VIBRIO - BACTERIA - CONTROLLING POTENTIAL OF A SEDIMENT BIOELECTROCHEMICAL SYSTEM INTEGRATED IN A TEN-LITER-SCALED BRACKISH AQUACULTURE MODEL - AN INITIAL UPSCALE STUDY

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ABSTRACT

Vibrio bacteria are among the most serious pathogens of aquatic creatures. Furthermore, they can cause diseases at any stage in the lifecycle of aquaculture animals with mortality up to 100%, which results in great economic losses. To control these agents, the currently widely-applied solution is to use antibiotics, which cause environmental pollution and drug resistance in microorganisms. Another solution is biological products, which have not yet been proven highly effective. In this context, there have been studies showing that a promising new solution is to use a sediment bioelectrochemical system (SBES) though only at small scales (<1 L). In this study, we evaluated the *Vibrio*-bacteria-controlling capability of a sediment bioelectrochemical system (SBES) integrated into a 10 L scale model that mimics a water column in a real-life brackish aquaculture pond. The viability of three strains *Vibrio harveyi* NBRC15634 (*Vh*), *Vibrio parahaemolyticus* NBRC12711 (*Vp*), and *Vibrio parahaemolyticus* CED (wild-type, isolated from actual ponds) when exposed to the filtrates obtained from the cathode solution and the anode solution of the system, was determined over time by plate counting on the specific TCBS medium. The results showed that the density of *Vibrio* bacteria did not change when exposed to the filtrate from the cathode but decreased significantly, up to 99% when treated with the filtrate from the anode of SBES, while no similar phenomenon occurred with the control. Even when the two *Vibrio* strains were present simultaneously, the SBES reduced the densities of *Vp* (in CFU/mL) by approximately 2 logs, and completely eliminated *Vh* after 3 hours. In summary, using the SBES will be a potential method to efficiently control pathogenic *Vibrio* bacteria in brackish aquaculture while offering simple implementation.

Keywords: *Sediment bioelectrochemical system, Vibrio harveyi, Vibrio parahaemolyticus.*

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1. INTRODUCTION

Aquaculture brings many benefits to Vietnam, such as creating millions of jobs, providing food, and earning great export value, thereby promoting our economy [1]. Nevertheless, this vital economic sector often faces several challenging issues, such as environmental pollution, inconsistent product quality, and aquatic diseases. One of the most common diseases is vibriosis caused by *Vibrio* bacteria [2]. For example, shrimp vibriosis is a highly destructive disease

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that affects many species of shrimp and is caused by many *Vibrio* species [3].

Vibrio bacteria are Gram-negative, have rod-shaped or comma-shaped cells, and usually inhabit environments with high salinity [4]. *Vibrio* species often interact in shared habitats, leading to *Vibrio* infections with high mortality rates both in the wild and aquaculture systems [5]. Negative changes in environmental factors can trigger the rapid multiplication of pathogenic bacteria already present at low levels in shrimp, leading to disease outbreaks and causing up to 100% production loss [6]. They cause various problems in shrimp during their different life stages. These problems may range from growth retardation to sporadic mortality and, eventually, mass mortality [7].

Among the *Vibrio* species, *Vibrio parahaemolyticus* and *Vibrio harveyi* are serious pathogens responsible for disease outbreaks, which infect from the first days of shrimp stocking [8]. Infection with *V. harveyi* can result in various diseases in shrimp, including red tail syndrome, luminous disease, …[9], while infection with *V. parahaemolyticus* causes acute hepatopancreatic necrosis disease (AHPND) in shrimp. The latter has affected the shrimp farming industry for nearly a decade, resulting in billions of dollars of losses [10].

Several measures have been taken to reduce the presence of *Vibrio* bacteria in aquaculture ponds, such as disinfectants, antibiotics, and microbial products [2]; however, each method has its disadvantages, the most serious of which are low efficiency and environmental pollution. Disinfectants can reduce the density of *Vibrio* bacteria but often cause secondary pollution and can affect the health of fish and shrimp, so they can be usually only applied before or after rearing [11]. Antibiotics are commonly used today, but the abusive use of antibiotics destroys beneficial bacteria in the animals' digestive tracts and the environment. In particular, the overuse of antibiotics leads to the widespread development of antibiotic-resistant bacteria, which becomes a significant threat to humans [12]. *V. harveyi* strains that are multi-resistant to streptomycin, erythromycin, and cotrimoxazole have been found to cause mass mortality in shrimp larvae [7]. In addition, recent studies have shown that most *V. parahaemolyticus* isolated from both wild and aquaculture environments are resistant to many drugs [13]. That negative consequence has led to the proposition for the use of probiotics. However, the reality shows that the improper use of probiotics and the loss of their quality control make this measure ineffective [14]. Therefore, viable, practical, economical, and environmentally safe alternatives are urgently required to control *Vibrio* bacteria in aquaculture.

