1 ^{ef} (1.2)	3.0 ± 0.1 ^h (2.1)
WAEW	4.4 ± 0.0 ^b (0.7)	4.0 ± 0.0 ^{de} (1.1)	4.2 ± 0.1 ^c (0.9)	3.8 ± 0.1 ^f (1.3)

¹Values are the means of three independent replicate trials ± standard deviation, with log₁₀ reductions (expressed in CFU/g) presented in parentheses.

² For each studied animal, treatment means without shared superscripts are significantly different (Tukey's HSD test, *P*<0.05).

^{3, 4} Mean viable counts of the inoculated untreated controls (0 h): 5.3 and 5.1 log₁₀ CFU/g for clam and mussel, respectively, and which selected as “a” in statistical analysis.

Table 5

Mean viable counts of *C. jejuni* ($\log_{10}$ CFU/g)^{1,2} inoculated into live clam and mussel

Treatment	Treatment time (h)			
	1		2	
	Nonselective media	Selective media	Nonselective media	Selective media
<i>Clam</i> ³				
(1% NaCl)	4.2 ± 0.0 ^b (0.7)	4.1 ± 0.1 ^b (0.8)	4.1 ± 0.0 ^b (0.8)	4.0 ± 0.1 ^b (0.9)
SAEW	3.4 ± 0.1 ^f (1.5)	3.1 ± 0.1 ^g (1.8)	3.2 ± 0.0 ^{fg} (1.7)	2.8 ± 0.1 ^h (2.1)
WAEW	4.1 ± 0.1 ^c (0.8)	3.9 ± 0.1 ^d (1.0)	3.9 ± 0.0 ^d (1.0)	3.7 ± 0.1 ^e (1.2)
<i>Mussel</i> ⁴				
(1% NaCl)	4.8 ± 0.0 ^b (0.5)	4.7 ± 0.1 ^b (0.6)	4.7 ± 0.0 ^b (0.6)	4.6 ± 0.1 ^b (0.7)
SAEW	3.2 ± 0.0 ^{de} (2.1)	3.0 ± 0.1 ^f (2.3)	3.1 ± 0.1 ^f (2.2)	2.6 ± 0.1 ^g (2.7)
WAEW	3.5 ± 0.1 ^c (1.8)	3.3 ± 0.1 ^d (2.0)	3.4 ± 0.1 ^d (1.9)	3.2 ± 0.1 ^{ef} (2.1)

¹Values are the means of three independent replicate trials ± standard deviation, with $\log_{10}$ reductions (expressed in CFU/g) presented in parentheses.

² For each studied animal, treatment means without shared superscripts are significantly different (Tukey's HSD test, $P < 0.05$).

^{3,4} Mean viable counts of the inoculated untreated controls (0 h): 4.9 and 5.3 $\log_{10}$ CFU/g for clam and mussel, respectively, and which selected as "a" in statistical analysis.