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Low-amperage pulsating direct current has a bactericidal effect on marine fish pathogens in circulating seawater

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ABSTRACT

The bactericidal effect of pulsating direct current (PC) generated at a very low amperage (0.01 A) with a frequency of 5 Hz or 14 kHz against two marine fish pathogens, *Edwardsiella tarda* and *Vibrio parahaemolyticus*, in circulating seawater at 15 and 25 °C in comparison with the effect of direct current (DC) of the same amperage was investigated. The bactericidal effect was directly correlated with the generation of active chlorine species (ACS) and the treatment duration. PC treatment at 14 kHz resulted in complete bacterial inactivation when the ACS level reached 0.11–0.12 mg/L after 45–60 min of treatment. PC treatment at 5 Hz required generation of only 0.03–0.07 mg/L ACS to achieve complete bacterial inactivation, although a slightly longer treatment duration (60–90 min) was needed. DC treatment resulted in complete disinfection within a shorter time (30 min) due to greater ACS production. The bactericidal effect and ACS generation were weaker at the higher temperature (25 °C) due to more rapid evaporation of Cl_2 gas. The pH of the seawater maintained at ~8.0. A disinfection study in circulating non-chloride Na_2SO_4 solution at pH 8.0 showed that the electric pulsation did not have notable bactericidal effect up to 14 kHz at 0.2 A.

1. Introduction

Fish pathogenic bacteria cause considerable damage to the aquaculture worldwide (Morohoshi, Inaba, Kato, Kanai, & Ikeda, 2004). *Edwardsiella tarda*, a gram-negative fish pathogen belonging to Enterobacteriaceae, infects not only marine and freshwater fishes but also many types of animals, such as reptiles, birds, and mammals, including humans (Swain & Nayak, 2003). *Vibrio parahaemolyticus*, a gram-negative marine and estuarine fish pathogen often found in oysters, shrimp, mussels, and many marine invertebrates worldwide, is of great concern as it causes acute foodborne gastroenteritis (Bej et al., 1999; Wong, Chen, Liu, & Liu, 1999). Effective disinfection of such fish pathogens is not only an important issue in the seafood industry, markets, and restaurants, where fishes are stored or transported in seawater tanks, but is also a key element in aquaculture, particularly the case in indoor fish farming, where the recirculation of seawater is often required to reduce seawater usage (to minimize environmental damage around farm sites) and enhance nutrient recycling (Martins et al., 2010; Turcios & Papenbrock, 2014).

Electrochemical disinfection is an effective seawater disinfection technology (Bullock et al., 1997; Jorquera, Valencia, Eguchi, Katayose, & Riquelme, 2002; Katayose, Yoshida, Achiwa, & Eguchi, 2007; Liltved & Cripps, 1999; Park et al., 2003, 2004; Rowan, MacGregor, Anderson, Cameron, & Farish, 2001; Tamplin & Capers, 1992; Urano, Ishikawa, & Fukuzaki, 2006). Electrochemical treatment of seawater with direct current (DC) results in production of diverse bactericidal factors. Active chlorine species (ACS) with high oxidation-reduction potential (ORP) are the primary bactericidal components: dissolved chlorine gas (Cl_2) generated by electrolysis at the anode, hypochlorous acid (HOCl) produced by the hydrolysis of Cl_2, and hypochlorite anions (OCl^-)

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formed by the dissociation of HOCl at pH > 5.0 (Jeong, Kim, Cho, Choi, & Yoon, 2007; Kim et al., 2006; Park et al., 2004; Urano et al., 2006). Short-lived reactive oxygen species (ROS) with high ORP, including the hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2), and oxidized halogen compounds other than ACS, such as hypobromous acid (HOBr) and hypobromite anions (OBr^-), which could be produced in trace amounts during the electrochemical treatment of seawater, were also suggested to be partly responsible for the bactericidal effect (Jeong et al., 2007; Kerwick, Reddy, Holt, & Chamberlain, 2005; Urano et al., 2006).

A major challenge in the application of electrochemical seawater disinfection for fish storage tanks or indoor fish farms is control of the ACS at low levels that result in sufficient disinfection, but do not exert harmful effects on humans or marine animals. It was suggested that the generation of ACS due to seawater electrolysis must not exceed 2.0 mg/L several hours after the disinfection process, and that for immediate use of seawater following disinfection, the ACS level must be <1.0 mg/L (Katayose et al., 2007). Our previous study showed that pulsating direct current (PC), a DC varying in intensity at regular intervals, was more useful than normal DC for control of ACS at an appropriately low level during the electrochemical disinfection of circulating brine solution (Pudtikajorn, Shin, Jeong, & Chung, 2009). This is because varying both the amperage and the frequency of the current pulse allows for greater control of the input of electric current. However, little is known about the bactericidal effect of PC on marine fish pathogens in seawater, especially in circulating systems widely used for fish storage or indoor fish farming; e.g. ACS concentrations and treatment durations required for complete bacterial inactivation and the bactericidal effect of electric current pulsation itself.