A promising new solution recently discovered is the use of bioelectrochemical systems (BESs), which are known to have antibacterial capabilities. In bioelectrochemical systems, energy-rich organic substances are often used as electron donors at the anode, where they are degraded by electrochemically active microorganisms; and the released electrons are transferred to the anode and then move through the external circuit to the cathode to be accepted by an electron acceptor, usually oxygen (thereby forming water), thereby completing a redox reaction [15]. Recent studies have shown that a sediment BES works well in brackish pond culture and enhances the ability of *in situ* treatment of organic pollution in the water and the sediment of aquaculture ponds [16]. Another promising application potential of BESs is their ability to treat pathogenic bacteria. Pioneering studies found that the bactericidal effect of BESs might be related to the production of H_2O_2, pH increases, or oxidant residues due to reactions at the cathode [17 – 19]. Subsequently, Ieropoulos *et al.* (2019) [20] discovered an apparent bactericidal effect of the anode medium. This is further demonstrated by the study of Vasieva *et al.* (2019) [21] on the microbial community in the anode of BESs, which showed a substantial reduction in the amount of DNA of common pathogenic bacteria such as *Vibrio*, *Shigella*, *Yersinia*, *Haemophilus*, and *Pseudomonas* bacteria.

The above-mentioned findings are the basis for the idea of integrating BESs in aquaculture

ponds to reduce pathogenic *Vibrio* bacteria. Such integration is practically possible using a sediment BES (SBES), which has a sediment anode and a floating cathode, as demonstrated in previous studies [16], [22]. Such an SBES integrated into a lab-scale brackish aquaculture model displayed an impressive ability of controlling *Vibrio* bacteria at a small scale (< 1 L), eliminating the *Vibrio* bacteria entirely after only 5 minutes; while the control did not have such an ability. We also discovered that such an effect was solely due to the SBES aqueous solution, rather than physical activities or biological contacts. In order to be able to apply such a promising application potential in practice, in this study, we have built and operated an integrated model of a 10-liter-scaled bio-electrochemical system with a sediment electrode and a height of 1.5 m, mimicking a water column in a brackish water aquaculture pond. We then evaluated the inhibitory ability of SBES against several reference strains of *V. harveyi* and *V. parahaemolyticus*, as well as a wild-type strain of *V. parahaemolyticus* isolated from nature. The results of this study could be the basis for an improved technique to control *Vibrio* bacteria in aquaculture.

2. MATERIALS AND METHODS

2.1. Microbial strains and growth media

In this study, the strains *Vibrio harveyi* NBRC15634 (abbreviated as Vh), *V. parahaemolyticus* NBRC12711 (abbreviated as Vp) (purchased from NBRC, Japan) and *V. parahaemolyticus* CED (abbreviated as Vp CED) (wild type, isolated from shrimp ponds, provided by Research Institute for Aquaculture No.1) were used as representatives of the respective species in the experiments.

The selective thiosulfate-citrate-bile salts-sucrose (TCBS) medium was used for counting the *Vibrio* bacteria. For culturing and maintaining *Vibrio*, Lubria-Bertani (LB) medium was used, with an exceptional NaCl concentration of 3% (w/v) (a concentration previously verified by the team to be the most favorable for the growth of the *Vibrio* strains used).

