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Escherichia coli Inactivation Using Pressurized Carbon Dioxide as an Innovative Method for Water Disinfection

Tsuyoshi Imai and Thanh-Loc Thi Dang

Abstract

Advanced water disinfection technologies that do not produce harmful by-products would be highly desirable. This study presents results for the use of pressurized carbon dioxide (CO_2) and a liquid-film-forming apparatus for disinfection of seawater. The sensitivity of *Escherichia coli* to the pressurized CO_2 was examined for various conditions of pressure, temperature, working volume ratios (WVRs), flow rates, and pressure cycling. Morphology of *E. coli* was observed by using scanning electron microscopy (SEM). A strong correlation between the *E. coli* inactivation efficiency and pressure cycling was detected ($p < 0.001$). The frequency and magnitude of pressure cycling were the key factors responsible for high rates of *E. coli* inactivation during the pressurized CO_2 treatment. The results from linear regression analyses suggest that the model can explain about 91% of the *E. coli* inactivation efficiency ($p < 0.001$). The pressurized CO_2 treatment (at 0.7 MPa, 20°C, 50% WVR) in the process involving pressure cycling ($\Delta P = 0.12$ MPa, 15 cycles) resulted in complete inactivation (5.2 log reduction) of *E. coli* within 3 min. These findings suggest that pressurized CO_2 could be a potentially useful disinfection method for water treatment.

Keywords: bactericidal performance, *Escherichia coli*, inactivation effect, pressurized carbon dioxide, water disinfection

1. Introduction

For more than a century, chlorination has been the most common method used worldwide for drinking water disinfection. Chlorine and chlorine-based compounds are widely used for the control of waterborne pathogens because of their high oxidizing potential, low cost, and

residual disinfectant properties that prevent microbial recontamination. Unfortunately, the chemical reaction between chlorine and organic compounds in water generates carcinogenic agents such as trihalomethanes and halogenic acetic acids [1, 2]. Furthermore, some resistant microorganisms may only be inactivated with very high chlorine doses, which can exacerbate the formation of disinfection by-products (DBPs) [3]. Presently, growing concerns about the potential hazards associated with DBPs have boosted efforts to develop chlorination alternatives. Ozonation is effective at inhibiting a variety of pathogens; however, its disadvantages include the high cost and the potential formation of DBPs such as bromate in seawater [4, 5]. Other water treatment methods such as ultraviolet (UV) radiation, ultrasound, cavitation, or heat application can be used for the inactivation of organisms. Although these methods do not produce DBPs or other problematic chemical residues, they require substantial energy consumption and have high operational costs [5]. Besides, the efficiency of UV disinfection is greatly dependent on water quality because the activity of UV light is substantially decreased by turbidity or organic matter present in water [5].

Sterilization by using pressurized CO_2 has been an active research field for decades [6, 7]. CO_2 has been used extensively to sterilize dried food and liquid products via a nonthermal sterilization method [8] because of its effectiveness in inactivating microbes, nontoxicity, and low cost [9]. Prior research on high-pressure CO_2 treatments has investigated the effects of several factors such as pressure, temperature, type of microorganisms, agitation speed, decompression rate, and pressure cycling on the inactivation capacity of this method [6, 8, 10–15]. Most studies have reported that high-pressure operating conditions (4–50 MPa) are required to inactivate significant numbers of pathogens [7, 9]. Subsequently, certain concerns involving high-pressure operations (i.e., the need for heavy-duty pressure equipment, high initial investment costs, energy consumption concerns, and pressure control and management issues) have hampered the implementation of high pressurized CO_2 preservation technology at a large scale within the food industry.

In recent years, pressurized CO_2 has shown great potential as a sustainable disinfection technology in water and wastewater treatment applications [16–22] largely because this method does not generate DBPs [9, 22]. Kobayashi et al. [16, 17] employed CO_2 microbubbles in the treatment of drinking water and succeeded in inhibiting *Escherichia coli* within 13.3 min. However, the pressure (10 MPa) and temperature (35–55°C) requirements for effective inactivation [16, 17] are still high from a practical standpoint. Our research group has developed a novel method that uses low-pressure CO_2 treatments (0.2–1.0 MPa) based on technology that produces high amounts of dissolved gas in water to inactive bacteria and bacteriophages in freshwater [19–21] and seawater [23, 24]. Cheng et al. [19] suggested that the sudden discharge and resulting reduction of pressure could cause cells to rupture via a mechanical mechanism, and further, that this would be lethal to cells at high levels of dissolved CO_2 at 0.3–0.6 MPa and room temperature. Vo et al. [20, 21] demonstrated that acidified water and cellular lipid extraction caused by pressurized CO_2 at 0.7 MPa and room temperature were major factors for efficient disinfection within a treatment time of 25 min.

Previous research has shown that pressure cycling is a potential means to improve bacterial inactivation during pressurized CO_2 treatments [8–10, 13, 15]; nevertheless, the inactivation

mechanism is still unknown for this process. Pressure cycling is defined as a repetitive procedure that involves the decompression and compression of CO_2 [9, 10]. Evidence so far suggests that the decompression process may lead to mechanically induced explosive cell ruptures [14], while the compression process may intensify the mass transfer of CO_2 across cell membranes [11–13]. In previous works, the pressure cycling procedure has been conducted with high-pressure operations (8–550 MPa) and with CO_2 discharges between each cycle of decompression and compression [8, 10, 13, 15]. Despite the good bactericidal performance of pressurized CO_2 technology enhanced by pressure cycling [11–13, 15], the high pressure and CO_2 release requirements are drawbacks owing to the costly and complex operating procedures. Presently, it is not clear whether pressure cycling with low-pressure CO_2 treatments (<1.0 MPa) will enhance the bactericidal activity. Therefore, in this study, we examined the effect of pressure cycling on the bactericidal performance of CO_2 at low pressures and with no release of CO_2 between each cycle of raised/lowered pressure.

This study investigated the use of pressurized CO_2 at less than 1.0 MPa for seawater disinfection applications such as ballast water treatment. Comparisons of *E. coli* inactivation caused by pressurized CO_2 and pressurized air were evaluated in both natural seawater and artificial seawater. The inactivation performance of pressurized CO_2 against *E. coli* was examined for various conditions of pressure, temperature, flow rates, and working volume ratios (WVRs). In particular, the influence of pressure cycling on *E. coli* inactivation was evaluated. Changes in cell morphology after pressurized CO_2 treatment were assessed by scanning electron microscopy (SEM). The research objective was to evaluate the bactericidal effectiveness of pressurized CO_2 for disinfecting water, with the goal of addressing the abovementioned emerging problems associated with water disinfection technology.

2. Novel idea: apparatus for forming highly dissolved gas in water

For pressurized CO_2 methods in the field of food preservation, the interaction efficiency between CO_2 and pathogens in the foodstuffs is probably limited at low pressures and ambient temperatures, and consequently, high-pressure (4–50 MPa) or ultra-high-pressure (200–700 MPa) conditions are vital for sufficient inactivation. However, to be more attractive in terms of its economic feasibility, pressurized CO_2 technology needs to be implemented at lower pressures. In this study, we employed the use of a liquid-film-forming apparatus, which enabled improvements in the interaction efficiency but with lower pressures (<1 MPa) for the water disinfection purposes.

