

Disinfection effect of adding slightly acidic electrolyzed water to artificial seawater under the condition of static hybrid

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Abstract: Mixed solution of slightly acidic electrolyzed water (SAEW) and artificial seawater was used to investigate the disinfection potential of SAEW in artificial seawater. Inoculated *Vibrio parahaemolyticus* (suspended in 3% sodium chloride alkaline peptone water and 0.85% sodium chloride water, respectively) was subjected to different mixed-SAEW and SAEW immersion treatments (5-20 mg/L available chlorine concentration (ACC)). In the presence of organic matter, 4.07 logCFU/mL significant reduction ($p < 0.05$) was achieved after treating with 20 mg/L mixed-SAEW for 15 min. There was 5.13 logCFU/mL reduction after treating with 15 mg/L SAEW for 15 min. For *V. parahaemolyticus* suspended in 0.85% sodium chloride solution, it was undetected after 30 s SAEW treatment (5 mg/L ACC) or 120 s mixed-SAEW treatment (10 mg/L ACC). At a ratio of SAEW and artificial seawater at 1:15 (V/V), SAEW could inactivate *V. parahaemolyticus* to undetectable level in artificial seawater in one minute, which was comparable with UV treatment of 10 W. The results indicated high sanitization potential of SAEW against *V. parahaemolyticus* in aquaculture seawater.

Keywords: slightly acidic electrolyzed water, disinfection, inactivation, *Vibrio parahaemolyticus*, artificial seawater, ultraviolet
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1 Introduction

Aquaculture is one of the fastest-growing food producing sectors, due to increasing demand and environmental considerations. Global aquaculture has grown dramatically over the past 50 years to 80 million tonnes, accounting for around 46% of the world's fish food supply^[1]. As water is filled in the place where fish and other aquatic product live in, water quality is significantly important. *Vibrio parahaemolyticus*, halophilic bacteria, is a prevalent food-borne pathogen and exists in a variety of raw seafood, such as oyster and shrimp. Consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* may cause gastrointestinal episodes^[2-4]. Therefore, elimination of *V. parahaemolyticus* at the origin of aquaculture system is of great importance. However, with consumers' increasing consciousness of food safety, it requires aquaculture free from the use of chemicals such as antibiotics. Therefore, developing eco-friendly disinfectant is necessary and promising to replace chemicals.

Slightly acidic electrolyzed water (SAEW) becomes a research focus in recent years as an effective, nontoxic and environmentally

friendly disinfectant with high bactericidal effect^[5]. It is also known as weakly acidic electrolyzed water (WAEW). It was reported that SAEW may have equivalent or higher bactericidal activity than NaClO solution with lower available chlorine concentration (ACC)^[6-9]. As an effective disinfectant, many studies have demonstrated the high bactericidal efficacy of SAEW against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.*, *Vibrio vulnificus* and *Vibrio parahaemolyticus*^[9-11]. SAEW contains a high concentration (approximately 95%) of hypochlorous acid^[12,13], which is more effective as a sanitizing agent than an equivalent concentration of the hypochlorite ion^[14]. Furthermore, with a near neutral pH of 5.0-6.5, it helps reduce the risk of human health and safety issues from Cl₂ off-gassing and decrease the risk of corrosion^[15].

There are many studies focused on the effect of acidic electrolyzed water (AEW, pH 2.3-2.7) or SAEW on seafood. As for the use of SAEW in purification of marine aquaculture, electrolyzation of seawater directly without a membrane was used to obtain the electrolyzed water for the disinfection in aquaculture^[16-18]. Katayose et al.^[18] reported that electrolyzed natural seawater is safe and effective with ACC at an adequate level in aquaculture. Jorquera et al.^[17] reported that electrolysis between 1.0-4.0 A was able to disinfect safely and electrolyzed seawater achieved higher microalga growth than traditionally autoclaved seawater or seawater treated with germicidal ultraviolet light (UV). It was reported that 0.2 mg/L of chlorine was safe for oysters and after depuration with electrolyzed seawater for 24 h, *E. coli* counts in artificially contaminated oysters decreased to detection limit^[16]. Seawater, especially natural seawater, has many ions, which may cause corrosion to the electrolysis device or react with oxide. Therefore, instead of electrolyzing seawater, adding SAEW directly into seawater to inactivate *V.*