2.2. Tank models and operation

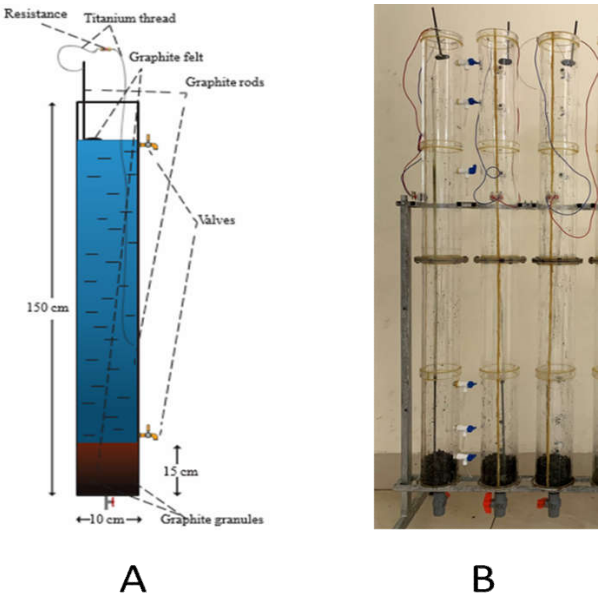

Figure 1. Drawing (A) and actual setups (B) of the column tank model integrated with an SBES in this study

Note: The control tank model is identical but not harboring the SBES components (graphite granules and graphite felt as the anode at the sediment, graphite rods, graphite felt cathode, external resistor, and wires).

Six cylindrical plexiglass columns, each of which is 150 cm high and has a 10 cm diameter, were used as aquaculture tank models (Figure 1A): three identical experimental columns with integrated SBESs (T_C1, T_C2, T_C3) for replication, three identical control columns without SBESs (C_C1, C_C2, C_C3) for replication. The installation of the SBES in each experimental tank was similar to that in previous studies by the group [16], [22]. An SBES generally consists of an anode electrode at the bottom of the column and a cathode electrode at the water's surface. The anode consists of a 10 cm thick layer of graphite particles (2 - 5 mm in diameter) (Xilong, China) evenly covering an underneath 8 cm diameter graphite cloth (0.5 cm thick) (Xilong, China) at the bottom of the tank. A 4 cm diameter graphite felt of the same type was used as the floating cathode (~50% in contact with water and ~50% with air as it floats). A graphite rod (5 mm diameter) was glued to the graphite felt of the electrode to collect the electrical current. The graphite rods of the anode and the cathode were connected by a titanium wire (having a negligible resistance) to an external circuit containing 10 Ω resistors to form a closed circuit. The graphite rod part of the anode and the titanium wire immersed in water were covered with inert acrylic tubing to prevent short circuits. Each test column (integrated with an SBES) has valves in positions corresponding to the anode and cathode for sampling and mounting the reference electrode (Figure 1A). Each model column was filled with 10 L of artificial brackish water (15‰ salinity) generated using Marinium Reef sea salt (Mariscience International Co. Ltd., Thailand).

To enrich electrochemically active bacteria in each SBES, the sediment of the respective test tank was inoculated with 300 g of a mixture of sediment mud samples collected in Quang Ninh province, Vietnam and from an operating SBES in the laboratory. Both experiment and control setups were operated under the conditions mimicking those when culturing 30-day-old white leg shrimp, with brackish water having a salinity ~15‰, and the amount of feed provided was 0.1 g/day (calculated as the equivalent of uneaten daily feed residue (~50% of total feed input) in actual aquaculture ponds, based on our previous calculations) [22]. The systems were operated at room temperature (27 ± 3°C) and pH = 7.

A real-time digital multimeter (model 2700, Keithley, USA) measured the voltage between the anode and the cathode of the SBES installed in each test setup. Electrical parameters (current I(A), voltage U(V)) were measured as previously reported [22]. pH of the water near the anode or the cathode was measured with a pen type digital pH meter (model pH-107, Total Meter Services Inc., Canada) during the operation.

2.3. Preparing bacterial suspensions for experiments, counting bacteria during experiments, and calculating their survival rates

Each *Vibrio* bacteria suspension was prepared by: (i) shaking incubation of the respective test strain in 3% NaCl LB broth for 4 hours, at 200 rpm (at 30°C for Vh or 37°C for Vp and Vp CED); (ii) harvesting the cells from that liquid culture by centrifugation (at ca. 4000 x g, 4°C, for 5 min.); and finally suspending the obtained cell pellet in an appropriate volume of artificial brackish water (having a 1.5% salinity, prepared as described above) so that the cell densities in the final suspension was approximately $1x10^7$ CFU/mL for Vp; approximately $1x10^9$ CFU/mL for both Vh and Vp CED.