The experimental apparatus for disinfection was a stainless steel chamber with an internal volume of 10 L and pressure tolerance up to 1.0 MPa. The device was designed with a solid stream nozzle and shield to enable vigorous agitation of the influent in such a way that produced liquid films along with fine bubbles (**Figures 1–3**). The device was supplemented with CO_2 pressure prior to the treatments. Sample water was then pumped into the device at high speed through a small nozzle and directed onto the shield. The highly pressurized fluid stream thus collided with the bubble-generating shield. Subsequently, numerous gas bubbles,
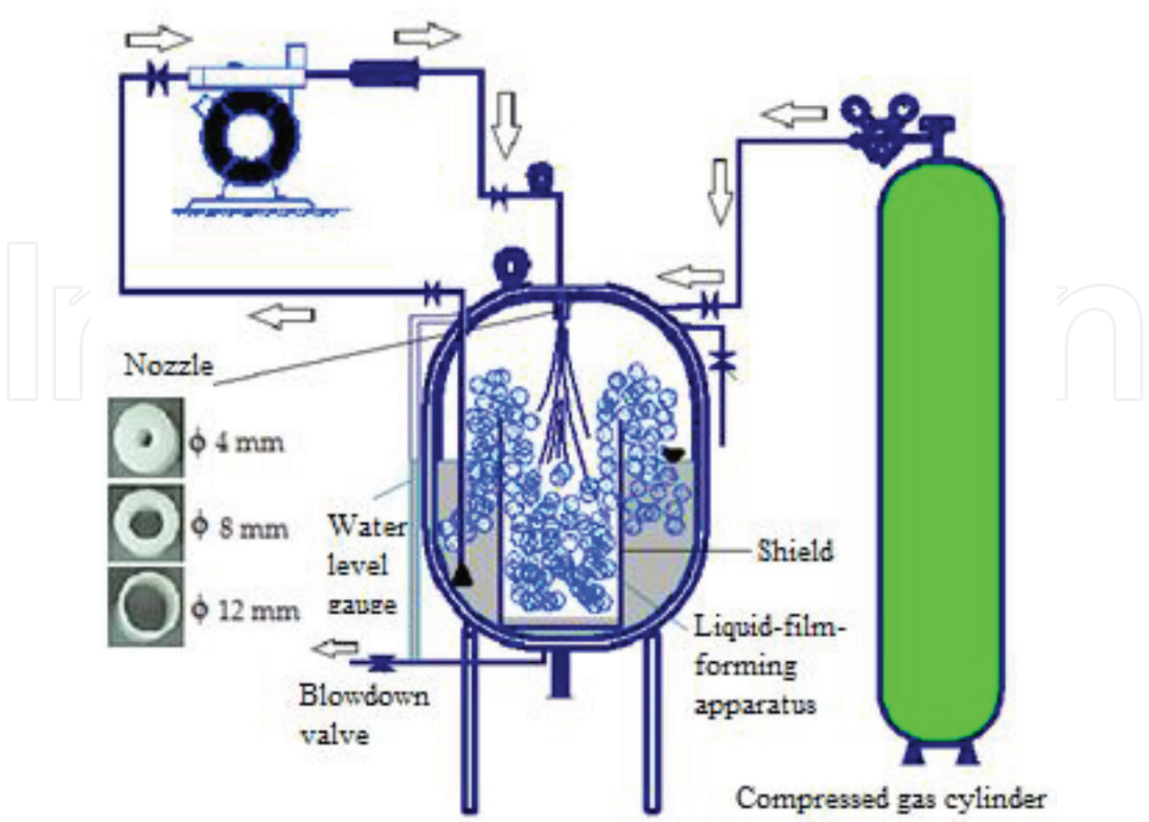
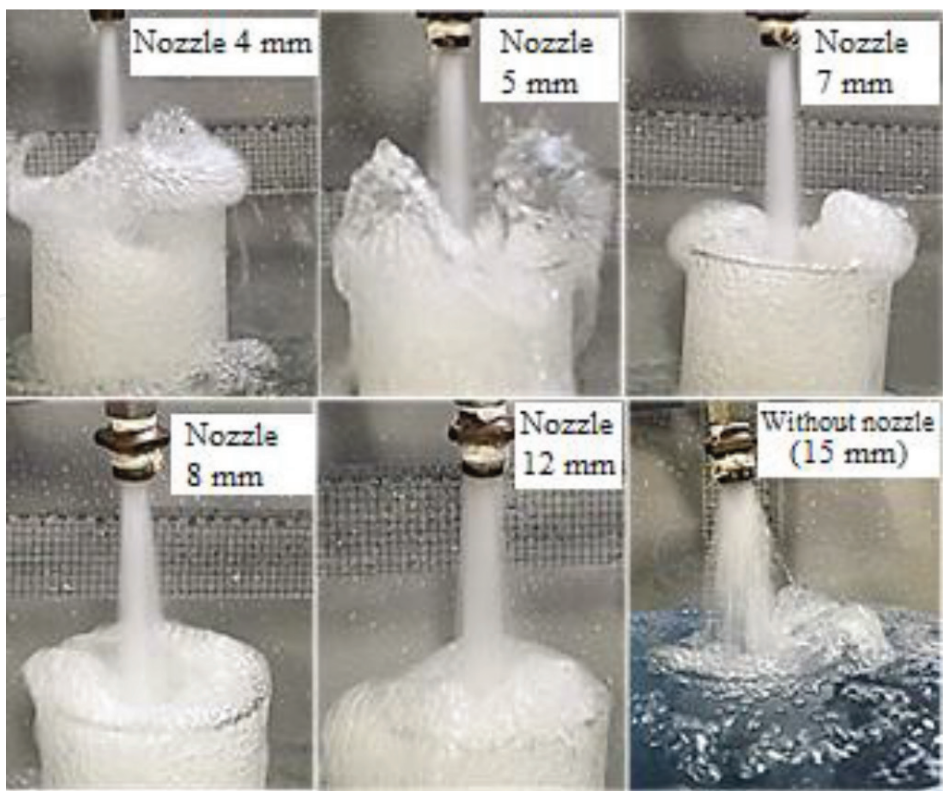**Figure 1.** Apparatus for forming highly dissolved gas in water.

Figure 2. Representative pictures of liquid film formation with various nozzle diameters at a normal pressure in the pipeline.
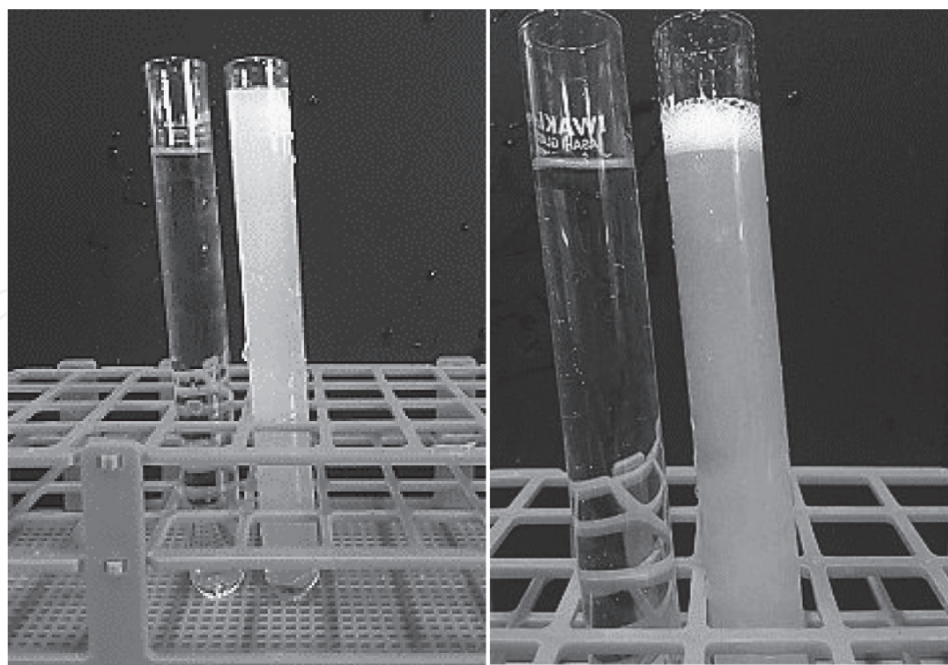
Figure 3. Pictures of an untreated sample and a CO_2-treated sample (the latter contains many small bubbles).

which were generated from inside the shield, were entrained by the ascending bubbles and overcame the shield; these bubbles then floated into the main chamber (outside the shield). Hence, CO_2 transfers took place both within the interior and exterior sides of the thin liquid films. The presence of numerous small bubbles also enhanced the contact area between gas and water and facilitated CO_2 dissolution into water. We hypothesized that the available interfacial contact area between CO_2 and the cell suspension was greatly multiplied in this setup and that the CO_2 transfer efficiency was high. Despite the lower pressures used, the high contact efficiency promoted by this apparatus enabled ample penetration of CO_2 into the cell membranes of *E. coli*.

3. Materials and methods

3.1. Microorganism preparation and enumeration

Stock cultures of *E. coli* (ATCC 11303) were propagated in Luria-Bertani (LB) broth (Wako Chemical Co., Ltd., Osaka, Japan) containing 30 g L^{-1} sodium chloride and incubated for 24 h at 37°C by using a reciprocal shaker set to rotate at 150 rpm. The initial enumeration was approximately 10^9–10^{10} CFU mL^{-1}. The permanent stock was maintained in 20% glycerol at −80°C.

The *E. coli* inoculum for each disinfection experiment was prepared by inoculating 100 µL of bacterial glycerol stock into 100 mL of LB broth containing 30 g L^{-1} sodium chloride. The culture was then incubated for 20 h at 37°C with continuous shaking at 150 rpm. Cells were harvested and washed three times with a 0.9% (w/v) saline solution followed by centrifugation (10 min at 8000 g at room temperature) in a CF15D2 centrifuge (Hitachi, Japan). The pellet was re-suspended in 100 mL saline solution.

E. coli were enumerated by using the plate count technique. Briefly, the samples were diluted into a series of 10-fold dilutions by using autoclaved artificial seawater at 3.4% salinity, and 100 μL of either a diluted or an undiluted sample was plated on LB agar (Wako). For samples with a low number of viable cells, 1 mL of the undiluted sample was poured into agar maintained at 45°C. Colonies growing on each plate were counted after incubating the plates overnight at 37°C. Each sample was analyzed in triplicate.

3.2. Seawater sample preparation

The artificial seawater was prepared by adding artificial sea salt (GEX Inc., Osaka, Japan) to distilled water to obtain a final salinity of 3.4%, as measured with a salinity meter (YK-31SA, Lutron Electronic Enterprice Co., Ltd., Taiwan). As for the preparation of filtered natural seawater, natural seawater (pH = 8.3, salinity 3.3%) was first filtered through a glass fiber filter (GA-100, Advantec, Toyo); then, the seawater was filtered through a membrane filter with a pore size of 0.45 μm (Millipore, Ireland). For all experiments, prepared *E. coli* cultures were added into the artificial/filtered seawater to obtain a bacterial concentration of 5–6 $\log_{10}$ CFU mL^{-1}. The solution was stirred for 30 min to acclimatize the bacteria before starting the experiments. For each batch mode operation, 12 L of samples were prepared, of which 4–5 L were used to restart the system. The pH and temperature of samples were measured with a pH meter (Horiba D-51, Japan).

