Disinfection effect of pressurized carbon dioxide on *Escherichia coli* and *Enterococcus* sp. in seawater

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ABSTRACT

Ship ballast water should be disinfected before being discharged into the ocean to avoid the dispersal of non-native species into the marine environment. This study presents the results of using pressurized carbon dioxide (CO₂) at less than 1.0 MPa for inactivating *Escherichia coli* and *Enterococcus* sp. in artificial seawater (3.4% salinity). The bactericidal effects of pressurized CO₂ were assessed using a liquid-film-forming apparatus under various conditions of pressure, temperature, and working volume ratio (WVR). Additionally, leakage of proteins and nucleic acids from cells was measured. Cell morphology of untreated cells and cells treated with pressurized CO₂ was assessed using scanning electron microscopy (SEM). Pressurized CO₂ treatment affected both strains; however, *Enterococcus* sp. exhibited higher resistance to pressurized CO₂ treatment than did *E. coli*. Under identical treatment conditions (0.7 MPa, 20 °C, and 50% WVR), more than 5.0 log reduction in the load of *E. coli* and *Enterococcus* sp. was achieved after treatments for 5 min and 20 min, respectively. Release of intracellular contents occurred during the treatment process and SEM images of *E. coli* and *Enterococcus* sp. revealed that morphological changes had occurred after the treatment with pressurized CO₂. Hence, pressurized CO₂ has potential applications for inactivating pathogens in ballast water.

**Key words** | bacterial inactivation, ballast water treatment, *Escherichia coli*, *Enterococcus* sp., pressurized carbon dioxide, water disinfection

INTRODUCTION

Ballast water is pumped-in to maintain the stability and maneuverability of ships, thus, it is essential to ensure safe operating conditions throughout a voyage. However, transfer of ballast water between different continents and oceans also transports aquatic species into a new ecosystem. Marine organisms can become invasive in new environments that support their growth, and their uncontrolled growth can destroy the non-native ecosystems (Ruiz *et al.* 1997; Molnar *et al.* 2008). For example, introduction of non-native aquatic species via ship ballast water can result in alteration of food webs, destruction of native aquatic habitat, loss of biodiversity, reduction of commercial fisheries, and increase in human health risk (Ruiz *et al.* 1997).

In response to these problems, in 2004, the International Maritime Organization (IMO) established standards and procedures for the management and control of ship ballast water and sediment (IMO 2004). These regulations require ships to limit the number of viable organisms in ballast water to meet the D-2 ballast water performance standard before the ballast water can be discharged into the sea (IMO 2004). The standard dictates that the effluent must not contain more than 250 colony-forming units (CFU) per
100 mL for *E. coli*, 100 CFU/100 mL for intestinal enterococci, and 1 CFU/100 mL for toxicogenic *Vibrio cholerae* (O1 and O139).

Several disinfection technologies have been applied for the treatment of ballast water. Chlorine or ozone has been commonly used to inactivate microorganisms in water owing to their high bactericidal efficiency. However, chlorine can combine with organic compounds in water to produce carcinogenic agents such as trihalomethanes and halogenic acids (Fabbricino & Korshin 2003). Ozone can react with bromide in seawater to produce bromate (Von Gunten 2003; Werschkun et al. 2012). Since toxic by-products generated with these disinfection treatments remain in the water, alternative methods of ballast water disinfection are sought after (Werschkun et al. 2012, 2014). Other water treatment methods such as ultraviolet (UV) radiation, ultrasound, cavitation, or heat application can be used for the mechanical disruption and inactivation of organisms. Although the mechanical disruption methods do not have problems associated with residual toxicity, these methods have high operational costs due to their large power requirement. In addition, the bacterial inactivation capability of UV light is reduced for waters with high turbidity or high concentration of dissolved organic matter (Werschkun et al. 2012). Currently, no single method can adequately fulfill the requirements of the D-2 ballast water performance standard (Tsokaki et al. 2010; Werschkun et al. 2014).