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parahaemolyticus is considerable. Several studies have demonstrated the efficacy of killing *V. parahaemolyticus* by WAEW or AEW in vitro^[9,19]. However, there are fewer studies about the effect of electrolyzed seawater on *V. parahaemolyticus* and researches related to the inactivation of *V. parahaemolyticus* in seawater using SAEW are even much scarcer.

Although some studies have been performed to study the electrolyzed seawater, no studies were reported on using SAEW to disinfect seawater. Therefore, the objects were: 1) to evaluate the bactericidal effect of SAEW diluted by distilled water and artificial seawater on *V. parahaemolyticus* with and without organic contents; 2) to determine the efficiency of SAEW for the elimination of *V. parahaemolyticus* in artificial seawater and compared with UV treatment.

2 Materials and methods

2.1 Bacterial culture and preparation of inoculums

The culture of *V. parahaemolyticus* (ATCC 33847) was obtained from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The culture was transferred on basis every 2 weeks to ensure their viability. To prepare the inoculation stock, several loops of isolated colonies of stock culture were transferred to 100 mL sterile 3% sodium chloride alkaline peptone water (30 g/L sodium chloride, 10 g/L peptone, pH 8.0±0.5) in 250 mL Erlenmeyer flasks and incubated at 37 °C for 24 h with agitation (150 r/min). Following incubation, 30 mL inoculation stock was transferred into a 50 mL sterilized centrifuge tube and centrifuged at 3200 g for 10 min at 20 °C (5810R, Eppendorf AG, Germany). The cell pellet was washed and resuspended in 30 mL 3% sodium chloride solution peptone water or 0.85% sodium chloride solution, respectively. Populations in individual cultures were determined by spread plating 0.1 mL portions of appropriately diluted cultures on thiosulfate citrate bile sucrose agar (TCBS) plates, respectively, and incubation at 37 °C for 24 h before counting. The initial *V. parahaemolyticus* inoculation was approximately 10⁸ to 10⁹ colony forming units (CFU)/mL.

2.2 Preparation of treatment solutions

SAEW was produced by electrolysis of 6% HCl solution using a flow-type electrolysis apparatus (Figure 1) equipped with an electrolytic cell without a separating membrane between anode and cathode at a voltage of 220 V (HD-240L, Wangpu Trading Co., Ltd, Shanghai, China). The SAEW was produced immediately prior to use for evaluating its bactericidal activities and SAEW with pH ~6.0, oxidation reduction potential (ORP) of ~880 mV and ACC of ~30 mg/L was collected for the experiments.

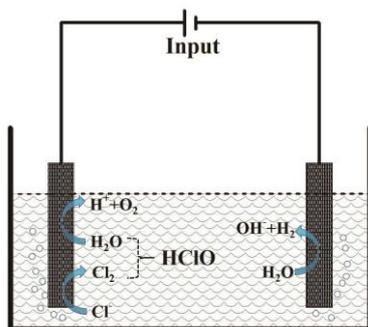


Figure 1 Schematic diagram of the electrolysis apparatus

The treatment SAEW solutions at low levels of ACC were diluted using distilled water prior to measurement. To investigate the inactivation ability of SAEW in seawater, concentrated artificial seawater was also used to dilute initial SAEW and these

solutions called mixed-SAEW. The formula of artificial seawater was modified according to Berges et al.^[20] and is shown in Table 1. The final salinity of the artificial seawater was 3.3% ±0.1% and pH was 8.00 ±0.10.