3.3. Experimental setup

Disinfection experiments were conducted in batch mode (**Figure 4**). Sample water, as the influent, was pumped in one shot into the device. Following the first influx of water, pressurized CO_2 was also injected into the main chamber. System pressure was adjusted by a gas pressure regulator and gas exhaust valve. The fluid was then circulated by pumping inside the system for 25 min. A pump was used to apply a higher pressure than that inside the main chamber to accelerate gas solubilization in water. During the treatment period, the outer wall of the device was kept in contact with cool water by using a water jacket to maintain the initial temperature of the sample at ±1.0°C. The treated water was then collected from a bottom valve of the device.

3.4. Procedure for disinfection experiments

3.4.1. Experimental procedure for investigating the effects of pressure and temperature

To investigate the effects of pressure and temperature, 7 L of sample were pumped into the main chamber by using a 0.2 kW pump (Iwaya-WPT-202), and the fluid was circulated inside the system at a flow rate of 14 L min^{-1} (hydraulic retention time, HRT = 0.5 min). The pump was used to apply 0.12 MPa higher pressure than that inside the main chamber. The sensitivity of bacteria to pressurized CO_2 treatments under different conditions was determined by varying the CO_2 pressure (0.2–0.9 MPa) and seawater temperature (11–28°C) for a 25-min treatment period [23]. Each experiment was conducted in triplicate.
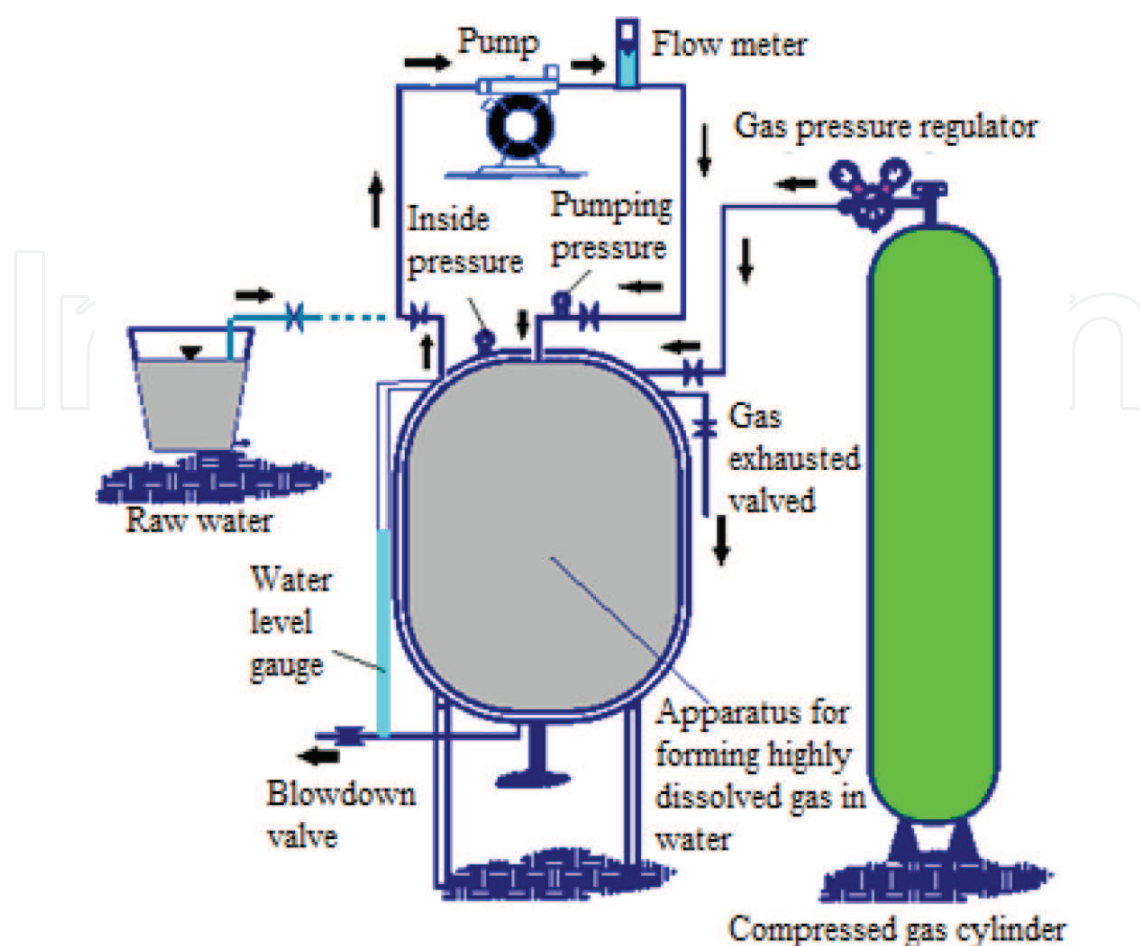
Figure 4. Setup of the water treatment apparatus.

3.4.2. Experimental procedure for investigating the effect of pressure cycling

In previous works, the pressure cycling procedure was conducted with high-pressure operations (8–550 MPa) and with CO_2 discharges between each cycle of decompression and compression [8, 10, 13, 15]. However, such high pressure and CO_2 release are undesirable from an economic standpoint. In order to overcome the above disadvantages, in the present study, we employed a process involving pressure cycling for *E. coli* inactivation but used lower pressures (<1 MPa) and no discharge of CO_2 between each cycle of raised and lowered pressure.

To investigate the effect of pressure cycling, two pumps (0.20 kW, Iwaya-WPT-202, Japan; 0.75 kW, 32 mm × 32 mm SUP-324 M, Toshiba, Japan) and nozzles with various sizes (15 mm height × 4–8 mm diameter) were used to change the flow rate and pressure power of the input (a treatment without a nozzle was also used, whereby the diameter of the pipeline inlet was 15 mm). Pumping pressure and system pressure were measured by pressure gages. The pressure difference ΔP = pumping pressure (MPa) − pressure inside the main chamber (MPa). The water flow rate was measured by a flow meter (GPI, Nippon Flow Cell Co., Ltd., Japan). The recycle number was calculated in relation to the treatment time and HRT, wherein HRT = sample volume/flow rate.

3.4.3. Experimental procedure for investigating the effect of the working volume ratio

The WVR is defined as the ratio between the sample volume and apparatus volume. To examine the effect of WVR, different sample volumes (5, 6, 7, and 8 L) were used to vary the sample volume ratios (50, 60, 70, and 80%). The experiment was conducted with the following two flow rate levels: 14 and 25 L min^{-1}. The water level was measured by using a gauge to evaluate the effect of WVR on the bubble-generating shield inside the main chamber. The HRT and recycle number were calculated as described in Section 3.4.2.

3.5. Scanning electron microscopy

Changes in cell morphology after pressurized CO_2 treatment were assessed by using SEM. The pellets of *E. coli* were immobilized with 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 3 h at 4°C and then rinsed with PBS three times. Next, the samples were soaked in 1.0% osmium tetroxide in cacodylate buffer for 90 min and then washed three times with cacodylate buffer for removal of the fixative. After fixation, the cells were dehydrated by consecutive soaking in increasing concentrations of ethanol solutions (50, 70, 80, 90, 95, and 100%), and this was followed by an ethanol/t-butyl alcohol (v/v = 1:1) treatment for 30 min. The prepared cells were then soaked in t-butyl alcohol two times for 1 h, freeze-dried for 2 h, and sputter coated with gold-palladium. Finally, the cells were examined by using a scanning electron microscope (QuantaTM 3D, FEI Co., USA) at 20 kV [23].

3.6. Statistical analysis

The statistical analysis was done by using the statistical computer program R (version 3.2.2, available at http://cran.R-project.org). Multicollinearity regression was performed to evaluate statistically significant variables of the system with a significance level of 0.05. Predicted values of inactivation efficacy were based on the following first-order regression model:

$$y_i = \beta_0 + \sum \beta_i x_i \qquad (1)$$

where y_i represents the predicted responses, x_i is a parameter, β_0 is the model intercept, and β_i is the linear coefficient.