The high-pressure carbon dioxide (HPCD) method has been widely used for food sterilization (Zhang et al. 2006; Garcia-Gonzalez et al. 2007). The potential benefits of CO₂ as a sterilizing agent over other agents include its nontoxicity, inexpensiveness, and availability (Zhang et al. 2006). HPCD has been used to successfully eradicate a variety of microorganisms in dried food and liquid products; however, the technique requires high-pressure conditions (4–50 MPa) to effectively inhibit pathogens (Hong et al. 1997; Zhang et al. 2006; Garcia-Gonzalez et al. 2007, 2009). Recently, HPCD has attracted renewed interest in the field of water treatment, owing to the fact that toxic by-products are not generated during the disinfection process (Kobayashi et al. 2007, 2009; Cheng et al. 2011; Vo et al. 2013a,b). Kobayashi et al. (2007) reported that at a high pressure of 10 MPa and temperature of 35 °C, supercritical CO₂ microbubble treatment eliminated *E. coli* and coliform bacteria in drinking water within 13.3 min. Vo et al. (2013a) showed that *E. coli* could be inactivated within 25 min by application of low-pressure CO₂ (below 1.0 MPa) at room temperature. Previous studies on HPCD treatment were conducted using distilled water or water with low salinity (<0.9% salinity) as the suspension medium. The efficacy of HPCD treatment for disinfecting seawater (~3.4% salinity) has not yet been studied.

This study determined whether CO₂ at low pressure (0.2–0.9 MPa) could be used to inactivate bacteria present in artificial seawater (3.4% salinity). *Enterococcus* sp. (ATCC 202155), which is a strain of enterococci, and *E. coli* (ATCC 11303) were used as representative Gram-positive and Gram-negative bacteria, respectively, in this study. Both *E. coli* and enterococci are pathogen indicators as included in the IMO D.2 ballast water performance standard. The bactericidal effect of pressurized CO₂ against these bacterial species was examined under various conditions of pressure, temperature, and working volume ratio (WVR) (defined as the ratio between the sample volume and apparatus volume). In addition, the release of bacterial intracellular contents and changes in cell morphology were evaluated to characterize the bactericidal efficacy of pressurized CO₂ against *Enterococcus* sp. and *E. coli* in seawater. The research objective was to evaluate the bactericidal effectiveness of pressurized CO₂ for disinfecting ship ballast water.

**MATERIALS AND METHODS**

**Microorganism preparation and enumeration**

The bacterial inoculums for *E. coli* (ATCC 11303) and *Enterococcus* sp. (ATCC 202155) were prepared by inoculation of 100 μL of bacterial glycerol stock into 100 mL of Luria-Bertani (LB) broth (Wako Chemical Co. Ltd, Osaka, Japan) and brain heart infusion (BHI) broth (Wako, Japan), respectively. Both LB and BHI broths were supplemented with sodium chloride to obtain a final concentration of 30 g L⁻¹. The bacterial cultures were incubated for 18 h at 37 °C by using a reciprocal shaker rotating at 150 rpm. Cells were harvested and washed three times with 0.9% (w/v) saline solution by centrifugation (10 min at 8,000 × g at room temperature) in a CF15D2 centrifuge.
The pellet was re-suspended in 100 mL saline solution. Permanent stocks were maintained in 20% glycerol at −80 °C.

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

**Artificial seawater samples**

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-51SA, Lutron Electronic Enterprise Co. Ltd, Taiwan). For all experiments, the bacterial preparations were diluted in the artificial seawater to obtain a bacterial concentration of 5–6 log10 CFU mL⁻¹, which was used as the initial concentration for all experiments. The solution was stirred for 30 min to acclimatize bacteria before starting the experiments.

**Apparatus and procedure for disinfection**

The experimental apparatus for disinfection was a stainless steel pressurized chamber with an internal volume of 10 L (Figure 1). The pressurization apparatus was designed to include a small nozzle and a shield to enable vigorous agitation of the influent for creating bubbles. Disinfection experiments were conducted in batch mode. Sample water, as the influent, was pumped in one shot into the device using a pump (0.2 kW, Iwaya-WPT-202). Following
the first influx of water, pressurized CO₂ was also injected into the main chamber. The fluid was then circulated by pumping inside the system at a flow rate of 14 L min⁻¹ for 25 min. A pump was used to apply a higher pressure (0.12 MPa) than that inside the main chamber. A high-pressure water stream was introduced into the main chamber through a nozzle such that it collided with a bubble-generating shield to promote CO₂ diffusion in the water. The fluid was mixed well by counter-current agitation (mixed by fluid recirculation) 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.