Table 1 Formula of artificial seawater

Solution I ^a	Concentration/g L ⁻¹	Solution II	Concentration/g L ⁻¹
NaCl	21.19	MgCl ₂ ·6H ₂ O	9.592
Na ₂ SO ₄	3.55	CaCl ₂ ·2H ₂ O	1.344
KCl	0.599	SrCl ₂ ·6H ₂ O	0.0218
NaHCO ₃	0.174		
KBr	0.0863		
H ₃ BO ₃	0.023		
NaF	0.0028		

Note: ^a Solution I and II were made separately and then mixed.

2.3 Determination of ORP, pH and ACC of SAEW

The ORP, pH and ACC of treatment solutions were measured in triplicate immediately after preparation and before the bactericidal experiments. The pH and ORP values of SAEW were measured with a dual scale pH/ORP meter (Mettler-Toledo International Co., Ltd., Zurich, Switzerland). The ACC in SAEW was determined by a colorimetric method using a digital chlorine test kit (RC-3F, Kasahara Chemical Instruments Co., Ltd., Saitama, Japan).

2.4 Mixed-SAEW and SAEW treatments of *V. parahaemolyticus*

Bacterial stocks approximately 8 logCFU/mL were prepared as previously described in section 2.1. For *V. parahaemolyticus* resuspended in sterilized 3% sodium chloride solution peptone water, one milliliter of the prepared *V. parahaemolyticus* culture solution was added into 9 mL of mixed-SAEW or SAEW with different ACC (treatment) and 0.85% sodium chloride solution (control). A timer was started and the tube was mixed immediately at (23±2) °C. At the end of each contact time, 1 mL of each sample was transferred to a tube containing 9 mL of sterilized neutralizer (0.5% sodium thiosulphate, 0.85% sodium chloride solution) followed by serial 10-fold dilution with 0.85% sodium chloride solution dilution blanks. Surviving population in each culture was confirmed by plating 0.1 mL portions of appropriately diluted cultures on TCBS plates in triplicate, respectively. Colonies of survived *V. parahaemolyticus* were enumerated on TCBS plates after incubation at 37 °C for 24 h. The reduction of *V. parahaemolyticus* was expressed as log(N_0/N_t), where N_0 is the count before treatment and N_t the count after treatment.

As for *V. parahaemolyticus* resuspended in 0.85% sodium chloride solution, one milliliter of the prepared *V. parahaemolyticus* was added into 9 mL of mixed-SAEW with different ACC (treatment), and the tube was mixed immediately at (23±2) °C. At the end of each contact time (30 s, 60 s and 90 s), 1 mL of each sample was transferred to a sterilized tube containing 9 mL of 3% sodium chloride solution peptone water and sealed with a silica gel stopper. Then incubate at 37 °C for 48 h with agitation (150 r/min). This is an enrichment to see visible growth of survived and injured *V. parahaemolyticus*. Tubes with negative growth were considered that *V. parahaemolyticus* in treated samples was completely inactivated.

2.5 SAEW treatment of *V. parahaemolyticus* in artificial seawater

Five milliliters of cultured *V. parahaemolyticus* stock was added into 245 mL and 220 mL artificial seawater in 500 mL beaker

with 1 min stirring and initial count was 7.60 ± 0.04 logCFU/mL. Then a volume of 25 mL, 15 mL, 10 mL SAEW (ACC=30 mg/L, ORP=858 \pm 2 mV, pH=5.69 \pm 0.04) was added into the contaminated artificial seawater (250 mL, 225 mL and 250 mL, respectively) and the proportion was 1:10, 1:15 and 1:25 (V/V), respectively. At the end of each contact time, 1 mL of each sample was transferred to a tube containing 9 mL of neutralizer (0.5% sodium thiosulphate, 0.85% sodium chloride solution) followed by serial 10-fold dilution in 0.85% sodium chloride solution dilution blanks. Surviving population in each culture was confirmed by plating 0.1 mL portions of appropriately diluted cultures on TCBS plates in triplicate, respectively. Colonies of survived *V. parahaemolyticus* were enumerated on TCBS plates after incubation at 37 °C for 24 h. In the meantime, 1 mL of each sample was also transferred to a tube containing 9 mL of 3% sodium chloride alkaline peptone water and sealed with a silica gel stopper. Then incubate at 37 °C for 48 h with agitation (150 r/min), which is the same enrichment step as section 2.4 described.