4. Results and discussion

4.1. Bactericidal performance of pressurized CO_2 and pressurized air against *E. coli* in seawater

Bactericidal effects of pressurized CO_2 in comparison with pressurized air against **E. coli** in seawater were investigated at three pressure conditions (0.3, 0.7, and 0.9 MPa) and at 20 ± 1°C (**Figure 5**). In general, the disinfection efficiency of the pressurized CO_2 treatment was not different between filtered seawater and artificial seawater. At every operating pressure, the *E. coli* inactivation efficiency of pressurized CO_2 was always higher than that of pressurized air.
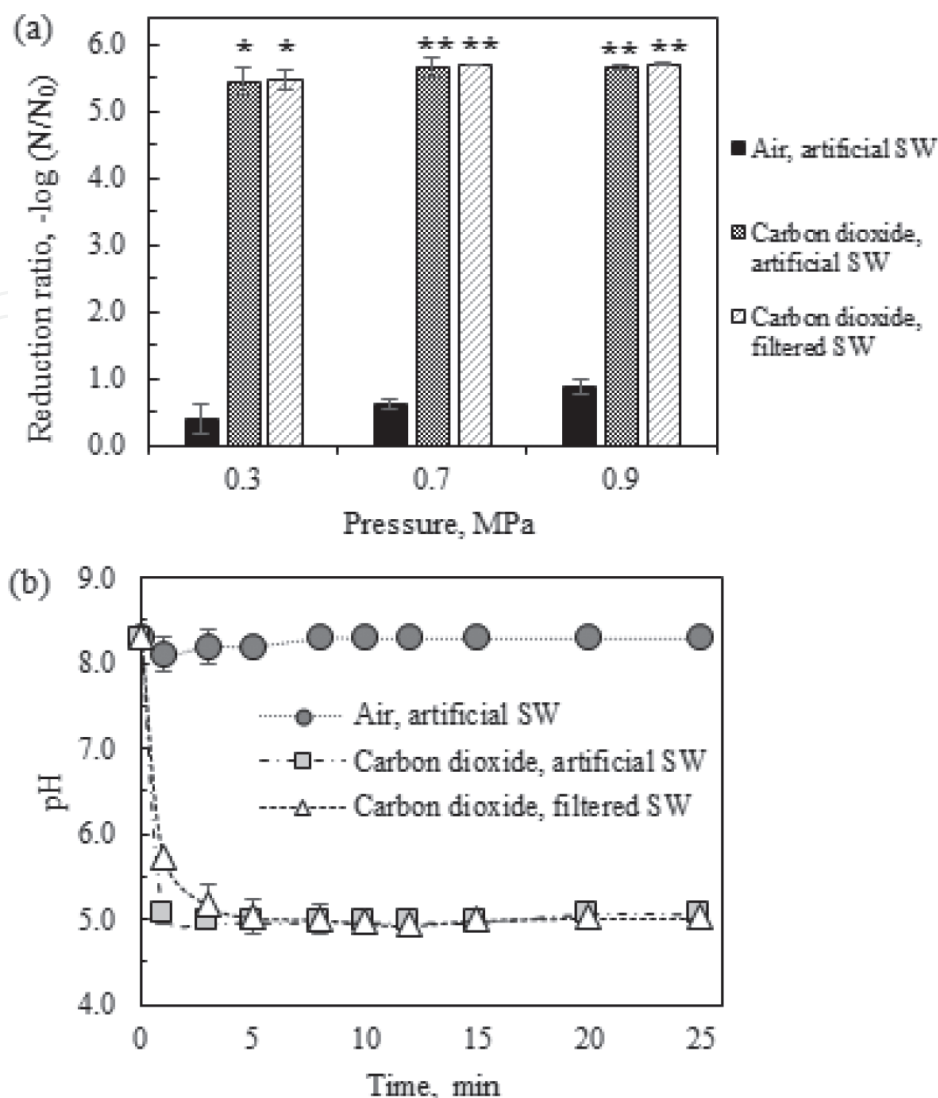
Figure 5. Effect of pressurized CO_2 and pressurized air on (a) *E. coli* inactivation and (b) the pH of seawater (SW). Operating conditions: 0.3–0.9 MPa, 20 ± 1°C, and a working volume ratio (WVR) of 70%. Asterisks (*) and (**) indicate that the *E. coli* load was completely inactivated after 25 and 10 min, respectively.

Approximately 5.4–5.7 log reductions of the *E. coli* load were achieved within 10–25 min by the pressurized CO_2 treatment (this involved complete inactivation of bacterial cells), whereas only 0.4–0.9 log reductions were achieved after 25 min by the pressurized air treatment; these tests involved pressures of 0.3–0.9 MPa (**Figure 5a**).

Pressurized CO_2 reduced the pH of both filtered seawater and artificial seawater to around 5.0 after the first few minutes of exposure time, whereas the pH of pressurized air-treated seawater remained around 8.3 during the treatment period (**Figure 5b**). It has been hypothesized that the decrease in pH caused by pressurized CO_2 is probably a major factor driving the bacterial inactivation process [12, 20, 21, 25]. However, Dang et al. [24] demonstrated that the low pH alone is not the main cause of the bactericidal activity. Perhaps with the concomitant presence of pressure and dissolved CO_2, the low pH prompted the *E. coli* cells to become more permeable, thereby stimulating the process of CO_2 penetration into the cells [24].

4.2. Effects of pressure and temperature

E. coli was disinfected in various pressure conditions (0.2–0.9 MPa) at 20°C (**Figure 6**). In general, *E. coli* inactivation significantly increased with increasing pressure, and higher pressures required shorter exposure times to achieve the same log reduction. For example, a treatment application period of 25 min was required to reduce the *E. coli* load by approximately 5.0 log with pressure applications of 0.2–0.4 MPa, whereas pressure applications of 0.5 and 0.6 MPa resulted in a reduction of the treatment period to 20 and 15 min, respectively. The treatment period was further reduced to 10 min with pressure applications of 0.7–0.9 MPa. However, the increased pressure application from 0.7 to 0.9 MPa did not result in significant increase in the of bacterial inactivation. These data indicated that the optimal CO_2 pressure for inactivating *E. coli* was in the range of 0.7–0.9 MPa, and hence, 0.7 MPa was chosen as the optimal pressure condition for effective bactericidal activity [23].

The disinfection efficiency of pressurized CO_2 substantially increased with increasing temperatures (11–28°C) at 0.7 MPa (**Figure 7**). The *E. coli* load was reduced by more than 5.0 log within 25 min of treatment at 11°C, whereas only 20, 12, and 10 min of pressurized CO_2 treatment at 15, 18, and 20–28°C, respectively, were required to reduce the *E. coli* load to a similar extent [23]. Taken together, these findings suggest that *E. coli* inactivation by pressurized CO_2 could be efficiently conducted at low-pressure (0.7 MPa) and ambient temperature
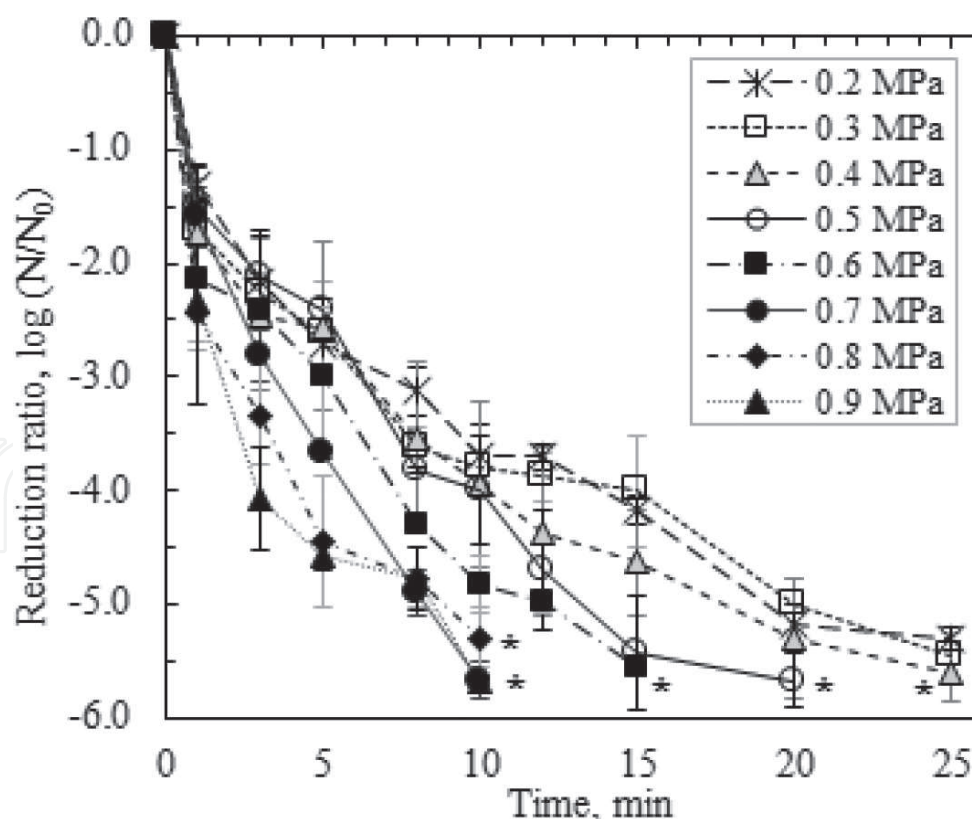