To investigate the effect of pressure, the sensitivity of the bacteria to pressurized CO₂ treatment was determined using varying pressures (0.2–0.9 MPa). The temperature of seawater varies seasonally and the temperatures range between 11 °C and 28 °C. To assess the effect of temperature, the disinfection cycle was performed at room temperature in different seasons. 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%). Each experiment was conducted in triplicate. Water level was measured by a gauge to evaluate the effects of WVR on the shield inside the main chamber. Water flow rate was measured by a flow meter (GPI, Nippon Flow Cell Co. Ltd, Japan). The recycle number performed in the 25 min period was calculated in relation to the treatment time and hydraulic retention time (HRT), wherein HRT = sample volume/flow rate.

**Measurement of intracellular material leakage**

To quantify intracellular material released from *E. coli* and *Enterococcus* sp. cells, pressurized CO₂-treated and untreated samples were centrifuged at 1,000 × g for 10 min at 4 °C in a centrifuge (CF15D2, Hitachi, Japan). Next, the nucleic acids and proteins in the supernatants were measured by assessing the UV absorbance at 260 nm and 280 nm, respectively (Kim et al. 2008). The absorbance was measured using a spectrophotometer (U1800, Hitachi, Japan). The absorbance values were evaluated using different UV-absorbing intensities and treatment times.

**Scanning electron microscopy**

The pellets of *E. coli* and *Enterococcus* sp. were immobilized with 2.5% glutaraldehyde (Wako, Japan) in 0.2 mol L⁻¹ Millonig’s phosphate buffer saline pH 7.4 (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 fixative. After fixation, the cells were dehydrated by consecutive soaking in increasing concentrations of ethanol solution (50%, 70%, 80%, 90%, 95%, and 100%), followed by ethanol/t-buty alcohol (v/v = 1:1) treatment for 30 min. The prepared cells were then soaked in t-buty alcohol two times for 1 h, freeze-dried for 2 h (JEE 4X vacuum evaporator, JEOL, Japan), and sputter coated with gold–palladium. Finally, the cells were examined using a scanning electron microscope (SEM; Quanta™ 3D, FEI Co., USA) at 20 kV.

**RESULTS AND DISCUSSION**

**Effect of pressure on bacterial inactivation**

The effect of using various pressure conditions (range: 0.2–0.9 MPa) for inactivating *E. coli* and *Enterococcus* sp. is shown in (Figure 2). Overall, the bactericidal activity of CO₂ on *E. coli* and *Enterococcus* sp. increased with increasing pressure. The reduction of bacterial load was 5.3–5.7 log for *E. coli* (Figure 2(a)) and 2.9–4.3 log for *Enterococcus* sp. (Figure 2(b)).

At higher CO₂ pressures, shorter treatment times were required to inactivate *E. coli* (Figure 2(a)). For example, 25 min of 0.2 MPa CO₂ treatment was required to reduce the *E. coli* load by 5.3 log, whereas only 20 and 15 min of 0.5 MPa and 0.6 MPa CO₂, respectively, were required to reduce *E. coli* load by 5.6–5.7 log. When pressures between 0.7 and 0.9 MPa were used, *E. coli* load was reduced to a similar extent with treatment periods of 10 min. However, the increased pressure application from 0.7 MPa to 0.9 MPa did not result in a significant increase in rate of bacterial inactivation. A similar relationship between CO₂ pressure and the efficacy of bacterial inactivation was observed with *Enterococcus* sp. (Figure 2(b)). The reduction
in bacterial load of *Enterococcus* sp. was 4.1–4.3 log using pressures of 0.7 MPa to 0.9 MPa and a treatment period of 25 min. These data indicate that the optimal CO₂ pressure for inactivating these bacteria is in the range of 0.7 to 0.9 MPa; therefore, 0.7 MPa was chosen as the optimal pressure for bactericidal activity.

The sensitivity of *Enterococcus* sp. to pressurized CO₂ treatment was lower than that of *E. coli* (Figure 2(b)). Under the experimental treatment conditions (0.7 MPa, 70% WVR, 20 ± 1.0 °C, and initial concentration of 5–6 logₐ CFU mL⁻¹), a treatment period of 25 min was required to reduce the bacterial load by approximately 4.1 log for *Enterococcus* sp.; whereas, the same treatment conditions completely inactivated *E. coli* within 10 min of treatment. Inactivation of *Enterococcus* sp. by pressurized CO₂ treatment is more difficult than inactivation of *E. coli* by the same treatment, possibly because of the differences between their cell wall structures. Gram-positive bacteria, such as *Enterococcus* sp., have a thick peptidoglycan layer that can make the cell wall structure more resistant to pressure treatment and thereby make Gram-positive bacteria more resistant to pressure inactivation than Gram-negative bacteria such as *E. coli* (Zhang et al. 2006).