2.6 UV treatment of *V. parahaemolyticus* in artificial seawater

Different power of UV light (10 W and 15 W) was used to inactivate *V. parahaemolyticus* in artificial seawater. UV light was set into the baker containing 250 mL artificial seawater (6.55 \pm 0.26 logCFU/mL). The enumeration and enrichment steps were the same as described in section 2.5.

2.7 Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Duncan test ($p < 0.05$) was applied to compare the average values using SPSS 20.0 (Statistical Package for the Social Sciences).

3 Results and discussion

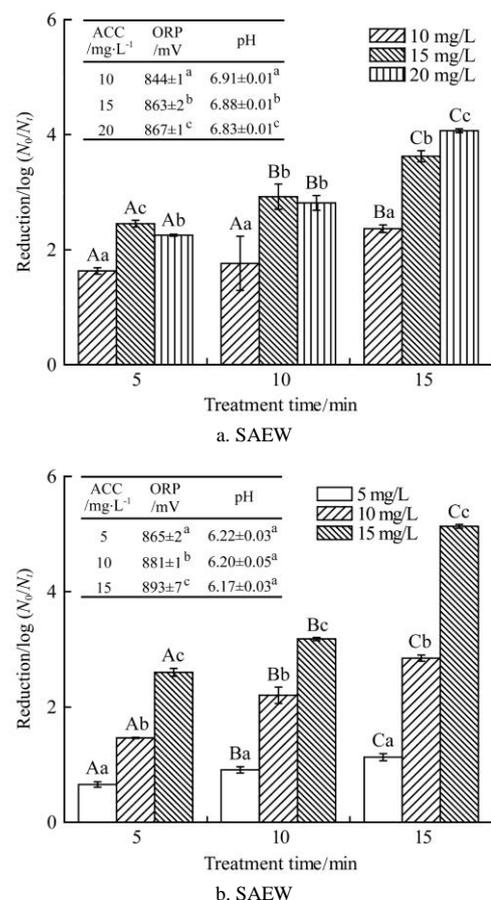
3.1 Inactivation effect of mixed-SAEW and SAEW on *V. parahaemolyticus* in broth

The results in Figure 2 show reduced *V. parahaemolyticus* when being suspended in 3% sodium chloride alkaline peptone water (0.1% peptone) treated by mixed-SAEW and SAEW solutions. The physicochemical properties of mixed-SAEW and SAEW were attached in the figure. The initial concentration of *V. parahaemolyticus* was ~ 8 logCFU/mL.

We can see from Figure 2 that reductions of *V. parahaemolyticus* significantly increased with increasing exposure time (except for 10 mg/L at 5 min and 10 min). The reductions of *V. parahaemolyticus* were 1.63 logCFU/mL, 2.46 logCFU/mL and 2.25 logCFU/mL after 5 min treatment of mixed-SAEW with ACC 10 mg/mL, 15 mg/mL and 20 mg/mL, respectively. It increased to 4.07 logCFU/mL after treating with 20 mg/mL ACC of mixed-SAEW for 15 min. As for samples treated by SAEW, reductions of *V. parahaemolyticus* also significantly increased with increasing treating time and ACC level. It increased from 0.65 \pm 0.05 logCFU/mL at 5 mg/L ACC, 5 min to 5.13 \pm 0.03 logCFU/mL at 15 mg/L ACC, 15 min.