The cell density of the tested *Vibrio* strain in any sample of interest was quantified by diluting the sample with sterile artificial brackish water (prepared as described above), plating on TCBS agar, incubating the plates (at 30°C for Vh and 37°C for Vp, Vp CED) and counting the colonies (usually after 24 hours). From the counting results, the cell density of each sample (in CFU/mL) was calculated using standard formulas [4].

2.4. Testing the inhibitory effect of the SBES aqueous filtrate on single *Vibrio* strains

A 100 µL volume of cell suspension containing approximately $1x10^7$ CFU/mL for Vp or approximately $1x10^9$ CFU/mL for both Vh and Vp

CED (prepared as described above) was added to 1.5 mL of a filtrate of interest. Therefore, the cell densities were reduced down to $6.25x10^5$ CFU/mL for Vp and $6.25x10^7$ CFU/mL for Vh, Vp CED. These are the densities that were actually tested. These cell densities are at the levels causing lethality for shrimp, which is at least 10^5 CFU/mL for Vp and 10^7 CFU/mL for Vh [23]. The filtrates of interest include those from the aqueous solutions at the cathode or the anode, in both the experimental and the control setups. For the control, the upper position near the water surface is considered to be equivalent to the cathode position, and the position at the bottom of the column sludge is considered to be equivalent to the anode position. To prepare a filtrate, the respective aquaeous solution was taken and filtered with a 0.22 µm filter (to remove microorganisms). We only tested the filtrates because our previous study results (at a scale of < 1 L) showed that the inhibitory effect on *Vibrio* was mainly due to the chemical components in the system fluid, rather than physical factors and direct biological contacts (Nhung, Phuong, *et al.*, manuscript to be accepted). Hereafter, the filtrates from the cathode and the anode of the experimental setups integrated with SBESs and the respective ones of the control are called the test/control cathode/anode filtrates, respectively. *Vibrio* survival in the samples was determined (as shown above) after 5 min, 30 min, 90 min, and 180 min upon the addition of the tested cell suspension.

2.5. Testing the inhibitory effect of the SBES aqueous filtrate on Vp and Vh when they were co-present

100 µL of cell suspension containing approximately $1x10^7$ CFU/mL of Vp and 100 µL of cell suspension containing approximately $1x10^9$ CFU/mL of Vh (prepared as described above) were added to 1.5 mL of a filtrate of interest. The survival of each strain in the samples was determined (as shown above) at 5 min, 30 min, 90 min, and 180 min.

3. RESULTS AND DISCUSSION

3.1. Installation and operation of SBES

Based on the design (Figure 1A), we successfully built six 10-litre column tanks, including three test models with an integrated SBES system and three control models without SBES.

The SBESs installed in the test column models (T_C1, T_C2, T_C3) started to generate electricity from the first days of operation (as described above). However, the currents gradually increased and only stabilized (about 2.5 mA) after 45 days (Figure 2). Regarding our previous studies on SBES, such electricity assures that electroactive bacteria were enriched in our SBESs and that the SBESs were functioning [22].

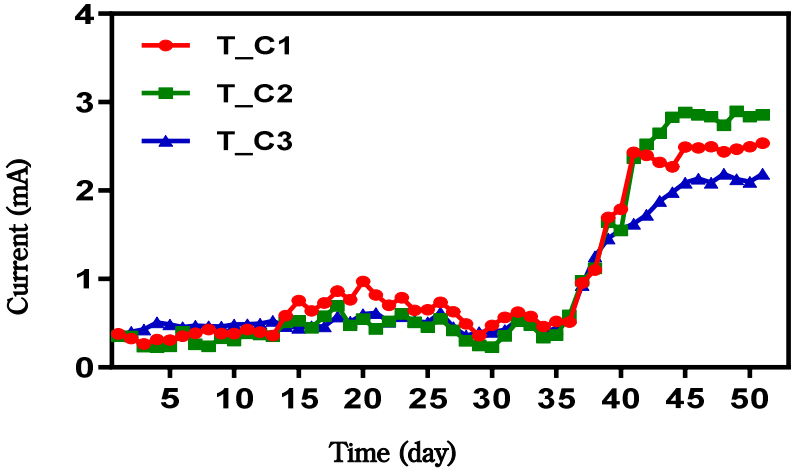

Figure 2. Electricity generation by the SBESs integrated in the test column models