**Effect of temperature on bacterial inactivation**

Figure 3 shows the bacterial inactivation efficiency of pressurized CO₂ treatment at different initial temperatures (11–28 °C), and at 0.7 MPa with 70% WVR for 25 min. The treatment efficiency for inactivating both *E. coli* and *Enterococcus* sp. substantially increased with increasing treatment temperature. As shown in (Figure 3(a)), the period required for complete inactivation of *E. coli* decreased as the temperature increased (25 min at 11 °C, 20 min at 15 °C, and 10 min at 20–28 °C). *Enterococcus* sp. also showed a similar trend of a decreased inactivation period at higher temperatures (Figure 3(b)), although the inactivation rate was lower than that of *E. coli*. The bacterial load reduced by approximately 2.0 log after treatment at 11 °C. Furthermore, 4.2 log reduction in bacterial load was recorded at 20 °C and more than 5.0 log reduction was achieved at 25–28 °C. These results indicate that pressurized CO₂ efficiently inactivated both bacterial species at temperatures in the range of 20 to 28 °C; therefore, subsequent experiments were conducted at 20 °C.

Thus, the disinfection efficiency of pressurized CO₂ increased with increasing temperatures for both *Enterococcus* sp. and *E. coli*. The disinfection efficiency could also be increased by enabling better contact between CO₂ and seawater in the liquid-film-forming apparatus to improve the solubility of CO₂ in seawater. Since CO₂ is both lipophilic and hydrophilic in nature, it can easily penetrate into the phospholipid bilayer of the cell membrane and accumulate there (Isenschmid et al. 1995). An increase in temperature may stimulate the diffusion of CO₂ into cells and may increase the fluidity of cell membranes (Hong et al. 1997; Oulé et al. 2006). Thus, we speculate that high temperature and pressure conditions may synergistically improve diffusion of CO₂ in water and enable its efficient penetration into the cells, thereby accelerating disinfection efficiency. Vo et al. (2013b) reported that 20 min was required for 5 log reduction of *E. coli* load with pressurized CO₂ microbubbles at 0.7 MPa and 26.6 ± 0.4 °C. Despite having the same pressurized CO₂, the treatment time obtained in the
present study (10 min for *E. coli*) was shorter than that obtained by Vo *et al.* (2013b).

**Effect of WVR on bacterial inactivation**

The effect of WVR was studied using four ratios (50%, 60%, 70%, and 80%) at 0.7 MPa and 20 ± 1 °C, with flow rate 14 L min⁻¹ for 25 min (Figure 4). During this period, there was a slight decrease in WVR (∼2%) due to withdrawal of samples. However, the WVR change was small and it was therefore assumed that the change does not have a significant influence on the treatment process. Figure 4(c) shows an increase in the water level (11 to 22 cm) and a decrease in recycle number (72 to 44 cycles) as a consequence of increase in WVR from 50% to 80%.

Remarkably, the disinfection efficacy of pressurized CO₂ against both *E. coli* and *Enterococcus* sp. greatly increased with decreasing WVR. The *E. coli* load was reduced by...
5.7 log within 5 min of treatment at 50% (±1%) WVR, whereas 15 min of treatment was required at 80% (±1%) WVR (Figure 4(a)). Similarly, 5.4 log reduction of Enterococcus sp. was achieved within 20 min at 50% (±1%) WVR, whereas only 1.7 log reduction was observed at 80% (±1%) WVR after 25 min of treatment (Figure 4(b)).