Tables attached in Figure 2 illustrate the properties of mixed-SAEW and SAEW. As the ACC of initially generated SAEW was approximate 30 mg/L, the ACC values of diluted mixed-SAEW and SAEW were ranged from 5 to 20 mg/L. These diluted mixed-SAEW solutions had a pH value ranging between 6.83 and 6.91, and an ORP value ranging from 844 to 867 mV, whereas, it was 6.17 to 6.22 and 865 to 893 mV for diluted SAEW solutions. When ACC was proportionally decreased as a result of dilution by artificial seawater and distilled water, ORP decreased and pH increased but not proportionally. However, the

inactivation effect of SAEW treatment increased with treating time and ACC level, which demonstrated that ACC is the main factor for the inactivation. It was consistent to other studies that the surviving population of microorganisms decreased with the increase of exposure time and ACC level^[9-11]. For mixed-SAEW treated samples, the reduction of *V. parahaemolyticus* had no significant difference between 20 mg/mL ACC, 10 min and 15 mg/mL ACC, 10 min. This may be due to the combination effect of ACC and ORP. Huang et al.^[21] reported that +200 to +800 mV was the best ORP extent for aerobic microorganisms. High ORP could cause the modification of metabolic fluxes and ATP production due to the change in the electron flow in cells^[9]. Therefore, similar reductions were obtained by mixed-SAEW 15 mg/L and 20 mg/L ACC with similar ORP of 863 mV and 867 mV. Whereas, diluted SAEW does not only reduce the levels of ACC but also its physico properties. The value of ORP was the ratio of oxidized to reduced species in solution^[22]. Though the decrease of ORP was not proportional, the dilution did decrease the concentration of oxide in solution and lowered the inactivation effect^[23]. This might be the reason that the reduction differences between 15 mg/L ACC and 20 mg/L ACC were much lower than those between 10 and 15 mg/L ACC, since ORP also plays an important role in inactivation combined with ACC.



Note: ORP and pH of mixed-SAEW and SAEW were attached respectively in the figure and different letters (a, b, c) indicate significant difference ($p < 0.05$) in each column. Vertical bars represent \pm SD, $n=3$. Different letters (a, b, c) above the bars indicate significant difference ($p < 0.05$) between different ACC at the same treating time, while A, B, C indicate significant difference ($p < 0.05$) between different treating times at the same ACC.

Figure 2 Reduction results of *V. parahaemolyticus* treated by mixed-SAEW and SAEW

Comparing between SAEW and mixed-SAEW (with the same ACC of 10 and 15 mg/L), SAEW showed better inactivation effect

than mixed-SAEW. The reductions were 2.85 and 5.14 logCFU/mL after treating 15 min with 10 mg/mL and 15 mg/mL ACC of SAEW respectively, while they were 2.37 logCFU/mL and 3.63 logCFU/mL for the same treating time and ACC of mixed-SAEW. These differences may mainly caused by different pH. *V. parahaemolyticus* is sensitive to acid and its optimal growing pH condition was ranging from 6.5 to 8.5. Due to the mixing with artificial seawater, the pH values of mixed-SAEW (approaching 7) were higher than SAEW (approaching 6). Low pH may sensitize the outer membrane of bacterial cells to the entry of hypochlorous acid into bacterial cells^[24]. On the other hand, the salinity of SAEW was zero, which is an unfavorable growth condition for *V. parahaemolyticus*. Bacteria are more resistance under ideal conditions. This may be the main reason that reductions in SAEW treated samples were higher than those in mixed-SAEW.

The inactivation effect of *V. parahaemolyticus* mainly increased with increasing ACC of both mixed-SAEW and SAEW. Though the inactivation effect of mixed-SAEW was lower than SAEW, it was efficient to inactivate *V. parahaemolyticus* with the presence of organic matter. As an effective, nontoxic and environmentally friendly disinfectant with high bactericidal effect, SAEW has large potential in practical use.