Figure 6. Effect of pressure on *E. coli* inactivation during the pressurized CO_2 treatment at 20 ± 1.0°C and a working volume ratio (WVR) of 70% [23]. Asterisks (*) indicate that no colonies were detected.
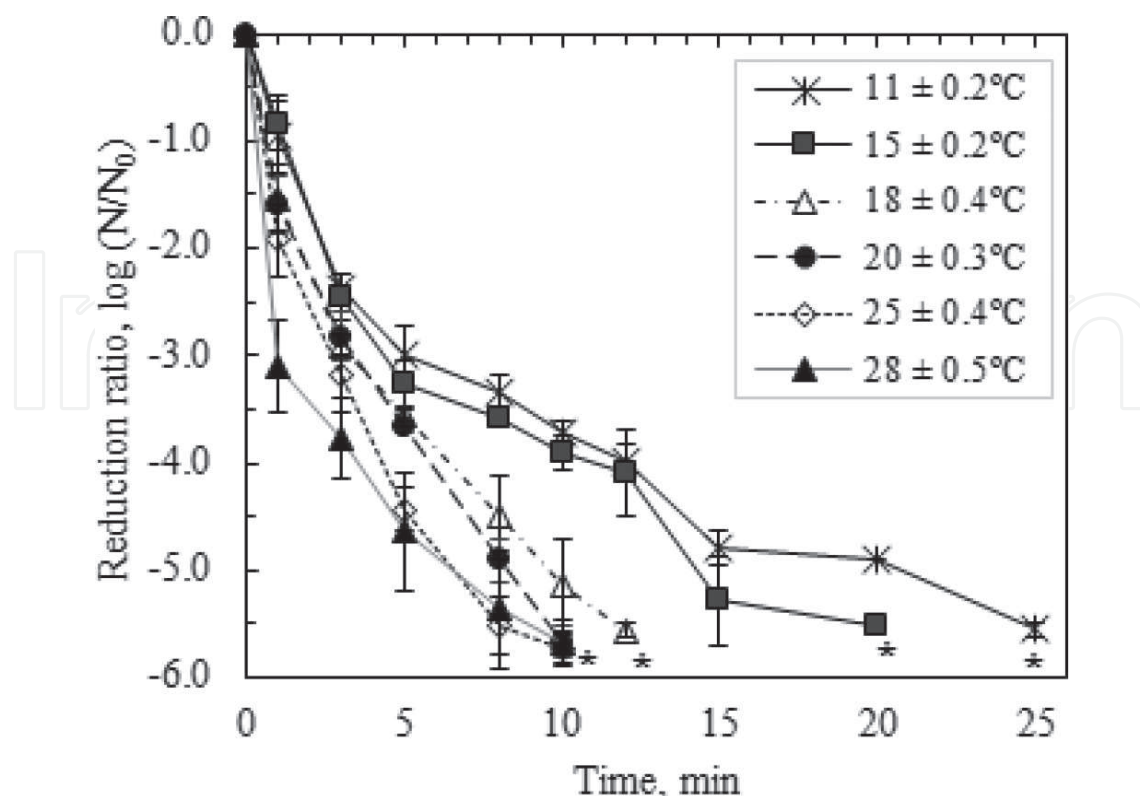
Figure 7. Inactivation of *E. coli* in seawater at various temperatures by using the pressurized CO_2 treatment at 0.7 MPa and a working volume ratio (WVR) of 70% [23]. Asterisks (*) indicate that no colonies were detected.

conditions. On the other hand, after disinfection and decompression, the pressurized CO_2-treated samples were placed at normal conditions to assess the viability of the remaining bacteria. After the 5-d holding period, the number of *E. coli* in the treated samples had not increased, i.e., no regrowth of bacteria was observed.

CO_2 is lipo-hydrophilic in nature, and it can easily penetrate into the phospholipid bilayer of cell membranes [26]. Thus, the increase in CO_2 pressure and temperature may stimulate the diffusion of CO_2 into cells and may increase the fluidity of cell membranes [11, 27]. In the present study, the solubility of CO_2 into seawater was considerably improved by using the liquid-film-forming apparatus. Hence, we speculate that simultaneous effects of pressure, temperature, and high efficiency of contact with this apparatus may have stimulated the process of CO_2 penetration into *E. coli* cells, thereby accelerating the efficiency of the pressurized CO_2 treatment [23].

4.3. Effect of pressure cycling

4.3.1. Effect of pressure cycling at various pump powers and nozzle diameters

The effect of pressure cycling on *E. coli* inactivation was investigated by using various nozzle diameters (4–8 mm) (a treatment without a nozzle was also tested, where the diameter of

the pipeline inlet was 15 mm) and two pump powers (0.20 and 0.75 kW) to change both the flow rate and ΔP of the input. The disinfection experiments were conducted under 0.7 MPa of pressurized CO_2 at $20 \pm 1°C$ with a WVR of 70% for a duration of 25 min (**Figure 8**). In general, larger nozzle diameters led to higher flow rates (**Figure 8c**) and faster fluid recycling in the treatment system (**Figure 8d**). In contrast, increases in the nozzle diameter reduced the pressure difference ΔP (**Figure 8c**). Furthermore, at the same nozzle diameter, stronger pumping powers improved not only the flow rate but also the pressure difference ΔP of the input (**Figure 8c**). At every nozzle diameter, operation of the pump with 0.75 kW of power (**Figure 8b**) yielded greater inactivation efficiencies than those with 0.20 kW of power (**Figure 8a**).
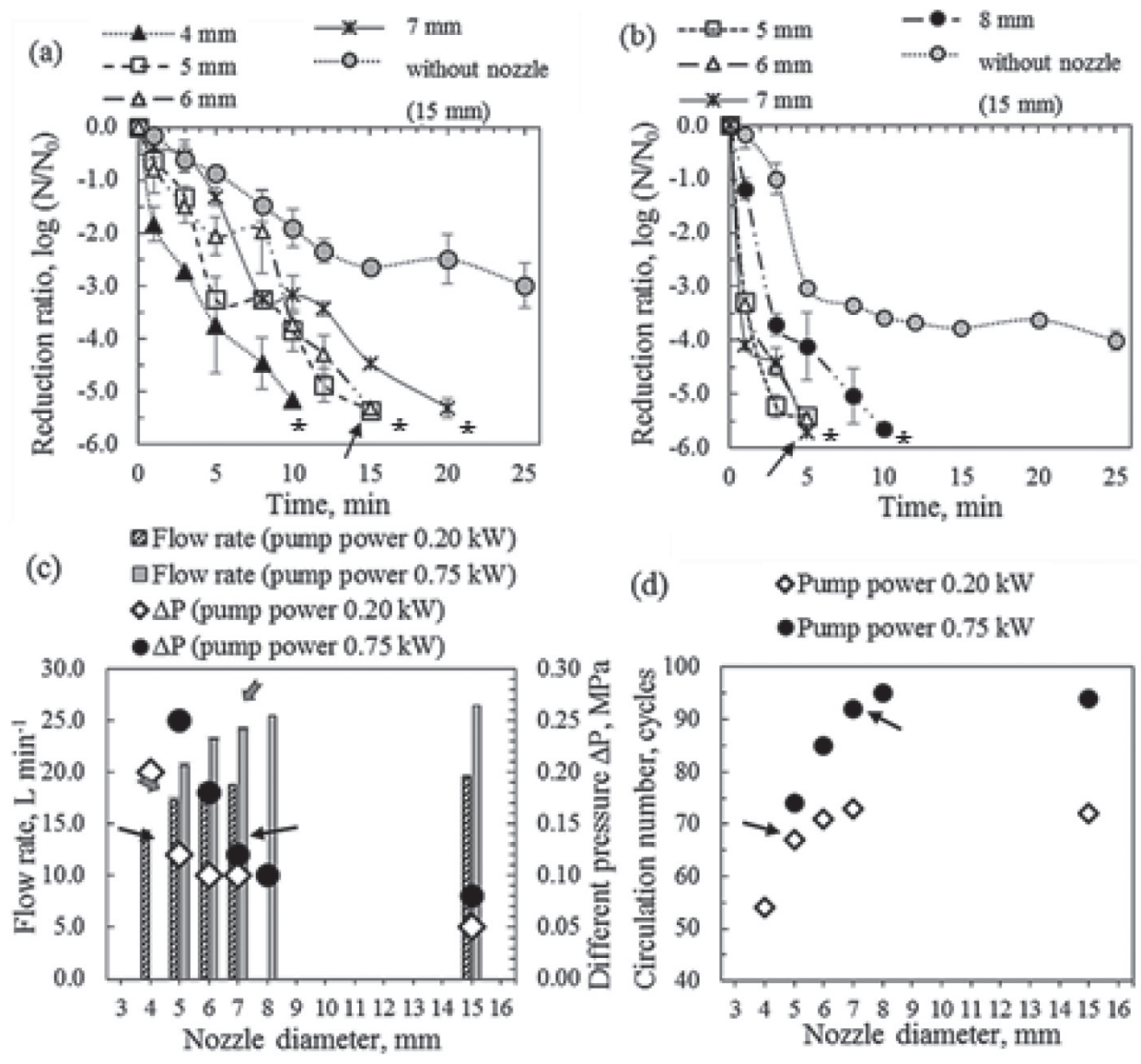

Figure 8. Effect of pressure cycling on the inactivation of *E. coli* in seawater. Effect of (a) 0.20 kW pump power and (b) 0.75 kW pump power along with various nozzle diameters on the inactivation with pressurized CO_2. Influence of different pump powers and nozzle diameters on the (c) flow rate and pressure difference ΔP, and (d) the circulation number. Operating conditions: 0.7 MPa, $20 \pm 1°C$, and a working volume ratio (WVR) of 70% within a duration of 25 min. Asterisks (*) indicate that no colonies were detected.