3.2. Effect of the SBES cathode filtrate on the viability of Vh, Vp, and Vp CED

Arends *et al.* (2014) [17], Gajda *et al.* (2020) [18] reported that the pH change in a biochemical system has a certain inhibitory effect on pathogenic bacteria in the cathode compartment. In addition, the study of Lu *et al.* (2012) [19] showed that the electrochemical system is capable of generating H_2O_2, a potent oxidizing agent produced at the cathode, which is toxic to bacterial cells. Therefore, we tested the inhibition of the cathode aqueous filtrate on the *Vibrio* strains.

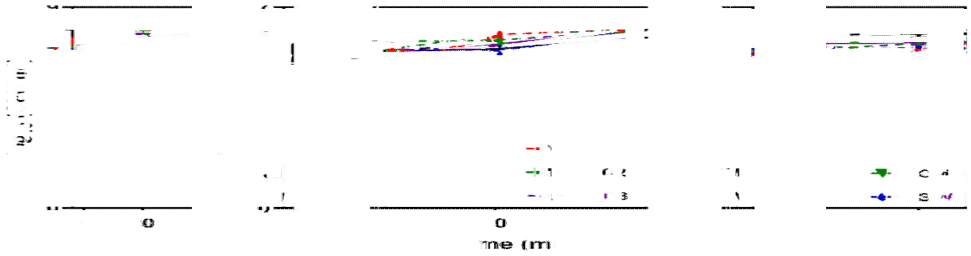

Figure 3. Effect of the SBES cathode filtrate on the viability of the *Vibrio* strains

Note: A: Vh, B: Vp, C: Vp CED; T_C1, T_C2, T_C3: filtrate samples from the 3 respective test models; CM: a mixture of the "cathode" filtrate from the 3 control models (C_C1, C_C2, C_C3); BW: artificial brackish water only (abiotic control).

The results (Figure 3) showed that, for all three tested strains, the bacterial density did not decrease after 180 min in direct contact with the cathode filtrates from both the test setups containing the SBESs and the controls. Even for Vh, the bacterial densities in all cases increased by more than 1 log (logarithm), which shows that Vh still can grow in the cathode filtrate. Thus, it can be seen that the cathode filtrate does not inhibit *Vibrio* bacteria.

Although the studies by Arend *el al.* (2014), Gajda *el al.* (2020), and Lu *el al.* (2012) showed an apparent bactericidal effect by H_2O_2 or high pH at the cathode, such an effect by the cathode was not strong [17 – 19]. In our study, we could not measure H_2O_2 so whether it is involved in the SBES effect is still questioned. Furthermore, the pH of the aqueous filtrates of both the test setup (even near the electrodes) and the controls did not change significantly during operation (Figure 4). Those phenomena may explain why in our study, the aqueous solutions at the cathode side of SBES could not inhibit *Vibro*. Probably, our SBES is at a much larger scale (compared to those in previous studies) and thus the amount of generated H^+ or H_2O_2 may be too small to have any significant effect on the bulk medium in the system.

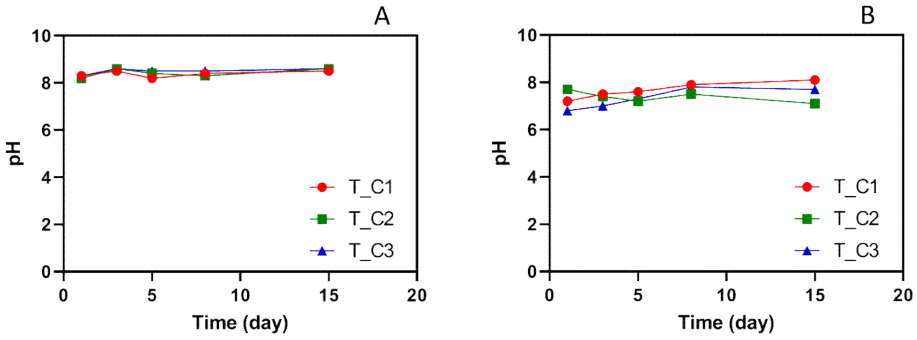

Figure 4. pH of SBES

Note: A: pH in cathode, B: pH in anode.