3.2 Inactivation of mixed-SAEW on *V. parahaemolyticus* without organic matter

Although many researchs have revealed the efficiency of SAEW to inactivate pure microb, we need to confirm the effect of mixed-SAEW on pure *V. parahaemolyticus*. Table 2 shows the enrichment results of *V. parahaemolyticus* suspended in 0.85% sodium chloride solution after being treated with different mixed-SAEW at different times, and corresponding physicochemical properties of mixed-SAEW are also shown in the table. These diluted mixed-SAEW solutions had a pH value ranging between 6.90 and 7.08, and an ORP value ranged from 746 to 856 mV.

Table 2 The *Vibrio parahaemolyticus* destruction and physicochemical properties of different mixed-SAEW

ACC/mg L ⁻¹	30 s	60 s	120 s	ORP/mV	pH
20	+ ^A	- ^B	-	856 ±4 ^{aC}	6.90 ±0.03 ^a
15	+	-	-	811 ±3 ^b	7.02 ±0.02 ^b
10	+	+	-	772 ±4 ^c	7.00 ±0.05 ^b
5	+	+	+	746 ±6 ^d	7.08 ±0.03 ^b

Note: ^A Negative for culture without visible growth, means completely inactivated. ^B Positive for culture with visible growth, means not completely inactivated. ^C Different letters (a, b, c, d) indicate significant difference ($p < 0.05$) in each column.

The inactivation trend in *V. parahaemolyticus* under organic free condition was similar to the results shown in section 3.1, which the inactivation effect was increased with increasing treating time and ACC level. After 48 h incubation, positive growth of *V. parahaemolyticus* observed after 2 min treatment of mixed-seawater with 5 mg/L ACC. However, no growth of *V. parahaemolyticus* was observed after 2 min treatment with 10 mg/L ACC. Time for negative growth reduced to 60 s when ACC increased to 15 mg/L and 20 mg/L. This indicates that SAEW was able to completely inactivate *V. parahaemolyticus*, but it had different minimum treating time with different ACC levels. The result was also consistent to the results in section 3.1 that the inactivation of SAEW was much more effective than mixed-SAEW. Negative growth was observed after 30 s under the SAEW

treatment with 5 mg/L of ACC (data not shown). It was due to the higher ORP and lower pH of SAEW than mixed-SAEW (ORP 838 VS 746 mV, pH 6.73 VS 7.08 at 5 mg/mL of ACC).

Quan et al.^[9] detected the minimum ACC of WAEW for bactericidal activity against cell suspensions of *Vibrio vulnificus* and *V. parahaemolyticus*, which was approximately 0.1 mg/L (volume ratio of cell suspension and WAEW was 1:19). However, for cell cultures (cell in the medium), minimum ACC was approximately 20 mg/L. These differences were probably due to different kinds of electrolyzed water and different volume ratios. Basically, it was due to different values of ACC, ORP and pH. These also confirmed the theory that the bactericidal effect of SAEW was the combined effect of ORP, ACC and pH. What's more, even different strains of the same serotype can differ in their resistance towards an intervention^[25].

These results indicated that it took longer time and higher ACC to achieve better inactivation results when *V. parahaemolyticus* was suspended in broth than suspended in 0.85% sodium chloride solution. Organic matter would decrease bactericidal effect of SAEW. Current research reports showed that organic matter could react with free chlorine to generate the combined chlorine with low antiseptic effect, thus reduced the bactericidal effect^[22,26]. The results in this paper were consistent with them. The inactivation effect increased with the increasing contacting time and the absence of organic matter, which is consisted with Issa-Zacharia et al.^[10]

As an effective, nontoxic and environmentally friendly disinfectant with high bactericidal effect, SAEW has large potential in practical use. Therefore, it's necessary to choose an appropriate ACC and treating time to get expected destruction. In the absence of organic matter or low organic matter content, we can choose low ACC electrolysis water or reduce processing time, in order to reduce waste and improve efficiency.