The objective of this study was to investigate *E. tarda* and *V. parahaemolyticus* inactivation in circulating seawater treated with PC generated at a very low amperage (0.01 A) in comparison to that by DC of the same amperage, and examine the effects of current pulse frequency and temperature on bacterial inactivation. Changes in the level of ACS, pH, and ORP were monitored, and the existence of bactericidal factors other than ACS was explored by examining inactivation in a circulating non-chloride solution at the pH of seawater (8.0).

2. Materials and methods

2.1. Materials

Natural seawater was collected from the seashore of Gangneung Province, Korea. Sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, and sodium sulfate were purchased from Showa Chemical Co., Ltd. (Tokyo, Japan). Sodium thiosulfate was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Brain-heart infusion (BHI) medium and bacto-agar were purchased from Becton, Dickinson and Co. (Sparks, MD, USA). Solid BHI agar was prepared and sterilized according to the manufacturer's instructions.

2.2. Cultivation of marine pathogens and preparation of inocula

E. tarda KCTC 12267 and *V. parahaemolyticus* ATCC 17802, obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea), were separately inoculated into 10 mL of BHI broth containing 3% (w/v) NaCl, followed by incubation at 37 °C with shaking at 250 rpm overnight. The pre-cultures were separately transferred to 100 mL of the same medium and incubated under the same conditions overnight. Bacterial cells were harvested by centrifugation at 8000*g* and 4 °C for 10 min, washed twice with filtered phosphate-buffered saline (PBS; pH 7.4), and then washed with sterile 3% (w/v) NaCl. The collected cells were resuspended in 10 mL of sterile 3% (w/v) NaCl to prepare inocula for disinfection experiments.

2.3. Electrochemical inactivation procedure

A laboratory-scale circulating electrochemical disinfection system previously constructed by our group (Pudtikajorn et al., 2009) was used in this study (Fig. 1). Natural seawater was filtered using a 0.45 μm mixed-cellulose-ester filter membrane (Advantec Toyo Kaisha, Ltd., Tokyo, Japan), and 20 L of the filtered seawater were decanted into a 40 L aquarium. No or very few viable cells were observed in the filtered seawater. The seawater was pumped out, transferred into an electrolytic cell, and then returned to the aquarium using a magnetic drive pump (Iwaki Co., Tokyo, Japan) at a flow rate of 4 L/min. A rectangular-shaped, non-membrane-type electrolytic cell containing two platinum-coated titanium electrodes (17 cm × 5.6 cm × 1.0 mm) 8 mm apart was provided by T.M.D. Co. (Pusan, Korea). The effective volume capacity of the electrolytic cell was 75 mL. The temperature of circulating seawater in the aquarium was set at 15 or 25 °C using a coiled water circulation tube connected to a heated/refrigerated circulator (RW-3025G, Jeio Tech Co., Ltd., Seoul, Korea). After seawater temperature equilibration, an appropriate volume of cell suspension was transferred into the aquarium to a final viable cell concentration of approximately 10^5 colony forming units (CFU)/mL. The inoculated seawater was circulated for 30 min before applying the electric current to ensure a homogeneous distribution of cells. Then, a rectangular PC of 0.01 A was applied to the electrolytic cell at a frequency of 5 Hz or 14 kHz. A current pulse with a 50% duty cycle was generated using a condenser of 2.2 μF capacity. A DC of 0.01 A was used for comparison. A volume of 100 mL of the seawater was sampled every 15 min for 2 h to enumerate viable cells. To investigate the existence of bactericidal factors other than ACS, bacterial inactivation was examined in circulating Na_2SO_4 (250 mM) at pH 8.0 and 15 °C with the following four electrical treatments: two PC treatments (PC/0.2 A/14 kHz and PC/0.02 A/14 kHz) and two DC treatments (DC/0.2 A and DC/0.02 A).

2.4. Enumeration of viable bacterial cells

A volume of 0.2 mL of 0.1 N sodium thiosulfate was added to seawater immediately after sampling (100 mL), and the mixture was stirred for 15 s to neutralize any remaining ACS (Urano et al., 2006). About 3 mL of the mixture were transferred to the sample cup of a spiral plater (Spiral Biotech, Norwood, MA, USA) and plated on BHI agar (1.5% agar with 3% NaCl). The plates were incubated at 37 °C for 24 h, and then colonies were counted.

2.5. Determination of ACS, pH, and ORP

Changes in the ACS concentration, pH, and ORP of the circulating seawater were monitored in separate experiments. The ACS concentration was determined using a Hach DPD (*N*,*N*-diethyl-*p*-phenylenediamine)-FEAS (ferrous ethylenediammonium sulfate) reagent set and digital titrator (Hach Co., Loveland, CO, USA) and expressed as the equivalent concentration of dissolved Cl_2 (mg/L as Cl_2). pH and ORP were measured using a digital pH-ORP meter equipped with a SenTix ORP electrode (Inolab level 1, WTW Co., Weilheim, Germany).

2.6. Statistical analysis

All the experiments were performed in at least triplicate, and all measurements were repeated at least three times. Data were