It is hypothesized that pressure cycling enhances the inactivation efficiency by facilitating the mass transfer of CO_2 into bacterial cell membranes [9, 10]. Thus, an increase in water flow rate can be expected to improve the *E. coli* inactivation. However, our results show that the *E. coli* inactivation efficiency did not increase with higher flow rates or faster recirculation. When 0.20 kW of pumping power was used (**Figure 8a**), the length of treatment periods required for complete inactivation of the *E. coli* load by more than 5.0 log increased with the greater nozzle sizes (i.e., 10 min with the 4-mm nozzle, 15 min with the 5–6-mm nozzles, and 20 min with the 7-mm nozzle, which corresponded to flow rates of 14, 17–19, and 19 L min^{-1}, respectively). Furthermore, the reduction in *E. coli* load was only 3.0 log after 25 min when the device was operated without a nozzle (flow rate = 20 L min^{-1}). A similar finding was found when the pump was operated at 0.75 kW of power (**Figure 8b**); at the higher power, more than a 5.0 log reduction was achieved within 5 min with the 5-mm nozzle (flow rate = 21 L min^{-1}), whereas only a 4.0 log reduction was obtained after 25 min in the treatment lacking a nozzle (flow rate = 26 L min^{-1}). These results indicate that the bactericidal performance of pressurized CO_2 associated with pressure cycling can probably not be attributed to the flow rate alone.

On the other hand, the disinfection efficiency substantially increased with the higher ΔP (**Figure 8**). A 5.4 log reduction in *E. coli* load was achieved within 5 min by the treatment with a ΔP of 0.25 MPa, whereas only a 3.0 log reduction was attained after 25 min by the treatment with a ΔP of 0.05 MPa. When operating the device with the same pump power, as noted above, a larger nozzle diameter resulted in higher water flow rates but weaker ΔP values. Hence, the reduction of ΔP may be considered as a key reason for the phenomenon of low inactivation efficiency at high flow rates. This suggests that the disinfection effect of pressure cycling might be influenced by not only by the frequency of circulation but also by the ΔP.

Noticeably, at the same ΔP value, a faster frequency of circulation substantially augmented the *E. coli* inactivation efficiency (**Figure 8**). For instance, at the same ΔP of 0.12 MPa (generated by a 5-mm nozzle and 0.20 kW pump, and a 7-mm nozzle and 0.75 kW pump), the periods required for complete inactivation of *E. coli* were reduced from 15 to 5 min when the frequency of pressure cycling was raised from 67 cycles/25 min to 92 cycles/25 min, respectively. A similar association between the disinfection efficiency and frequency of pressure cycling was found at ΔP = 0.10 MPa (generated by a 6-mm nozzle and 0.20 kW pump and a 8-mm nozzle and 0.75 kW pump); the associated treatment periods were 15 and 10 min for the recycle numbers corresponding to 71 cycles/25 min and 95 cycles/25 min, respectively. These results affirm the effect of pressure cycling on *E. coli* inactivation during pressurized CO_2 treatment.

Table 1 summarizes the coefficients of correlation for the inactivation efficiency and parameters associated with pressure cycling, including the nozzle diameter (x_1), pressure difference ΔP (x_2), flow rate (x_3), and recycle number (x_4). Based on the Pearson matrix correlation results, *E. coli* inactivation efficiencies were correlated with ΔP values ($r = 0.63$, $p < 0.0001$) and recycle numbers ($r = 0.66$, $p < 0.0001$). The flow rate showed a weak correlation with the inactivation efficiency ($r = 0.09$, $p = 0.3$). Meanwhile, an inverse correlation ($r = -0.35$, $p = 0.0004$) was found between the nozzle diameter and disinfection efficiency. These data indicate that operations with a high flow rate, high ΔP value, large recycle number, and small nozzle diameter will yield greater inactivation efficiencies.

Factor	Symbol code	Unit	r	t-statistic	p-value
Nozzle diameter	x_1	mm	−0.35	−3.64	0.0004*
Pressure difference ΔP	x_2	Pa	0.63	8.08	1.69e-12*
Flow rate	x_3	L min^{-1}	0.09	1.05	0.30
Recycle number	x_4	cycles	0.66	8.73	6.928e-14*

*$p < 0.05$ (significant at the 95% confidence level); df = 98.

Table 1. Correlation coefficients among various operating parameters associated with pressure cycling and the *E. coli* inactivation efficiency.

Regression coefficients, *t*-values, and *p*-values were analyzed for the four factors as shown in **Table 2**. The outcome of the multicollinearity regression model analysis ($R^2 = 0.77$, $p < 0.001$) suggests that the model can explain 77% of the inactivation efficiency of *E. coli*. With bootstrap analysis, the results of multivariate regression analyses were validated. The variables of x_1, x_2, x_3, and x_4 that were found to be associated with pressure cycling in the original analyses were significantly associated with pressure cycling in approximately 8, 28, 3, and 37%, respectively, of the 1000 iterations of the multivariate analyses. Taken together, these findings suggest that the frequency of recirculation (x_4) and the ΔP magnitude of the input (x_2) were key factors that drove the effectiveness pressure cycling.

Although the use of small nozzle diameters was associated with effective inactivation, operating conditions at high ΔP values and low flow rates may be more complex and of lesser economical interest. The highest inactivation efficiency was observed when 5–7 mm nozzle diameters and the 0.75 kW pump were used (**Figure 8b**). Since a large processing capacity is of great commercial interest, the 7 mm nozzle and 0.75 kW pump were used for subsequent experiments.

4.3.2. Effect of pressure cycling at various WVRs

The effect of WVR was investigated at four ratios (50, 60, 70, and 80%) by applying a pressure of 0.7 MPa at a temperature of 20 ± 1°C and two flow rates (14 and 25 L min^{-1}) for 25 min (**Figure 9**). As shown in **Figure 9c**, decreasing WVR from 80 to 50% resulted in a decrease in

Source	Coefficient	t-statistic	p-value
Intercept	−0.63	−0.99	0.33
x_1	−0.13	−3.59	0.0005*
x_2	0.01	7.32	7.8e-11*
x_3	0.10	3.40	0.001*
x_4	0.05	11.29	<2e-16*

*Significant at the 95% confidence level; multiple $R^2 = 0.77$; adjusted $R^2 = 0.76$.
F-statistic = 78.77 with 4 and 95 degrees of freedom, $p < 2.2e-16$.

Table 2. Regression results showing the influence of operating parameters associated with pressure cycling on the inactivation efficiency (at 20 ± 1°C, system pressure = 0.7 MPa, and working volume ratio (WVR) = 70%).
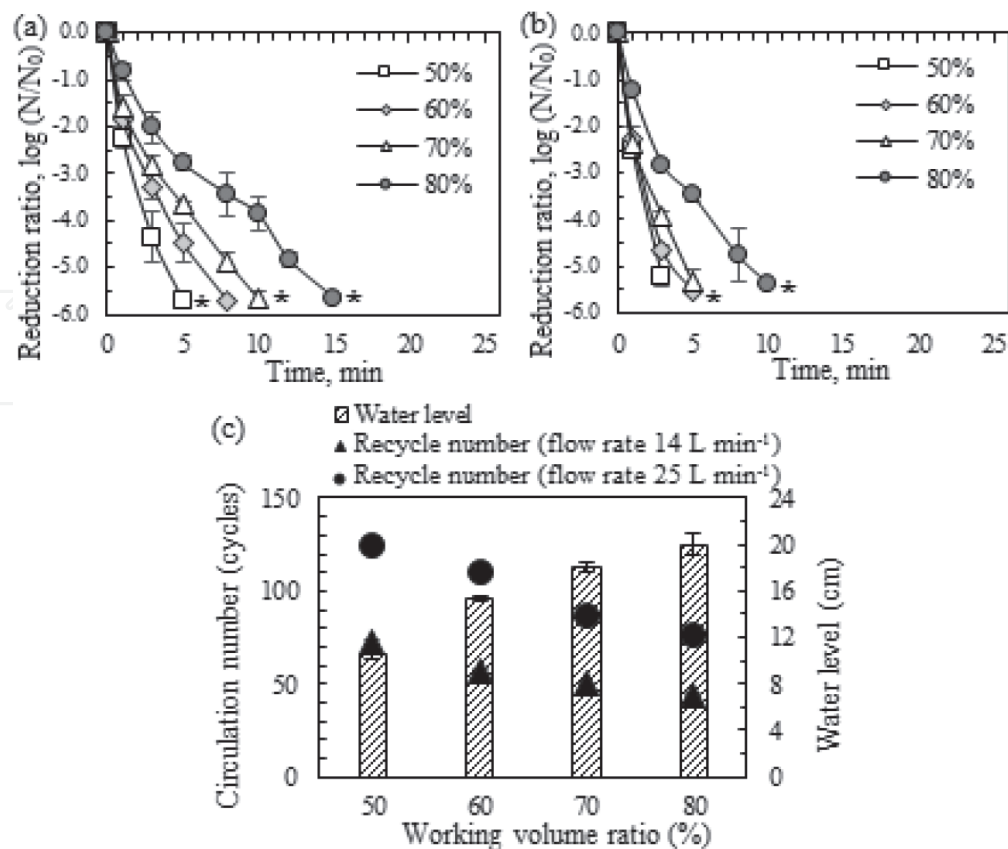
Figure 9. Effect of the working volume ratio (WVR) on the inactivation of *E. coli* in seawater by pressurized CO_2 at 0.7 MPa and $20 \pm 1°C$ with (a) a flow rate of 14 L min^{-1} [23] and (b) a flow rate of 25 L min^{-1}. (c) Influence of the WVR on the circulation number and water level in the main chamber. Asterisks (*) indicate that no colonies were detected.

the water level (22–11 cm) and a faster frequency of pressure cycling. In regard to pressure cycling, the circulation number increased from 44 to 72 cycles with the flow rate of 14 L min^{-1}, and from 78 to 125 cycles with the flow rate of 25 L min^{-1}.