3.3 Inactivation effect of *V. parahaemolyticus* in artificial seawater by SAEW and UV

Preliminary experiment indicated that *V. parahaemolyticus* could survive for a long time in artificial seawater and the reduction was less than 1.5 logCFU/mL after 10 d (data not shown). Previous results showed good inactivation effect of SAEW and mixed-SAEW on *V. parahaemolyticus* suspended in broth. The inactivation time and ACC level could reduce further when *V. parahaemolyticus* was suspended in 0.85% sodium chloride solution. We suppose it may have good sanitization potential as a kind of disinfectant additive to treat seawater.

Table 3 and 4 show the inactivation effect of SAEW and UV on *V. parahaemolyticus* inoculated in artificial seawater, according to the enumeration and enrichment results. The final ACC values of different seawater with ratio of 1:10, 1:15 and 1:25 (V/V) was 3 mg/L, 2 mg/L and 1 mg/L, respectively. When the ratio of SAEW and artificial seawater was 1:25(V/V), average count of *V. parahaemolyticus* enumeration was less than 10 CFU/mL and enrichment broth showed positive growth after 5 min treatment. Survival *V. parahaemolyticus* was detected even after 30 min (data not shown). However, they showed negative growth both on plates and broth after 3 min and 1 min treatment when the ratios were 1:15(V/V) and 1:10(V/V), respectively.

While treated with UV of 10 W, positive growth was observed both on enumeration plates and enrichment tubes after 5 min UV treatment. The results of 15 W UV treatment were similar to SAEW treatment with a ratio of 1:15 that no *V. parahaemolyticus* colonies were formed on TCBS plates after 1 min treatment.

Theoretically the ACC of 1:15 SAEW-seawater solution was approximately 1.9 mg/L. In fact, it would be less than 1.9 mg/L due to the effect of dilution and inactivation, which is in the safe zone of 2.0 mg/L reported by Katayose et al.^[18]

Therefore, with a ratio of 1:15 of SAEW and artificial seawater, SAEW was efficient and safe to inactivate *V. parahaemolyticus* in artificial seawater. It is promising to use SAEW as an additive to treat seawater in aquaculture.

Table 3 Inactivation results of different SAEW ratio on *V. parahaemolyticus* in artificial seawater

Ratio (V/V)	1:25		1:15		1:10	
Time	5 min	1 min	3 min	5 min	1 min	3 min
Survivals /log CFU mL ⁻¹	<1 ^a	<1	0 ^b	0	0	0

Note: ^a verage enumeration of plates was less than 10 CFU/mL and enrichment tubes showed positive growth. ^b egative growth both on plates and tubes.

Table 4 Inactivation results of different UV on *V. parahaemolyticus* in artificial seawater

Power	10 W			15 W		
Time	3 min	5 min	10 min	1 min	3 min	5 min
Survivals /log CFU mL ⁻¹	<1 ^a	<1	0 ^b	<1	0	0

Note: ^a verage enumeration of plates was less than 10 CFU/mL and enrichment tubes showed positive growth. ^b egative growth both on plates and tubes.

4 Conclusions

SAEW has high bactericidal efficacy on *V. parahaemolyticus* and the higher the ACC, the shorter time it takes. The performance of SAEW is promising against *V. parahaemolyticus* inoculated in artificial seawater. With an appropriate ratio about 1:15, SAEW inactivated *V. parahaemolyticus* to undetectable level in artificial seawater. The bacterial destruction effect of SAEW was different at different ACC levels and within or without organic condition. Therefore, we can choose relatively high ACC of SAEW to reduce the time, or under some chlorine rigorous conditions we can use low ACC of SAEW and prolong the inactivating time. More research should be done for the further use of SAEW as an additive in aquaculture industry, for example, the sanitization potential against other foodborne pathogens in aquaculture seawater and efficiency under dynamic hybrid condition.

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