3.3. Effect of the SBES anode filtrate on the viability of Vh, Vp and Vp CED

Several research groups have previously reported that the reduced redox potential at the anode can induce the formation of redox agents capable of inhibiting the metabolic activity of pathogenic bacteria [23, 24]. Therefore, we tested the viability of *Vibrio* bacteria when exposed to the SBES anode filtrate.

The obtained results (Figure 5) showed that when exposed to the anode filtrates of the test setups having the SBESs, the cell densities of the *Vibrio* strains decreased over time, while they seemed to survive well in the "anode" filtrate (from the bottom position) of the control. Specifically, the Vh densities decreased after 180 min by 2.5 log, 1.6 log, and 2.6 log (equivalent to 99%), respectively, when exposed to the anode filtrates of T_C1, T_C2, and T_C3 (Figure 5A). The ability to inhibit Vp of T_C3 anode filtrate seemed to be weaker (with only 0.6 log reduction) compared to that of Vh; however, when Vp exposed to T_C1 and T_C2, this ability is strong, with the cell densities decreased by 1.6 log and 1.7 log (equivalent to 99%) (Figure 5B). For Vp CED, a wild-type strain isolated from actual ponds, which is generally more resistant, the cell density of this bacterium in the anode filtrate of T_C2 was not significantly reduced. However, it slightly decreased in T_C1 (0.8 log) and decreased by 90% in T_C3 (1.3 log) (Figure 5C). These results generally demonstrate an evident inhibition of the SBES aqueous environment against *Vibrio* bacteria.

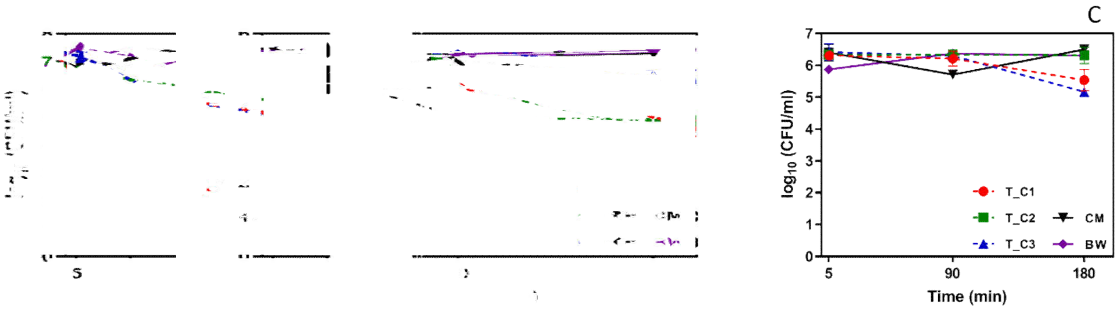

Figure 5. Effect of the SBES anode filtrates on the viability of the *Vibrio* strains

Note: A: Vh, B: Vp, C: Vp CED; T_C1, T_C2, T_C3: filtrate samples from the 3 respective test models; CM: a mixture of the "anode" filtrate from the 3 control models (C_C1, C_C2, C_C3); BW: artificial brackish water only (abiotic control)

In a previous study, Ieropoulos *et al.* (2019) [20], Ieropoulos *et al.* (2017) [24] hypothesized that the formation of redox agents reduces redox capacity at the anode and, at the same time, may create an inhospitable environment for pathogenic bacteria. Accordingly, the anode medium of their BES could strongly reduce the number of bacteria *Salmonella enteritidis* (up to 10 - 100 times) [24], *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (100 - 1000 times on average) [20]. This bactericidal ability is further demonstrated by the research results of Vasieva *et al.* (2019) [21], which showed a sharp decrease in the proportion of DNA of common pathogenic bacteria such as *Shigella*, *Yersinia*, *Vibrio*, *Haemophilus*, and *Pseudomonas* in the metagenome of all bacteria in the anode medium of the studied BESs. In addition, to create biofilms, electrochemical bacteria such as *Pseudomonas aeruginosa* are capable of producing mediators such as the antibiotic pyocyanin, which can kill other competing bacteria [25]. Those are the possible explanations for the inhibitory effect on *Vibrio* bacteria of the SBES anode filtrate.