E. coli inactivation efficacy of pressurized CO_2 significantly increased with decreases in the WVR (**Figure 9**). Besides, at every WVR, operations with a high flow rate greatly enhanced the disinfection efficiency. When operating the device with a flow rate of 14 L min^{-1}, an approximate 5.7 log reduction of *E. coli* was achieved within 15 min at 80% WVR, whereas only 5 min was required at 50% WVR to reduce the *E. coli* load to a similar extent (**Figure 9a**; [23]). A similar tendency was found in the case of the 25 L min^{-1} flow rate (**Figure 9b**). The durations required for complete inactivation of *E. coli* were 10 min at 80%, 5 min at 60–70%, and 3 min at 50%.

Pressure cycling boosts the inactivation efficiency by providing a driving force for CO_2 transfer efficiency [9–13]. Recall that at the same flow rate and ΔP, a decrease in WVR increased the frequency of pressure cycling. Hence, it is hypothesized that a smaller WVR may have stimulated the CO_2 transfer across cell membranes and thus improved the bactericidal performance of pressurized CO_2 [11, 28, 29]. In this study, the low inactivation efficiency with a large WVR (i.e., 80%) may be related to the high water level (20–22 cm; **Figure 9c**), which led to submergence of the shield inside the device; this may have in turn decreased bubble formation via

shield interactions [23, 24]. In contrast, the operations with smaller WVRs helped not only to promote a greater efficiency for CO_2 bubble generation but also increased the speed of the pressure cycling. Consequently, CO_2 supported by the high pressure and high efficiency of interactions in the apparatus easily penetrated into the cell membranes, thereby accelerating the *E. coli* inactivation efficiency.

Regarding the effect of WVR in pressure cycling treatments, Pearson regression tests showed that *E. coli* inactivation efficiency was strongly correlated with the recycle number ($r = 0.95$, $p < 0.001$). The regression coefficient, *t*-value, and *p*-value were analyzed with regard to the recycle number at various WVRs and flow rates (**Table 3**). According to the regression analysis, the experimental results fit the linear model shown in the following equation:

$$Y = 0.736 + 0.285 \times x_4 \tag{2}$$

Here, x_4 is the recycle number (cycles), and Y is reduction ratio ($-\log N/N_0$) of *E. coli* caused by pressurized CO_2.

As shown in **Table 3**, the *t* values of the regression model were positive and significant ($p < 0.05$), thus indicating that the model result was significant. The outcome of the linear regression model analysis ($R^2 = 0.91$, $p < 0.001$) suggests that 91% of the variation in the *E. coli* inactivation efficiency was explained by the frequency of pressure cycling ($\Delta P = 0.12$ MPa, flow rate = 14–25 L min^{-1}). Predicted values of *E. coli* reduction ratios were calculated based on Eq. (2), and the data are summarized in **Table 4** along with the experimental results. The predicted values were fairly similar to the experimental results, thus suggesting that the model could adequately describe the strong relationship between pressure cycling and bactericidal activity ($p < 0.05$). Taken together, these findings affirm that at the same ΔP, faster pressure cycling can achieve a greater *E. coli* inactivation efficiency.

Dillow et al. [13] reported that an increase of pressure cycling from 3 to 6 cycles using supercritical CO_2 (at 20.5 MPa and 34°C) within 0.6 h increased the inactivation from 3 to 9 log reductions. Silva et al. [10] found that an 8.0 log reduction could be achieved with pressure cycling (5 cycles/140 min) and supercritical CO_2 at 8 MPa, whereas a 5.0 log reduction was observed with 1 cycle/28 min and 8 MPa. However, high pressure and CO_2 discharge are not interesting from both economic and practical viewpoints. As demonstrated in the present study where CO_2 discharge was eliminated during the treatment process, pressure cycling at a low pressure (0.7 MPa) is a promising method to enhance the bactericidal activity of pressurized CO_2.

Coefficients	Estimate	Standard error	*t*-statistic	*p*-value	R^2
Intercept	0.736	0.195	3.77	0.0009*	
x_4	0.285	0.019	15.30	7.2e-14*	0.91

*95% confidence level.

Table 3. Regression results showing the influence of pressure cycling on the inactivation efficiency (at 20 ± 1°C, system pressure = 0.7 MPa, $\Delta P = 0.12$ MPa, flow rate = 14 to 25 L min^{-1}, and initial bacterial concentration = 5–6 log$_{10}$ CFU mL^{-1}).

Flow rate, L min^{-1}	HRT, min	Variables		Responses Y: Reduction ratio, $-\log(N_t/N_0)$	
		WVR, %	x_4, cycles	Experimental	Predicted
25[a]	0.20	50	15[c]	5.2 ± 0.2	5.0*
25[a]	0.24	60	21[d]	5.5 ± 0.0	6.4*
25[a]	0.28	70	18[d]	5.3 ± 0.2	5.8*
14[b]	0.36	50	14[d]	5.7 ± 0.1	4.7*
14[b]	0.43	60	19[e]	5.7 ± 0.0	6.1*
14[b]	0.50	70	20[f]	5.7 ± 0.2	6.5*

HRT, hydraulic retention time

*Predicted values calculated based on Eq. (2).

[a, b]Generated by a 7-mm nozzle and 0.75 kW pump, and a 5-mm nozzle and 0.20 kW pump, respectively.

[c, d, e, f]Exposure times were 3, 5, 8, and 10 min, respectively, when bacteria were completely inactivated.

Table 4. Validation of model regression for the inactivation efficiency responses to pressure cycling as a function of various working volume ratios (WVRs) and flow rates (at 20 ± 1°C, system pressure = 0.7 MPa, ΔP = 0.12 MPa, and initial bacterial concentration = 5–6 $\log_{10}$ CFU mL^{-1}).

4.4. SEM analyses

Comparative SEM images of untreated samples and samples treated with pressurized CO_2 (0.7 MPa and 20°C for a duration of 25 min) revealed changes in the morphology of *E. coli* cells (**Figure 10**). The *E. coli* cells treated with pressurized CO_2 presented several small vesicles on the cell surface, and some treated cells appeared to be lysed (**Figure 10b**); in contrast, the untreated *E. coli* cells did not have such structures on the surface (**Figure 10a**) [23]. These results suggest that the pressurized CO_2-treated *E. coli* cells may have been disrupted [19, 20, 23], and that intracellular substance may have leaked out, possibly because of the alterations in cell permeability [20, 23, 30]. The findings also affirm the excellent bactericidal performance of the pressurized CO_2 treatment.
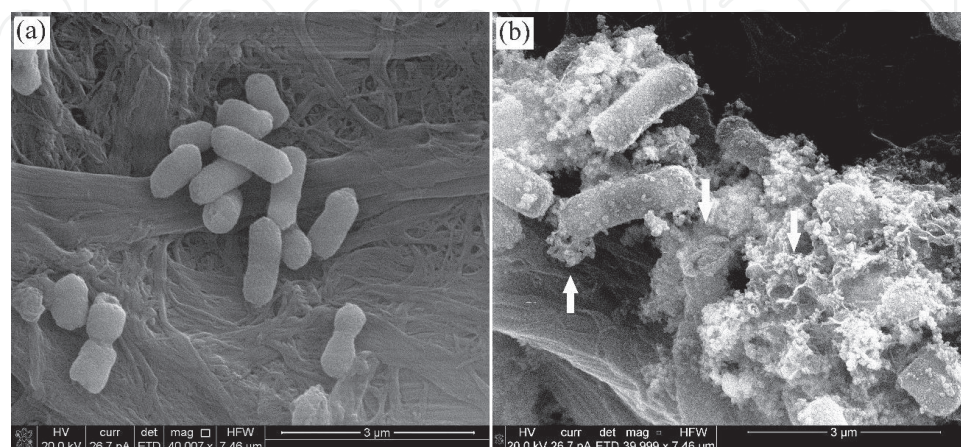

Figure 10. Representative scanning electron microscopy (SEM) images of *E. coli* cells that were (a) untreated and (b) treated by pressurized CO_2 at 0.7 MPa and 20°C for a duration of 25 min [23].