It is hypothesized that operation at a smaller WVR results in a greater inactivation efficiency, which is related to the influence of mass transfer rate of CO2 in water (Lin et al. 1993; Hong et al. 1997; Garcia-Gonzalez et al. 2009). In our study, the disinfection efficacy decreased dramatically with operation at 80% WVR, suggesting that CO2 mass transfer was limited at that WVR under identical treatment conditions. The reduced disinfection efficiency at 80% WVR may be related to a reduction in recycle number (44 cycles/25 min) and an increase in the water level (22 cm; Figure 4(c)). Particularly, when operating at 80% WVR, the water level submerged the shield inside the device, which might have ultimately reduced the formation of bubbles and limited CO2 mass transfer. In contrast, smaller WVRs (70% to 50%) provided a larger space to generate CO2 bubbles, and increased the recycle number (50 to 72 cycles within 25 min, respectively; Figure 4(c)). In particular, the pump was used to apply 0.12 MPa higher pressure than that inside the main chamber. Therefore, the circulation cycles were characterized by repetitions of raised and lowered pressure. An increase in the circulation number, thus, resulted in more rapid repetitions of raised and

Table 1  | Disinfection times for several different pathogenic microorganisms with chlorinated water compared to pressurized CO2

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Experimental conditions</th>
<th>Microorganism (Initial concentration)</th>
<th>Time, min</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monochloramine (0.5 mg L(^{-1}))</td>
<td>Pure culture in 0.05 M KH(_2)PO(_4), pH 7.0, at 5 °C</td>
<td>E. coli (6.2 log(_{10}) CFU mL(^{-1}))</td>
<td>30(^a)</td>
<td>Rice et al. (1993)</td>
</tr>
<tr>
<td>Chlorine (0.5 mg L(^{-1}))</td>
<td>Natural seawater, 3.5% salinity, 20–25 °C</td>
<td>E. coli (6.0 log(_{10}) CFU mL(^{-1}))</td>
<td>12(^a)</td>
<td>Azanza et al. (2004)</td>
</tr>
<tr>
<td>CO2</td>
<td>Artificial seawater, salinity = 3.4%, pressure = 0.7 MPa, WVR = 50%, at 20 °C</td>
<td>E. coli (5.7 log(<em>{10}) CFU mL(^{-1})) Enterococcus sp. (5.4 log(</em>{10}) CFU mL(^{-1}))</td>
<td>5(^a)</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\)Approximately 1 CFU mL\(^{-1}\) was detected after the treatment period, and 6.13 log reduction of E. coli was achieved.

\(^b\)Approximately 5 CFU mL\(^{-1}\) was detected after the treatment period, and 4.56 log reduction of Enterococcus faecium was achieved.

\(^c\)Values were calculated from decimal reduction times (\(D\)-values) and initial bacterial concentration. \(D\)-value is the time required to inactivate 90% of the treated microbial population.

\(^d\)Values were estimated from Rice et al. (1993).

\(^e\)No viable microorganism was observed.

Figure 5  | Quantitation of proteins and nucleic acids that leaked out of E. coli and Enterococcus sp. after pressurized CO2 treatment (at 0.7 MPa, 20 ± 0.3 °C, and 70% WVR). The UV absorbance values of supernatants obtained from pressurized CO2-treated samples were measured at 260 nm for determining nucleic acid content (a) and at 280 nm for determining protein content (b). OD is optical density.

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lowered pressure, which in turn enhanced the solubility of CO₂ in seawater and probably increased the bactericidal efficiency. Hence, further research on the effects of pressure cycling on this disinfection would be necessary.

Table 1 compares the exposure times required for inactivating bacteria present in water by chlorine and chloramine, as previously reported (Rice et al. 1993; Azanza et al. 2001), and by pressurized CO₂, as reported in this study. Rice et al. (1993) observed that disinfection using monochloramine (0.5 mg L⁻¹) resulted in 6.13 log reduction of *E. coli* within 30 min. Compared to *E. coli*, *E. faecium* was more resistant to monochloramine, *E. faecium* in pure culture (0.05 mmol L⁻¹ KH₂PO₄, pH 7.0, at 5°C) was reduced by 4.56 log after treatment for 60 min. Azanza et al. (2001) reported that only 12 min of chlorine disinfection (0.5 mg L⁻¹) was required to achieve 6.0-log reduction of *E. coli* in seawater (3.5% salinity and 20–25°C). It is noteworthy that use of pressurized CO₂ (at 0.7 MPa, 20°C and 50% WVR) resulted in complete inactivation of both bacterial species tested: 5.7 log reduction of *E. coli* and 5.4 log reduction of *Enterococcus* sp. within 5 min and 20 min, respectively. These findings demonstrate the excellent bactericidal activity of pressurized CO₂, and suggest that this method could be further developed as a sustainable technology for disinfecting ship ballast water.