One may notice that in some experiments, the three replicates of SBES-containing columns did not always display the same inhibitory activities against the *Vibrio* bacteria (sometimes one of

them did not show a clear inhibitory activity). We still can not explain this observation but according to previous studies, sometimes a slight change in the internal resistance (due to disconnection or micro-environments in such large volumes) may lead to unstable performance of BESs [15]. Such a change can even significantly change the redox potential of the system, which may severely affect the bactericidal effect of the SBES, as mentioned above [24]. In this study, we were not be able to check the internal resistances and the redox potentials of our SBESs, and thus further tests are needed to evaluate the stability of the system. Nevertheless, in general, at least 2 of 3 experimental models always displayed significant inhibitory activities against the *Vibrio* strains, which supports our hypothesis that the SBES does have bactericidal effects on *Vibrio* bacteria.

3.4. Effect of the SBES anode filtrate on the viability of Vh and Vp when they are co-present

Different species of *Vibrio* may be found to co-infect on the same host leading to more severe infections, or they may co-occur during vibriosis outbreaks [26]. The question is whether this coexistence makes them more resistant. Therefore, we tested the viability of Vh and Vp when they were present simultaneously and in contact with the SBES anode filtrate.

The results showed that the anode filtrates of all three test models could still inhibit Vh and Vp when they co-existed, while they seemed to live well in the control "anode" filtrate and the abiotic control solution (artificial brackish water) (Figure 6). Noticeably, in this experiment, the inhibition of Vh was more substantial than in the above-mentioned experiment when it was tested individually. Specifically, Vh cells were completely eliminated after 180 minutes in all cases when tested with the SBES anode filtrates. The inhibition was less for the Vp strain; however, it was still comparable to those in the individual strain test, with the bacterial densities significantly reduced by 1.2 log, 2.2 log, and 1.9 log when exposed to the T_C1, T_C2, and T_C3 filtrates, respectively.

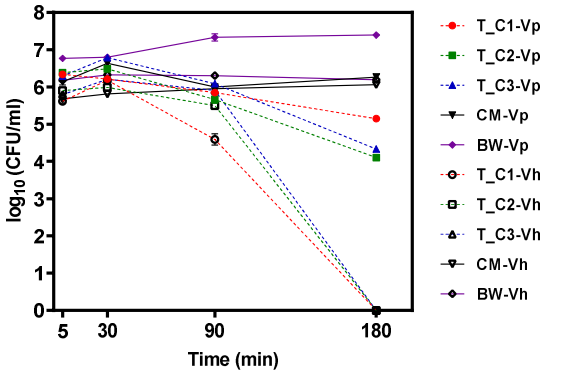

Figure 6. Effect of the SBES anode filtrate on the viability of Vh and Vp when they are co-present

Note: T_C1, T_C2, T_C3: filtrate samples from the 3 respective test models; CM: a mixture of the "anode" filtrate from the 3 control models (C_C1, C_C2, C_C3); BW: artificial brackish water only (abiotic control).

Most previous studies reported that different *Vibrio* species often occur together in habitats, such as *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. cholerae*, … [27]. However, there is little research interest in the co-infection of aquatic animals by different *Vibrio* species, although this is common. During co-infection, interactions between infectious agents lead to different outcomes: the virulence of one or both pathogens may be increased, one or both may be inhibited, or one can be favored while the other is inhibited [28]. Thus, interactions between concomitant pathogens can be supportive or antagonistic. The supportive interactions can lead to increased agent resistance [28]. In our study, the inhibition of the SBES to Vh in the co-presence of Vp was even stronger than that when the strains were tested alone. Thus, the interactions between the *Vibrio* species (if any), at least for the strains in this study, do not seem to affect the ability of SBES to inhibit these bacteria.

4. CONCLUSION

With the potential ability to inhibit some representative strains of *V. harveyi* and *V. parahaemolyticus* in our study, SBES can be a promising new technology in aquaculture to control pathogenic *Vibrio* bacteria. Integrating

SBESs in aquaculture ponds to combat *Vibrio* diseases would also be a potential alternative to using antibiotics and other chemicals, which are currently not supported in many countries. This offers the prospect of reducing the burden of aquaculture water treatment and enables longer operations of the ponds. Thus, the application of SBES should be considered in aquaculture to ensure more economical operation and sustainability. Obviously, further studies in actual conditions are needed to realize this fascinating prospect.

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