5. Summary

Pressurized CO_2 treatments can be used to eliminate *E. coli* from seawater. In this study, the inactivation efficiency was substantially enhanced by pressure cycling, which was conducted at a low pressure (0.7 MPa) and without CO_2 release during the treatment period. Bactericidal performance of pressure cycling was concomitantly influenced by two key factors involving the frequency of recirculation and ΔP ($p < 0.001$). At the same ΔP, an increase in the frequency of pressure cycling significantly improved the *E. coli* inactivation efficiency ($p < 0.001$). Additionally, the sensitivity of *E. coli* to pressurized CO_2 treatments substantially increased with increased pressures (0.2–0.9 MPa) and temperatures (11–28°C). Under identical treatment conditions (0.7 MPa, 20°C, 25 L min^{-1}, and 50% WVR), more than 5.0 log reductions in the load of *E. coli* were achieved after treatments for 3 min by using pressure cycling ($\Delta P = 0.12$ MPa, 15 cycles). Overall, these findings suggest that pressurized CO_2 technology would be feasible for water disinfection applications such as those used in ballast water treatment.

Acknowledgements

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References

[1] Boorman GA, Dellarco V, Dunnick JK, Chapin RE, Hunter S, Hauchman F. Drinking water disinfection byproducts: Review and approach to toxicity evaluation. Environmental Health Perspectives. 1999;**107**(Suppl 1)217-217

[2] Fabbricino M, Korshin GV. Formation of disinfection by-products and applicability of differential absorbance spectroscopy to monitor halogenation in chlorinated coastal and deep ocean seawater. Desalination. 2005;**176**69-69. DOI: 10.1016/j.desal.2004.10.026

[3] LeChevallier MW, Au K, editors. Water Treatment and Pathogen Control: Process Efficiency in Achieving Safe Drinking-water. WHO Drinking-water Quality Series. World Health Organization: IWA Publishing; 2004. p. 112. DOI: 10.2166/9781780405858

[4] Von Gunten U. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. Water Research. 2003;**37**1487-1487. DOI: 10.1016/S0043-1354(02)00458-X

[5] Werschkun B, Sommer Y, Banerji S. Disinfection by-products in ballast water treatment: An evaluation of regulatory data. Water Research. 2012;**46**4901-4901. DOI: 10.1016/j.watres.2012.05.034

[6] Haas GJ, Prescott HE, Dudley E, Dik R, Hintlian C, Keane L. Inactivation of microorganisms by carbon dioxide under pressure. Journal of Food Safety. 1989;**9**265-265. DOI: 10.1111/j.1745-4565.1989.tb00525.x

[7] Garcia-Gonzalez L, Geeraerd AH, Spilimbergo S, Elst K, Van Ginneken L, Debevere J, Van Impe J, Devlieghere F. High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. International Journal of Food Microbiology. 2007;**117**28-28. DOI: 10.1016/j.ijfoodmicro.2007.02.018

[8] Spilimbergo S, Elvassore N, Bertucco A. Microbial inactivation by high-pressure. The Journal of Supercritical Fluids. 2002;**22**63-63. DOI: 10.1016/S0896-8446(01)00106-1

[9] Zhang J, Davis TA, Matthews MA, Drews MJ, LaBerge M, An YH. Sterilization using high-pressure carbon dioxide. The Journal of Supercritical Fluids. 2006;**38**372-372. DOI: 10.1016/j.supflu.2005.05.005

[10] Silva JM, Rigo AA, Dalmolin IA, Debien I, Cansian RL, Oliveira JV, Mazutti MA. Effect of pressure, depressurization rate and pressure cycling on the inactivation of *Escherichia coli* by supercritical carbon dioxide. Food Control. 2013;**29**81-81. DOI: 10.1016/j.foodcont.2012.05.068

[11] Hong SI, Park WS, Pyun YR. Inactivation of *Lactobacillus* sp. from kimchi by high pressure carbon dioxide. LWT-Food Science and Technology. 1997;**30**685-685. DOI: 10.1006/fstl.1997.0250

[12] Hong SI, Pyun YR. Inactivation kinetics of *Lactobacillus plantarum* by high pressure carbon dioxide. Journal Food Science. 1999;**64**733-733. DOI: 10.1111/j.1365-2621.1999.tb15120.x

[13] Dillow AK, Dehghani F, Hrkach JS, Foster NR, Langer R. Bacterial inactivation by using near and supercritical carbon dioxide. Proceedings of the National Academic of Sciences of the United States of America. 1999;**96**10348-10348. DOI: 10.1073/pnas.96.18.10344

[14] Fraser D. Bursting bacteria by release of gas pressure. Nature. 1951;**167**34-34. DOI: 10.1038/167033b0

[15] Ferreira EHdR, Rosenthal A, Calado V, Saraiva J, Mendo S. *Byssochlamys nivea* inactivation in pineapple juice and nectar using high pressure cycles. Journal of Food Engineering. 2009;**95**669-669. DOI:10.1016/j.jfoodeng.2009.06.053

[16] Kobayashi F, Hayata Y, Kohara K, Muto N, Osajima Y. Application of supercritical CO_2 bubbling to inactivate *E. coli* and coliform bacteria in drinking water. Food Science and Technology Research. 2007;**13**22-22. DOI: 10.3136/fstr.13.20

[17] Kobayashi F, Yamaza F, Ikeura H, Hayata Y, Muto N, Osajima Y. Inactivation of micro-organisms in untreated water by a continuous flow system with supercritical CO_2 bubbling. Journal of Water and Environment Technology. 2009a;**7**250-250. DOI: 10.2965/jwet.2009.241

[18] Kobayashi F, Hayata Y, Ikeura H, Tamaki M, Muto N, Osajima Y. Inactivation of *Escherichia coli* by CO_2 microbubbles at a lower pressure and near room temperature. Transactions of the ASABE. 2009b;**52**1626-1626. DOI: 10.13031/2013.29113

[19] Cheng X, Imai T, Teeka J, Yamaguchi J, Hirose M, Higuchi T, Sekine M. Inactivation of *Escherichia coli* and bacteriophage T4 by high levels of dissolved CO_2. Applied Microbiology and Biotechnology. 2011;**90**1500-1500. DOI: 10.1007/s00253-011-3163-0

[20] Vo HT, Imai T, Teeka J, Sekine M, Kanno A, Le TV, Higuchi T, Phummala K, Yamamoto K. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. Water Research. 2013a;**47**4293-4293. DOI: 10.1016/j.watres.2013.04.053

[21] Vo HT, Imai T, Yamamoto H, Le TV, Higuchi T, Kanno A, Yamamoto K, Sekine M. Disinfection using pressurized carbon dioxide microbubbles to inactivate *Escherichia coli*, bacteriophage MS2 and T4. Journal of Water Environment Technology. 2013b;**11**505-505. DOI: 10.2965/jwet.2013.497

[22] Vo HT, Imai T, Ho TT, Dang TTL, Hoang SA. Potential application of high pressure carbon dioxide in treated wastewater and water disinfection: Recent overview and further trends. Journal Environmental Science. 2015;**36**47-47. DOI: 10.1016/j.jes.2015.04.006

[23] Dang TTL, Imai T, Le TV, Vo HT, Higuchi T, Yamamoto K, Kanno A, Sekine M. Disinfection effect of pressurized carbon dioxide on *Escherichia coli* and *Enterococcus* sp. in seawater. Water Science and Technology: Water Supply. 2016a;16(6)1735-1744. DOI: 10.2166/ws.2016.086

[24] Dang TTL, Imai T, Le TV, Nishihara S, Higuchi T, Nguyen KDM, Kanno A, Yamamoto K, Sekine M. Effect of pressure and pressure cycling on disinfection of *Enterococcus* sp. in seawater using pressurized carbon dioxide with different content rates. Journal of Environmental Science and Health, Part A. 2016b;51(11)930-937. DOI: 10.1080/10934529.2016.1191309

[25] Hutkins RW, Nannen NL. pH homeostasis in lactic-acid bacteria. Journal of Dairy Science. 1993;**76**2365-2365. DOI: 10.3168/jds.S0022-0302(93)77573-6

[26] Isenschmid A, Marison IW, von Stockar U. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. Journal of Biotechnology. 1995;**39**237-237. DOI: 10.1016/0168-1656(95)00018-L

[27] Oulé MK, Tano K, Bernier AM, Arul J. *Escherichia coli* inactivation mechanism by pressurized CO_2. Canadian Journal of Microbiology. 2006;**52**1217-1217. DOI: 10.1139/w06-078

[28] Lin HM, Yang Z, Chen LF. Inactivation of *Leuconostoc dextranicum* with carbon dioxide under pressure. The Chemical Engineering Journal. 1993;**52**:B29-B34. DOI: 10.1016/0300-9467(93)80047-R

[29] Garcia-Gonzalez L, Geeraerd AH, Elst K, Van Ginneken L, Van Impe JF, Devlieghere F. Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization. The Journal of Supercritical Fluids. 2009;**51**82-82. DOI: 10.1016/j.supflu.2009.06.020

[30] Kim SR, Rhee MS, Kim BC, Lee H, Kim KH. Modeling of the inactivation of *Salmonella typhimurium* by supercritical carbon dioxide in physiological saline and phosphate-buffered saline. Journal Microbiology Methods. 2007;**70**141-141. DOI: 10.1016/j.mimet.2007.04.003