**Leakage of intracellular materials after pressurized CO₂ treatment**

The amount of UV-absorbing substances released from *E. coli* and *Enterococcus* sp. cells increased steadily during the
Pressurized CO₂ treatment process (Figure 5). The result demonstrates that *E. coli* and *Enterococcus* sp. cells were disrupted and that intracellular material had leaked out during the treatment process. Remarkably, the UV-absorbance values of *E. coli* supernatant that were measured using 260 nm wavelength (Figure 5(a)) and 280 nm wavelength (Figure 5(b)) were higher than those obtained for *Enterococcus* sp. supernatant. These findings suggest that the leakage of intracellular materials of *Enterococcus* sp., a Gram-positive bacterium, was lower than that of *E. coli*, a Gram-negative bacterium. These data support the findings from the previous section, which showed that Gram-positive bacteria such as *Enterococcus* sp. were less susceptible to pressurized CO₂ treatment owing to the presence of a thicker peptidoglycan layer (Zhang et al. 2006).

**SEM analyses**

To examine the effect of pressurized CO₂ treatment on bacterial morphology, SEM assessment was performed using *E. coli* (Figure 6(a) and 6(b)) and *Enterococcus* sp. (Figure 6(c) and 6(d)) samples treated with pressurized CO₂ at the determined optimal conditions (0.7 MPa, 20 °C, and 50% WVR) for 25 min. Comparative SEM images of untreated samples and samples treated with pressurized CO₂ did not reveal dramatic changes in the cell shape of *Enterococcus* sp.; however, some *E. coli* cells that were treated with pressurized CO₂ did not retain the original shape and appeared to be lysed. Notably, the treated cells of both strains had several small vesicles on the cell surface, whereas the untreated cells did not present such structures on the cell surface.

Previous studies have used SEM and transmission electron microscope imaging to show that *Salmonella typhimurium* and *E. coli* cells treated with supercritical CO₂ present small vesicles on the surface (Kim et al. 2007; Garcia-Gonzalez et al. 2010) and that the vesicles are indicative of cytoplasm leakage due to altered cell permeability (Garcia-Gonzalez et al. 2010). Despite lower pressurized CO₂ (0.7 MPa and 20 °C), this study showed that the formation of several extracellular small vesicles was not only observed in *E. coli*, a Gram-negative bacterium with a thin peptidoglycan layer (Zhang et al. 2006), but also in *Enterococcus* sp., a Gram-positive bacterium with a thick peptidoglycan layer (Zhang et al. 2006). These findings were supported by the results presented in previous section, which shows that the leakage of intracellular materials occurred during the treatment period. The data also affirmed the superior performance of pressurized CO₂ treatment.

**CONCLUSIONS**

Pressurized CO₂ treatment can be used to eliminate both *E. coli* and *Enterococcus* sp. from artificial seawater. The Gram-positive bacterial strain, *Enterococcus* sp., had lower susceptibility to pressurized CO₂ treatment than did the Gram-negative bacterial strain, *E. coli*. The seawater disinfection efficiency can be considerably improved by enhancing the solubility of CO₂ into seawater to increase penetration of CO₂ into bacterial cells. Disinfection substantially increased with increased pressure (0.2 to 0.9 MPa) and temperature (11 to 28 °C). Conversely, the bactericidal efficiency increased with decreasing WVR (80% to 50%). Treatment application at 0.7 MPa, at room temperature (20 °C), and at 50% WVR resulted in complete inactivation: 5.7 log reduction of *E. coli* and 5.4 log reduction of *Enterococcus* sp. within 5 min and 20 min, respectively. Taken together, these data indicate that pressurized CO₂ could be potentially used for treatment of ballast water. Further research is required to elucidate the effects of pH and pressure cycling on bactericial activity of pressurized CO₂. Studies are also required to elucidate the kinetics of bacterial inactivation for optimization of the treatment process.

**ACKNOWLEDGEMENTS**

This study was supported by a cooperation agreement between Yamaguchi University (Japan) and the Ministry of Education & Training of Vietnam. The authors also would like to thank Prof. Koichi Udo and Mr Mitsuaki Kimoto (Medical School of Yamaguchi University) for providing kind guidance for performing SEM analysis.

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First received 4 February 2016; accepted in revised form 6 May 2016. Available online 6 June 2016